THESIS

TEMPERATURE AND WINTER DURATION REQUIREMENTS FOR REPRODUCTIVE SUCCESS IN JOHNNY DARTER *ETHEOSTOMA NIGRUM* IN THE SOUTH PLATTE RIVER BASIN, COLORADO

Submitted by

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ABSTRACT

TEMPERATURE AND WINTER DURATION REQUIREMENTS FOR REPRODUCTIVE SUCCESS IN JOHNNY DARTER *ETHEOSTOMA NIGRUM* IN THE SOUTH PLATTE RIVER BASIN, COLORADO

Changes in water temperature and its seasonal timing influences the physiological processes of many aquatic ectotherms. Wastewater treatment plants (WWTP) along Front Range streams of Colorado have contributed to warmer and more consistent water temperatures throughout the year, particularly in winter months. Reduced variation in seasonal temperatures may have adverse effects on fishes that rely on temperature fluctuations or sustained periods of specific overwinter temperatures for reproductive cues and proper gonadal development. Assessing thermal requirements for reproduction is necessary for the conservation of native warmwater fishes residing in WWTP effluent-impacted streams. Johnny Darter *Etheostoma nigrum* are used as a sentinel species to assess winter water temperature regulations in Colorado because they are a thermally sensitive native species; however, their winter temperature requirements for successful reproduction are not known. Therefore, I evaluated the effects of winter stream temperature and winter duration on Johnny Darter reproductive success in the laboratory. Winter duration and temperature treatments simulated warmed effluent-impacted streams as well as streams with a natural thermal regime. Data indicated winter temperature and duration influenced timing of reproduction and egg development. Earlier spawning initiation was observed in fish exposed to warm winters and along with longer development time of eggs spawned at cooler water temperatures. Egg and larval production was similar among treatments

and indicates that the current winter water temperature standard may be adequate. However, reproductive output needs to be evaluated in the context of seasonal timing because spawning timing has the potential to effect overall production, egg development and survival.

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INTRODUCTION

Long-term trends of increasing water temperatures have been observed globally in many streams and rivers and are typically associated with increases in air temperature and urbanization (Kaushal et al. 2010). Webb and Nobilis (2007) reported long-term increases in water temperature in three Austrian rivers throughout the 20th century, with more accelerated rates occurring after 1970, likely due to increasing air temperature associated with climate change. However, the most rapid rates of increase in water temperature have been observed in highly urbanized areas (Nelson and Palmer 2007; Kinouchi et al. 2007; Kaushal et al. 2010). Water temperature on the Ara River near central Tokyo, Japan, increased 3.2-4.2°C over a 20-year period associated with thermal input from urban wastewater facilities (Kinouchi et al. 2007). Reaches of the Delaware River near Chester, Pennsylvania, USA showed large increases in water temperature over a 42-year period and the most rapid rates of increase occurred downstream of urban areas (Kaushal et al. 2010). Thus, urbanization can have similar or more severe effects on stream temperatures than climate change alone (Nelson and Palmer 2007).

Several components of urbanization affect water temperature, including runoff from impervious surfaces (Nelson and Palmer 2007), loss of shade and canopy cover (Burton and Likens 1973), water storage and diversion (Webb and Nobilis 2007), and thermal discharge (Kinouchi et al. 2007). Effluents of wastewater treatment plants (WWTPs) are responsible for a substantial proportion of thermal input to streams and can decouple the relationship between air and stream temperature (Kinouchi et al. 2007). Residential and commercial water use increases the temperature of water to WWTPs, which are often poorly equipped to regulate effluent water temperature entering streams and rivers (Kinouchi 2007). Water temperatures of wastewater

effluent sites are typically warmer than natural stream temperatures and can remain elevated several kilometers downstream of the effluent, particularly in winter (Lewis and McCutchan 2012).

The effect of WWTP thermal discharge on stream temperature is exacerbated in arid regions with naturally low winter flows and shallow channels, which are common hydrologic characteristics in the Great Plains ecoregion. The South Platte River Basin sits on the western edge of the Great Plains and is one of five major drainages that traverse the transition zone between montane headwater streams to lower elevation Great Plains streams on the Colorado Front Range. Transition zone streams are known to have high habitat heterogeneity and historically featured a high diversity of fishes (Haworth and Bestgen 2017). Specialized fish assemblages in Great Plains transition zones are highly susceptible to environmental changes and are at increased risk of extirpation (Fausch and Bestgen 1997). Most transition zone streams in the South Platte River Basin along the Colorado Front Range run through one or more major urban landscapes and are subject to high levels of anthropogenic influence (Figure 1). For instance, the mainstem of the South Platte River runs through several municipalities in the Denver metropolitan area where it accumulates substantial input from WWTP discharge. Although snowmelt contributes a large proportion of flow to the South Platte River seasonally, WWTP effluent comprises 69% of the annual streamflow and reaches downstream of urban areas are dominated by effluent eight months out of the year (Dennehy et al. 1993). The South Platte River Basin is also the most populous basin in Colorado, comprising 68.2% of Colorado's total population, and is projected to continue growing rapidly (Watson and Davies 2011). Historically, the South Platte River Basin naturally experienced a wide range of seasonal temperatures (Dennehy et al. 1993). However, rapid urbanization and development of WWTPs

have contributed to warmer and more consistent water temperatures throughout the year, particularly in winter months.

Water temperature is one of the most influential environmental factors for aquatic ectotherms and changes in water temperature and its seasonal timing affects physiological processes (Brett 1956; Vinson 2001; Kinouchi 2007; Nelson and Palmer 2007; Isaak et al. 2010; Hester and Doyle 2011). Understanding the effects of elevated stream temperatures on the physiological processes of sensitive native fishes in urbanized areas is crucial to the development of effective conservation and management plans. Previous studies have suggested that earlier onset of warm spring temperatures and delayed onset of winter temperatures from climate change and urbanization will lengthen the growing season and have positive effects on warmwater fishes' growth rates (Magnuson et al. 1997; Rypel 2009; Pease and Paukert 2014). Increased growth could benefit fecundity and survival, since each may be length related. However, other studies have suggested that a period of sustained cooler overwinter temperatures (<12°C) is critical for reproductive development in spring-spawning fishes (Jones et al. 1974; Hokanson 1977; Ciereszko et al. 1997). Cold winter temperatures influence gonad maturation, timing of spawning, egg quality and production, larval health, and recruitment (Jones et al. 1974; Hokanson 1977; Ciereszko et al. 1997; Farmer et al. 2015, Firkus et al. 2018). The potential for reduced reproduction and recruitment has negative implications for population growth and sustainability. Understanding temperature thresholds and winter duration requirements for reproduction by warmwater fishes is necessary to guide management and conservation actions in urbanized areas.

To facilitate a more natural thermal regime in Front Range streams, the Colorado Department of Public Health and Environment (CDPHE) implemented a winter water

temperature standard. However, it is uncertain whether the standard is protective of native fishes' physiological processes. My study attempts to address whether or not the current winter water temperature standard is protective for reproduction in Johnny Darter *Etheostoma nigrum*, a thermally sensitive species native to the South Platte River Basin. My specific objectives were to evaluate the effects of overwinter water temperatures and winter duration on Johnny Darter reproductive success, as measured by egg and larval production, egg success and development, and seasonal timing of spawning.

METHODS

Temperature Standard and Stream Classification

The first Colorado temperature standard was adopted in 1978 and has since undergone multiple revisions to incorporate species-specific temperature tolerance classification tiers of warm stream and cold stream fishes (Colorado Department of Public Health and Environment 2017). Warm Stream Tier I encompasses the most thermally sensitive warmwater species in the South Platte River Basin along the Front Range of Colorado, including Johnny Darter, Common Shiner *Luxilus cornutus*, Orangethroat Darter *Etheostoma spectabile*, and Stonecat *Noturus flavus* (Table 1; Colorado Department of Public Health and Environment 2008; Colorado Parks and Wildlife data, M. May, personal communication). The current CDPHE Warm Stream Tier I winter water temperature standard for the State of Colorado states that WWTP effluent cannot exceed a Maximum Weekly Average Temperature of 12.1°C from December 1st to February 28th (Colorado Department of Public Health and Environment 2016).

Focal Species

I chose Johnny Darter for my study because it was designated as a focal species by CDPHE to address the efficacy of the current winter water temperature standard in protecting fish reproduction. Among the four native sensitive species in Warm Stream Tier I, Johnny Darter is the most relevant study species because they are present in most streams in the South Platte River Basin (Figure 1), are regularly exposed to elevated winter temperatures from WWTP effluent, and have limited movement (Mundahl and Ingersoll 1983). Additionally, Johnny Darter are a spring-spawning species that may require cold winter water temperatures for reproduction

(Winn 1958; Propst and Carlson 1989; Farmer et al. 2015). Research conducted on other percids suggests specific winter temperatures are essential for proper reproductive development (Jones et al. 1974; Hokanson 1977; Ciereszko et al. 1997; Farmer et al. 2015). For example, female Yellow Perch *Perca flavescens* exposed to short warm winters produced smaller eggs, had reduced hatching success, and produced smaller larvae, which resulted in lower recruitment (Farmer et al. 2015). Firkus et al. (2018) demonstrated that elevated winter water temperatures negatively affected Johnny Darter fecundity by triggering out-of-season spawning, supporting the notion that the species is thermally sensitive and a good candidate for understanding effects of altered water temperatures.

Experimental Rationale and Design

I designed my experiment to encompass both winter duration and temperature relative the current CDPHE standard (Table 1) and natural conditions. Based on Colorado thermograph data, natural streams minimally impacted by WWTP effluent exhibit winter temperatures at or near 4°C (Firkus et al. 2018; M. May, Colorado Parks and Wildlife, personal communication). An initial analysis of minimally impacted Colorado streams suggested that these streams have approximately 110 days per year at or below 4°C (range = 90-127 days; Colorado Parks and Wildlife data, M. May, personal communication).

I used two winter temperatures (12°C and 4°C) and three winter durations (60-, 90-, and 120-days) in a factorial design of six treatments (Table 2). Winter durations of 60 and 120 days were chosen to simulate a "short" winter and a "long" winter within a 30-day bracket of the 90-day winter CDPHE standard. The 12ºC 90-day winter treatment (12_{90}) was representative of the current CDPHE winter water temperature standard and was used for assessing the standard. The

4°C 120-day winter treatment (4_{120}) mimicked winter water temperatures of streams minimally impacted by WWTP effluent and served as a natural stream comparison to the other treatments.

Two laboratory experiments were conducted: Experiment 1 in 2018-2019 and Experiment 2 in 2019-2020. In Experiment 1, I intended to run all six treatments but bacterial septicemia resulted in high mortality of darters collected from St. Vrain Creek in September 2018. All remaining fish were euthanized due to poor condition. Additional fish were collected from the same location on December 19th, 2018 when the average water temperature was 3.6°C. Due to the cold water temperature at the collection site, I only ran the 4°C temperature treatment at all durations in Experiment 1 because I felt it was not appropriate to increase water temperature to 12ºC for fish already acclimated to lower winter temperatures (Figure 2). In Experiment 2, all six treatments were executed and occurred from September 2019 through August 2020 (Figure 3). Experiment 1 had nine replicate tanks for each treatment (27 total tanks); high replication was possible because only the 4°C treatments were conducted and more space was available. Tanks were arranged on two racks in close proximity with six replicate tanks on one rack and three replicate tanks on the other. Experiment 2 had six replicate fish tanks per treatment arranged as three replicate tanks on each rack (36 total tanks).

All combinations of temperature and duration had 60-day transition periods in both the spring and autumn to mimic the natural thermograph of streams in the South Platte River Basin (Colorado Parks and Wildlife data, M. May, personal communication). Autumn transition started on October 1st, and water temperature in all treatments was decreased 0.1-0.3°C/day, with the rate dependent on the temperature condition, from the initial holding temperature of 17°C until their target winter temperature of 12°C or 4°C was reached on December 1st. Tanks were then held at that temperature for their assigned winter duration period of 60, 90, or 120 days. The

beginning of each treatment's spring transition was staggered by 30 days due to the differing durations of winter: the two 60-day winter treatments began spring conditions on January 30th, the two 90-day winter treatments began spring on February 29th (leap year), and the two 120-day winter treatments began spring on March 30th (Figure 3). During the spring transition period, water temperature was increased 0.1-0.4°C/day until the summer temperature of 21°C was reached. Fish were held for 90 days at 21°C to allow for extended spawning.

Field Collection

Approximately 250 wild adult Johnny Darter (45-70 mm total length) were collected for each experiment. It was beyond the scope of the project to assess differences among populations, so to minimize potential effects due to local variation in thermal tolerances, all fish were collected from St. Vrain Creek near Sandstone Ranch in Longmont, Colorado (UTM: 13 T 497293.6 E, 4444292.2 N). This location was chosen as the collection site for Johnny Darter based on recommendations from local biologists (A. Treble and B. Wright, Colorado Parks and Wildlife, personal communication). Collection began in late September for both experiments (Experiment 1 recollection in December 2018) before the start of the autumn temperature decline when the stream temperature was still relatively warm at about 17°C. Water temperature data of the collection site showed relatively natural winter temperatures (3-5ºC) and fish were not regularly exposed to altered thermal regimes from WWTP effluent. During collection, the date, time, and water temperature were recorded daily. Fish were captured via backpack electrofishing and settings were determined using the automatic calibration function to adjust for water conductivity differences. Fish behavior, recovery, and damage were monitored during electrofishing operations and settings were adjusted accordingly to minimize injury. Upon

capture, Johnny Darter were placed in multiple oxygenated holding tanks to reduce density-related stress and transported to the Foothills Fisheries Laboratory at the Colorado State University Foothills Campus in Fort Collins, Colorado. After transportation, fish were monitored for short-term mortality or injury and no mortalities were observed during the one-week acclimation period.

Fish Care and Aquaculture Setup

Fish were measured and placed in 37.9-L glass aquaria. Secondary sexual characteristics to distinguish males and females during the non-spawning season were not obvious. Therefore, I assigned fish to their tanks sequentially to make the size distribution similar in each tank, increasing the probability of having similar sex ratios in each tank because adult male Johnny Darter are generally larger than adult females. All adults were euthanized with tricaine mesylate (MS-222) at the end of each experiment and dissected to ascertain sex; undeveloped gonads were histologically stained and sectioned to confirm sex. Each tank contained six fish and four spawning tiles made from half-pipes of 50.8-mm diameter PVC pipe. PVC tiles provided cover, a defendable spawning location for males, and spawning substrate for females. To reduce the likelihood of stress and disease, fish in each tank were initially treated with a 0.2% (1852 ppm) solution of dissolved sodium chloride for a 25-minute static bath and then a 0.02% (185 ppm) solution of formalin and malachite green (Rid Ich Plus disease treatment) for a 60-minute static bath. In Experiment 1, fish acclimated in the field to 3.9°C and were placed directly into their respective 4°C winter treatments. In Experiment 2, all fish were held at their collection temperature of 17°C for a one-week acclimation period until the beginning of their autumn transition period on October 1st. All fish were fed a thawed mixture of two-thirds bloodworms

and one-third brine shrimp (*Artemia* spp.) once daily to satiation. Food weight was recorded every day to adjust amounts and minimize food waste. Fish tanks were siphoned once daily to remove uneaten food from the previous day and all inner surfaces were cleaned once weekly to reduce disease potential. Fish in all tanks were exposed to the same weekly-adjusted photoperiod for Longmont, Colorado (wild collection site).

Water from College Lake, Fort Collins was pumped into the Foothills Fisheries Laboratory where it was mechanically filtered and treated with ultra-violet light to reduce external biological contamination before use in the experiments. Desired water temperature was achieved using solenoid valves (ASCO 2-way 8210 Series) and a temperature controller (Love 1/16 DIN Temperature/Process Controller Series 16B) that mixed hot and cold water in separate insulated head tanks for each treatment. One data logger per head tank (HOBO Water Temperature Pro v2 Data Logger) recorded water temperature at 15-minute intervals (Appendix 1). Each head tank was connected to an alarm that indicated if water level or temperature was not in the desired range. Head tank water was gravity-fed via siphon in insulated PVC pipes to tanks using a flow-through system with standpipe drains (Figure 4). Flow rates into fish tanks were set to replace 1.5 tank volumes per hour (675 mL/min) and tanks were insulated on three sides to maintain desired water temperatures. All tanks were supplemented with oxygen using medium-pore diffusers.

Reproductive Monitoring

The tank bottom, walls, and PVC tiles in each tank were checked daily for eggs throughout the duration of both experiments. All newly observed eggs laid on the tank bottom, walls, and tiles were counted and recorded daily along with the corresponding water temperature.

There was no observed parental care for eggs spawned on the tank bottom and walls, thus I assumed that they were mortalities because unattended eggs would become infected with fungus or eaten. These eggs were siphoned from the tanks and discarded. Eggs deposited on tiles were counted from daily photographs (Figure 5). Tiles with eggs were removed from the tank, replaced by a fresh tile, and placed in a 4-L aerated incubation container labeled by treatment and filled with water from the corresponding treatment head tank (Figure 4). Each tile had a unique identification number to distinguish it from other tiles in the incubation container and labeled egg photographs enabled monitoring of development, mortality, and hatching (Figure 5). To replicate male parental care, eggs infected with fungus were manually picked from tiles daily. A preventive fungal treatment of 50 ppm of 35% hydrogen peroxide was also applied daily in a 15-minute static bath to all incubation containers. Hydrogen peroxide treatments reduced fungal mortality and increased my ability to evaluate temperature effects. Once the eggs were counted, tiles were placed in a temporary holding container while the water from the original incubation container was filtered through a fine screen to search for hatched larvae. All live and dead larvae were counted. Containers were filtered and rinsed at least four times to reduce chances of missing any larvae. After larval collection, tiles were placed back into their original incubation container with fresh aerated water from the corresponding treatment head tank.

Measured Response Variables

My reproductive monitoring approach enabled evaluation of four main reproductive elements: overall production, egg success, egg development, and spawning initiation. First, I measured overall production as the number of eggs per gram of female per week (egg production) and the number of live larvae per gram of female per week (larvae production) in

each tank. The number of females per tank was not known until fish were examined after the experiments concluded, therefore egg and larvae production were standardized by the biomass of females in each tank (per gram of female). Similarly, the length of spawning season varied among tanks so production was also standardized by spawning season length (per week). Second, egg success was measured three ways. I defined fertilization success as the total proportion of eggs that were fertilized, indicated by "eyed" eggs (McGree et al. 2010). Hatching success was defined as the proportion of live larvae versus total larvae collected. Egg to larva survival was defined as the proportion of eggs laid that hatched into live larvae. Third, I analyzed egg development among treatments from daily egg counts and larvae collection to examine how water temperature at spawning affected the number of days from egg laying to larval hatching (hereafter referred to as "days to hatch"). Spawning temperature refers to the specific water temperature on the day the eggs were laid and does not necessarily correspond to the winter treatment temperature of 4°C and 12°C. Fourth, spawning initiation date was defined as the number of days from beginning of winter (December 1st) to date of first egg observation in each tank. I also informally described the date of first egg observation in each treatment relative to water temperature.

Statistical Analyses

Normality assumptions were checked prior to statistical analyses for each variable of both experiments by examining residuals versus fitted values and quantile-quantile plots. I looked for outliers using Grubbs' and Rosner tests. Normality assumptions were satisfied for all modeled variables and I proceeded with parametric statistical procedures (A. Hess, Colorado State University Department of Statistics, personal communication). Only two outliers were found in

Experiment 1 and were not omitted from analyses (one outlier in eggs per gram of female per week and one outlier in live larvae per gram of female per week). No outliers were found in Experiment 2.

In Experiment 1, the means of seven response variables (females per tank, eggs per gram of female per week, live larvae per gram of female per week, fertilization success, hatching success, egg to larva survival, and spawning initiation date) were compared using a one-way Analysis of Variance (ANOVA) with winter duration as the main effect. In Experiment 2, means of the same seven response variables were analyzed using a two-way ANOVA with winter temperature and winter duration as the main effects and the winter temperature x winter duration interaction was also investigated. To compare consistency of the 4ºC treatment effects in Experiment 1 and Experiment 2, means of the seven variables were compared using a two-way ANOVA with winter duration and experiment as the main effects along with the winter duration x experiment interaction. For egg development in both experiments, a linear regression was used to evaluate if there was a statistically significant relationship between days to hatch and spawn water temperature for each treatment and with all treatments combined. For all analyses, statistical significance was defined by a p-value of less than 0.05. Statistically significant results were followed with a post-hoc Tukey's Honest Significant Difference test and pairwise comparisons to identify specific differences between treatments.

RESULTS

Females per Tank

In Experiment 1, the number of females per tank did not differ among the three 4ºC winter durations ($p = 0.4513$; Table 3; Figure 6). Similarly, the number of females per tank in Experiment 2 did not differ among winter temperature ($p = 0.3919$) or duration treatments ($p = 0.2820$) and there was no interaction ($p = 0.1031$; Table 3; Figure 6). All replicate tanks in both experiments had at least one female and one male present (range = 1-5 females).

Overall Production

Egg production in Experiment 1 was not statistically different among winter durations ($p = 0.3323$; Table 3; Figure 7). In Experiment 2, egg production did not differ among winter temperature ($p = 0.5772$) or winter duration ($p = 0.3743$), and no interaction was present ($p = 0.4503$; Table 3; Figure 7). The larvae production in Experiment 1 did not differ among winter durations ($p = 0.5869$; Table 3; Figure 8). In Experiment 2, larvae production did not differ among winter temperatures ($p = 0.6584$) or durations ($p = 0.2865$) and there was no interaction ($p = 0.5957$; Table 3; Figure 8).

Egg Success

In Experiment 1, I did not find statistical evidence of differences in fertilization success ($p = 0.0778$) and egg to larva survival ($p = 0.1157$) among winter duration treatments. Hatching success in Experiment 1 was significantly different among winter durations because hatching success in treatment 4_{120} was significantly lower than in treatment 4_{90} ($p = 0.04911$; Table 4). In

Experiment 2, fertilization success ($0.1749 < p < 0.4755$), hatching success ($0.0612 < p < 0.5538$), and egg to larva survival ($0.3056 < p < 0.5900$) were not statistically different among winter temperatures or winter durations and there was no interaction (Table 4).

Egg Development

In Experiment 1, the spawning temperature of all 4ºC treatments ranged from 15.7ºC to 21ºC and spawning temperature did not influence the days to hatch (Table 5; Figure 9). The mean days to hatch on each end of the spawning temperature range were relatively similar: 7.5 days (range = 7-9 days) for eggs spawned from 15.7-16.7ºC and 7.1 days (range = 5-10 days) for eggs spawned at the warmest temperatures 20-21ºC (Figure 9).

In Experiment 2, days to hatch was negatively related to spawning temperature (Table 5; Figure 9). Five of the six winter treatments in Experiment 2 displayed a significant negative relationship between days to hatch and spawn temperature (Table 5; Figure 9). Treatment 4_{90} had a positive slope, likely due to the lower numbers of spawning events across a narrow temperature range (Table 5; Figure 9). The spawning temperature of all Experiment 2 treatments ranged from 12ºC to 21ºC. The mean days to hatch was 18.4 days (range = 12-24 days) for eggs spawned at the coldest temperatures 12-13°C and 6.7 days (range = 4-9 days) for eggs spawned at the warmest temperatures 20-21°C. (Figure 9).

Spawning Timing

Spawning Initiation Date. In Experiment 1, spawning initiation date did not differ among winter duration treatments ($p = 0.4054$), likely due to the high variability in treatment 4_{60}. Treatments 4_{90} and 4_{120} both had a narrow range of days between their earliest and latest

spawning initiation date among tanks (11 and 9 days respectively), however treatment 4_{60} had the widest range of 92 days between spawning initiation dates of tanks (March 29th – June 29th, 2019; Figure 10).

In Experiment 2, spawning initiation date of fish in the three 12ºC treatments was significantly earlier than the 4ºC treatments ($p = 0.0007$; Figure 10). The spawning initiation date did not differ among winter duration treatments in Experiment 2 ($p = 0.4448$). Winter duration and temperature showed a significant interaction and spawning initiation date depended on both effects ($p = 0.0007$; Figure 10). Pairwise comparisons suggested winter duration had a clear sequential effect on the spawning initiation date of fish in the 4°C treatments with the 60-day winter spawning earliest, followed by the 90-day duration, and the 120-day duration was the last to initiate spawning ($0.0002 < p < 0.0399$). However, pairwise comparisons showed no evidence that winter duration affects spawning initiation date among the 12°C treatments ($0.0670 < p < 0.9842$), resulting in the observed interaction (Figure 10).

I observed an unusual gap in spawning duration of fish in all the 60-day winter treatments across both experiments. Fish in the 4_{60} treatments of both experiments briefly began spawning at 19.9ºC and 20°C and then ceased spawning for 56 days in Experiment 1 and 40 days in Experiment 2. The 12_{60} treatment began spawning at 16.4°C and ceased spawning for 76 days. Fish in all three treatments resumed spawning at a water temperature of 21°C. Fish in all other treatments across both experiments exhibited a relatively continuous spawning pattern (Figure 11).

Date of First Egg Observation. In Experiment 1, fish in the 4_{60} treatment spawned first on March 29th, 2019 when the water temperature was 19.9ºC, followed by treatment 4_{90} on April 18th at 17.0ºC, and 4_{120} fish were the last to spawn on May 13th at 15.7ºC (Figure 11). In

Experiment 2, fish in the 12_{120} and 12_{90} treatments spawned first in early February 2020 when they were still experiencing winter temperatures of 12°C (Figure 11). The first eggs were observed in 12_{120} and 12_{90} on February 5th and 6th, respectively. First eggs in the 12_{60} treatment were spawned later on February 27th when fish were approximately halfway through their spring transition and the water temperature was 16.4°C. Fish in the three 4°C treatments all spawned first eggs later in their spring transition: 4_{60} on March 26th at a water temperature of 20°C, 4_{90} on April 8th at 15.3°C, and 4_{120} on May 5th at 14.5°C (Figure 11).

Comparison of 4ºC Treatments in Experiment 1 and 2

Experiment 1 had significantly higher number of females per tank in the 4°C treatments compared to Experiment 2 ($p = 0.0001$; Table 3; Figure 6). However, the number of females per tank among winter duration treatments did not differ between the experiments ($p = 0.7714$) and the interaction was not significant ($p = 0.5744$; Table 3; Figure 6). Similarly, the overall egg production was significantly higher in Experiment 1 ($p = 0.0240$), but did not differ among winter duration treatments between the experiments ($p = 0.5724$) and there was no interaction ($p = 0.2990$; Table 3; Figure 7). Despite the higher number of females and egg production in Experiment 1, larvae production was not significantly different between the experiments ($p = 0.1030$) or among winter duration treatments ($p = 0.9601$) and there was no significant interaction ($p = 0.3457$; Table 3; Figure 8).

Fertilization success and egg to larva survival did not differ between the experiments ($p = 0.9595$, $p = 0.5554$, respectively) or among winter duration treatments ($p = 0.2495$, $p = 0.8150$, respectively) and no interaction was present ($p = 0.1707$, $p = 0.1116$, respectively; Table 4). Although there was a significant interaction between winter duration and experiment for

hatching success ($p = 0.0357$), hatching success did not differ between experiments ($p = 0.6631$) or winter duration ($p = 0.2596$) and no evidence of differences were detected in the post-hoc Tukey's Honest Significant Difference test or pairwise comparisons ($p \geq 0.1756$).

Spawning initiation dates in Experiment 1 and 2 were not statistically different ($p = 0.1054$; Figure 10). Winter duration significantly affected the spawning initiation date of all 4ºC treatments in both experiments ($p = 0.0181$) likely because pairwise comparisons showed treatment 4_{60} of Experiment 2 spawned significantly earlier than treatment 4_{120} of Experiment 2 ($p = 0.0162$; Figure 10). No interaction between winter duration and experiment was present for spawning initiation date ($p = 0.0946$). The date and water temperature of first eggs observed in Experiment 1 treatments were relatively similar to the corresponding treatment in Experiment 2: fish in treatment 4_{60} of Experiment 1 and 2 began spawning within 3 days and 0.1ºC of each other, the 4_{90} treatments within 10 days and 1.7ºC, and the 4_{120} treatments within 8 days and 1.2ºC of each other (Figure 11).

DISCUSSION

Water temperature strongly influences ectotherm biology and is considered a master variable regulating fish physiology (Brett 1956; Nelson and Palmer 2007; Isaak et al. 2010; Hester and Doyle 2011). My data indicated that winter conditions significantly influenced spawning initiation date and the rate of egg development. However, Johnny Darter egg and larvae production in the laboratory were not substantially different among the winter conditions examined, indicating that production is not critically dependent on winter temperature or duration. Although winter conditions did not appear to influence egg and larvae production in the laboratory, spawning timing has the potential to affect overall production in the wild through temperature mediated effects on egg development and survival. Therefore, it is crucial that production results be evaluated within the context of seasonal timing of spawning.

Johnny Darters exposed to longer warm winters began spawning three months earlier than fish experiencing long cold winters, and they spawned extensively in winter and minimally in spring. Early winter spawning has also been reported in another study that evaluated elevated winter water temperatures on Johnny Darter reproduction where fecundity was negatively affected by early spawning (Firkus et al. 2018). Although early spawning at 12ºC was sustainable under laboratory conditions, winter spawning in the wild leads to longer egg development time that could potentially affect egg survival, which may be cause for concern in Johnny Darter residing in effluent-impacted streams in the South Platte River Basin.

My data clearly indicated that eggs spawned at lower temperatures took longer to develop and hatch, which was consistent with other studies on Johnny Darter egg development (Speare 1965; Paine and Balon 1986). Decreased egg survival has been linked to longer development

times at lower incubation temperatures (Van der Kraak and Pankhurst 1997). Johnny Darter exposed to the longest cold winter first spawned at 14.5ºC whereas fish in the longest warm winter first spawned at 12ºC. Eggs spawned at temperatures cooler than 14.5ºC in warmer winter treatments took over twice as long to hatch compared to eggs spawned at or above that threshold. A large proportion of eggs (62-84%) in the longer warm winter treatments were produced at temperatures below 14.5ºC. If longer developemental time influences survival, eggs spawned in winter could be subject to lower survival and total production could be substantially reduced (Figure 12). However, egg to larval survival did not differ in my laboratory experiments, potentially because I used a standard fungal treatment (Marking et al. 1994) as a precaution to avoid mortality associated with isolating eggs from parental care and any stress related to counting eggs. If fungal infection is temperature dependent, then my data may underestimate temperature effects on egg to larval survival. Assessing the effects of winter temperature on Johnny Darter egg and larval survival requires further research, particularly in the wild, but Johnny Darter eggs spawned at temperatures lower than 14.5ºC could be subject to increased mortality from factors associated with longer developmental times.

Longer developmental time could also result in increased parental care for males that may affect their condition and survival or a mismatch with critical larval resources. Males in the laboratory were fed to satiation daily and had the energetic resources to maintain and defend a nest. However, parental care in the wild reduces male condition and survival because fitness benefits can outweigh the costs of extensive parental care (Klug and Bonsall 2014). Males in the wild are also subjected to increased predation risk while guarding a nest (Winkelman 1996; Klug and Bonsall 2014). Additionally, potential disjunction of phenology between early-hatched larvae and required resources could negatively affect survival and may lead to recruitment failure

(Durant et al. 2007). For instance, upon the onset of exogenous feeding, Johnny Darter larvae require adequate prey and it is unclear if those prey resources are available in the winter. Furthermore, early life stages of Johnny Darter may be dependent on certain flows, and winter flows are typically much lower than spring and early summer in the South Platte River Basin (Dennehy et al. 1993), and it is unclear whether low winter flows or high spring runoff flows are more challenging to young darters. Overall, the potential combined effects of physiological, behavioral, and environmental factors during winter may be problematic for early production and recruitment to the population.

Winter duration had unexpected influences on seasonal spawning patterns. Johnny Darter exposed to short winters initiated spawning but then quickly stopped and waited approximately 1.5-2.5 months before resuming spawning. The spawning gap for short winters occurred in both experiments regardless of winter temperature. I do not have an explanation for this spawning pattern exhibited in shorter winters, but the spawning pattern may be unfavorable due to the long cessation of egg production during the majority of normal spring spawning. For instance, one tank in the 12_{60} treatment did not reinitiate spawning until the end of May after a 76-day spawning gap. Larvae that are spawned later in the reproductive season may have less time to grow and smaller individuals can exhibit higher overwinter mortality (Shuter et al. 1980; Post and Evans 1989). Conversely, there could be benefits to spawning later; these larvae may avoid high flows from spring runoff and their long-term survival may improve. Further research is required to assess potential factors that result in the observed spawning gap in fish experiencing short winters because the repercussions for Johnny Darter reproduction are unknown.

Experiment 1 had a higher mean number of females per tank and higher mean egg production compared to Experiment 2, suggesting tanks with more females had higher egg

production. Standardizing the data by female biomass was not sufficient to eliminate differences in egg production, indicating other factors may be responsible for my observations. One possible explanation is sex ratio per tank could affect female oviposition and courtship rates via behavioral interactions of territorial males (Spence and Smith 2005). Throughout both experiments I observed agonistic behaviors in Johnny Darter males such as chasing and biting other fish (male or female) while defending their chosen spawning tile. Although the number of females varied between the experiments, they did not differ among treatments within an experiment and did not influence my inferences regarding winter conditions. Despite differences in egg production and number of females, the larvae production, fertilization success, egg to larva survival, hatching success, and spawning initiation date were similar between experiments.

Clearly, laboratory conditions differ from field conditions and this must be considered before using my results to inform criteria development of water temperature standards. Many environmental factors in the field influence fish reproduction, especially in urban streams. My fish in the laboratory experienced steady conditions in a controlled environment with a consistent food supply, high water quality, and no predators. The availability of food can affect the amount of energy available for spawning (Fausch and Bestgen 1997) and fish in the wild likely do not have access to unlimited food, especially in the winter (Foy and Paul 1999). Additionally, fish residing near WWTP effluents are also exposed to many wastewater components, especially endocrine-disrupting compounds that may exacerbate the effects of environmental conditions and compromise reproduction (Vajda et al. 2008; Jin et al. 2010; McGree et al. 2010; Schwindt et al. 2014; Schwindt and Winkelman 2016). In addition to reduced water quality and lack of consistent food, adults and juveniles in the wild are vulnerable to a high risk of predation during spawning (Winkelman 1996; Klug and Bonsall 2014). Due to the combination of these factors,

wild fish exposed to elevated winter water temperatures in effluent-impacted streams may not be able to sustain the same amount of egg production observed under laboratory conditions.

My study suggests that the current CDPHE winter water temperature standard of 12ºC and a duration of 90 days appears to be adequate for egg and larval production in Johnny Darter. However, it is important to recognize that egg and larval production are influenced by spawning timing through its effects on egg development. Adverse effects on reproduction from early spawning in effluent-impacted streams could reduce overall production and lead to recruitment failure and affect population sustainability (Farmer et al 2015; Firkus et al 2018). Therefore, spawning timing must be considered during criteria evaluation and management decision-making for water temperature standards in the South Platte River Basin. I would also argue against shortening the duration of the winter standard because short winters could be disadvantageous to reproduction based on the long cessation of spawning seen in all 60-day winter treatments.

As the human population expands and more areas become developed, the need for empirical data of urbanization effects on stream alteration become more relevant and necessary. Although my project has contributed to evaluating the efficacy of the current winter water temperature standard in protecting fish reproduction, critical questions and possible next steps have arisen from the findings. First, support from field studies is essential to develop a more comprehensive understanding of WWTP effluent effects on fish reproduction. For example, investigating hatch date distribution of wild Johnny Darter larvae along with seasonal prey abundance is necessary to address whether early and late spawning are detrimental in effluent-impacted and non-impacted areas. Second, additional laboratory studies are necessary to address the role of temperature, seasonal duration, and temperature transition rates on important population parameters, such as post-hatch larval survival and recruitment. Overall, fish residing

in areas near WWTPs are exposed to many stressors, and the best approach is to evaluate both laboratory and field data to better inform our management actions and policy development.

TABLES

Table 1. Seasonal water temperature standard specifications of Warm Stream Tier I. Species expected to be present are Johnny Darter *Etheostoma nigrum*, Common Shiner *Luxilus cornutus*, Orangethroat Darter *Etheostoma spectabile*, and Stonecat *Noturus flavus*. Maximum Weekly Average Temperature (MWAT), and Daily Maximum (DM) are shown (adapted from Colorado Department of Public Health and Environment 2008).

Applicable months	Water temperature standard (ºC)	
	MWAT	DM
March - November	24.2	29.0
December - February	12.1	14.5

Table 2. Experimental design for the Johnny Darter laboratory study. Treatment 12_{90} (90 day winter of 12ºC) represents the current CDPHE Warm Stream Tier I Maximum Weekly Average Temperature standard in winter. Treatment 4_{120} (120 day winter of 4ºC) represents the natural winter temperature regime of streams minimally impacted by WWTP effluent. Experiment 1 consisted of only the 4ºC temperature at three durations, while Experiment 2 consisted of temperatures 4ºC and 12ºC at three durations.

	Winter duration		
Winter temperature	60-day	90-day	120-day
12°C	12_{60}	12_{90}	12_{120}
4°C	4_{60}	4_{90}	4_{120}

Table 3. Mean and standard deviation (SD) of the number of females per tank and overall egg and larvae production for all treatments in both experiments. Sample size *n* refers to the number of replicate tanks per winter treatment.

Experiment	Winter treatment	*n*	Number of females per tank		Number of eggs per gram of female per week		Number of live larvae per gram of female per week	
			Mean	SD	Mean	SD	Mean	SD
1	4_{60}	9	3.1	0.9	15.4	14.7	4.3	4.9
1	4_{90}	9	3.6	1.0	11.0	10.3	6.1	6.7
1	4_{120}	9	3.6	0.7	6.6	9.7	3.3	4.8
2	4_{60}	6	2.3	0.5	4.0	4.1	1.8	2.1
2	4_{90}	6	2.3	1.0	1.4	2.4	1.0	1.8
2	4_{120}	6	2.2	0.8	6.2	8.4	3.6	5.2
2	12_{60}	6	2.0	0.0	2.9	5.2	1.3	2.3
2	12_{90}	6	2.3	0.8	5.6	5.1	3.0	3.0
2	12_{120}	6	3.2	1.0	6.1	5.3	3.5	3.4

Table 4. Mean and standard deviation (SD) of fertilization success, hatching success, and egg to larva survival for all treatments in both experiments. Sample size *n* refers to the number of replicate tanks per winter treatment.

Experiment	Winter treatment	*n*	Fertilization success (%)		Hatching success (%)		Egg to larva survival (%)	
			Mean	SD	Mean	SD	Mean	SD
1	4_{60}	9	67.2	39.4	71.1	41.1	49.2	37.9
1	4_{90}	9	49.6	42.2	83.7	31.8	56.5	29.0
1	4_{120}	9	20.6	38.5	34.1	47.1	22.8	32.0
2	4_{60}	6	54.4	28.3	78.3	38.8	43.1	19.7
2	4_{90}	6	30.0	46.4	30.2	47.0	32.1	41.6
2	4_{120}	6	54.8	43.8	63.3	49.2	47.1	40.0
2	12_{60}	6	43.7	35.7	64.5	50.1	36.9	31.2
2	12_{90}	6	69.1	16.8	90.5	10.6	52.1	21.3
2	12_{120}	6	73.0	17.0	91.3	8.5	52.7	22.1

Table 5. Linear regression parameters for days to hatch versus spawn water temperature (ºC) for all treatments in both experiments. Sample size *n* is the number of tiles spawned on in each treatment. Significant *p*-values ($p < 0.05$) are marked by an asterisk.

Experiment	Winter treatment	*n*	Slope	Intercept	R^2	*p*-value
1	4_{60}	19	-1.1538	31.5271	0.1458	0.1304
1	4_{90}	28	-0.2937	13.2572	0.0708	0.1796
1	4_{120}	14	-0.3339	13.2825	0.2034	0.1056
1	all	61	-0.1778	10.8864	0.0441	0.1130
2	4_{60}	18	-1.8235	44.4706	0.2307	0.0436*
2	4_{90}	5	0.444	-0.8393	0.0777	0.6500
2	4_{120}	16	-0.519	17.3919	0.6493	0.0002*
2	12_{60}	19	-1.4424	36.6199	0.6757	1.58e-05*
2	12_{90}	25	-0.99	28.156	0.5596	1.71e-05*
2	12_{120}	70	-1.52493	36.10769	0.7818	<2e-16*
2	all	153	-1.2317	31.8055	0.8052	<2e-16*

FIGURES

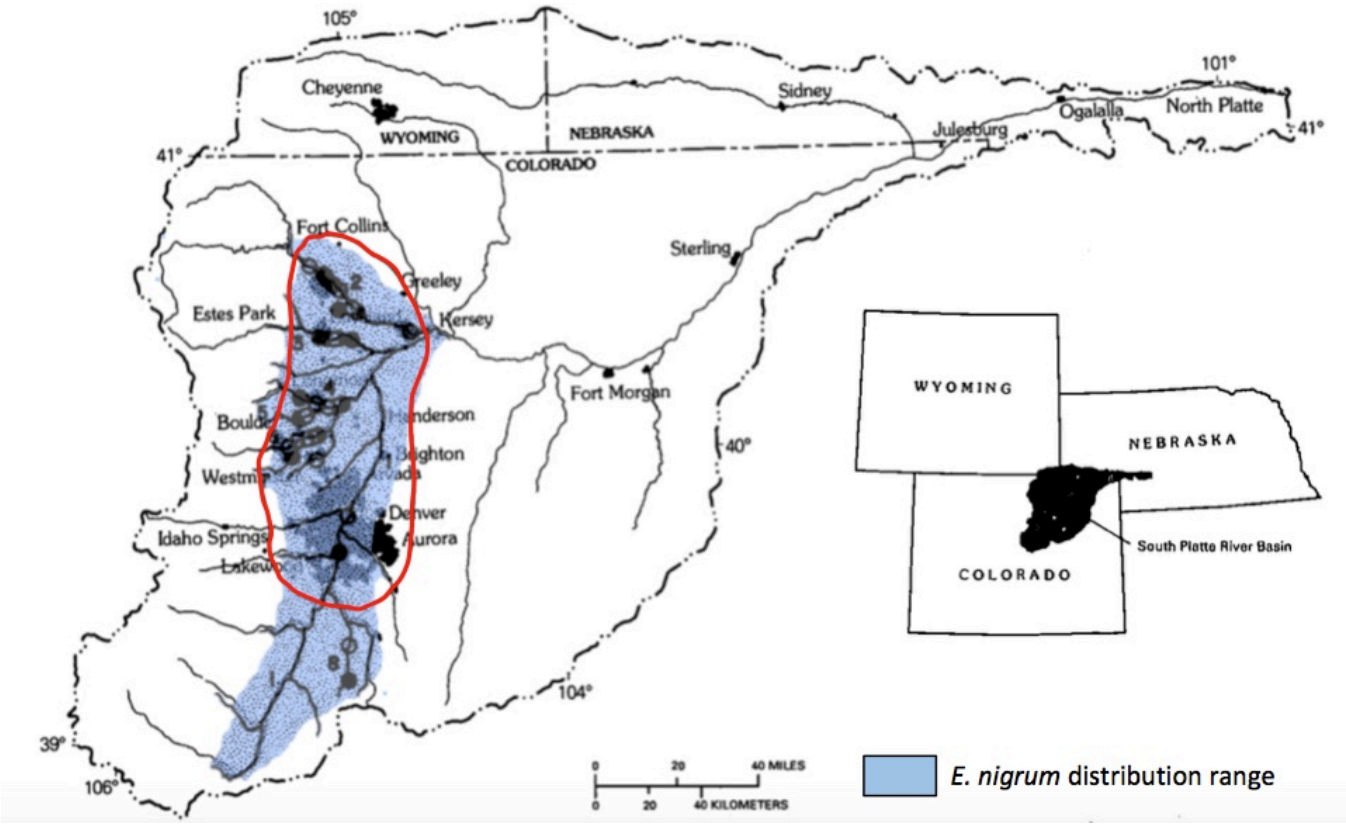

Figure 1. Map of the South Platte River Basin and the Colorado distribution of Johnny Darter *Etheostoma nigrum*. The urban center of the Colorado Front Range is outlined in red (adapted from Propst and Carlson 1989 and Dennehy et al. 1993).

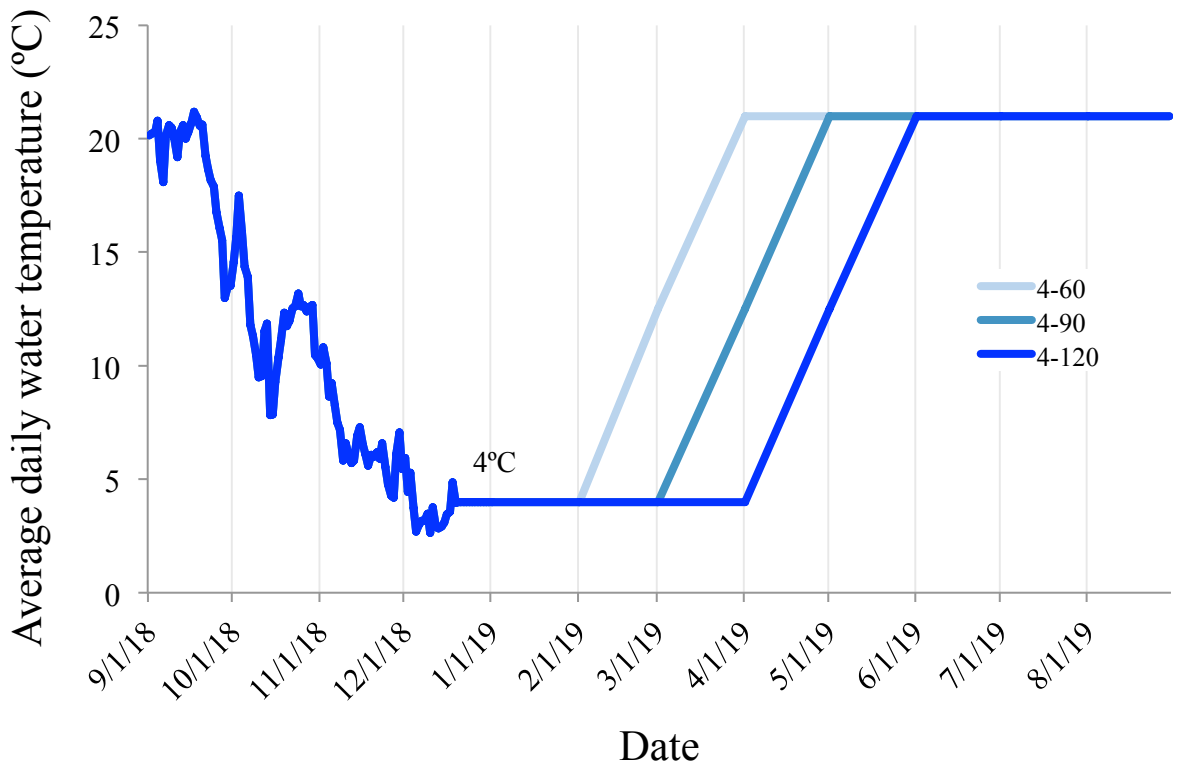

Figure 2. Timeline of winter duration treatments for Johnny Darter at 4ºC in Experiment 1 (2018-2019). Fish experienced a natural autumn temperature decline in St. Vrain Creek (actual temperature data from collection site shown from September to December 2018) and were collected on December 19th, 2018. After collection, fish in all treatments experienced temperatures in the laboratory.

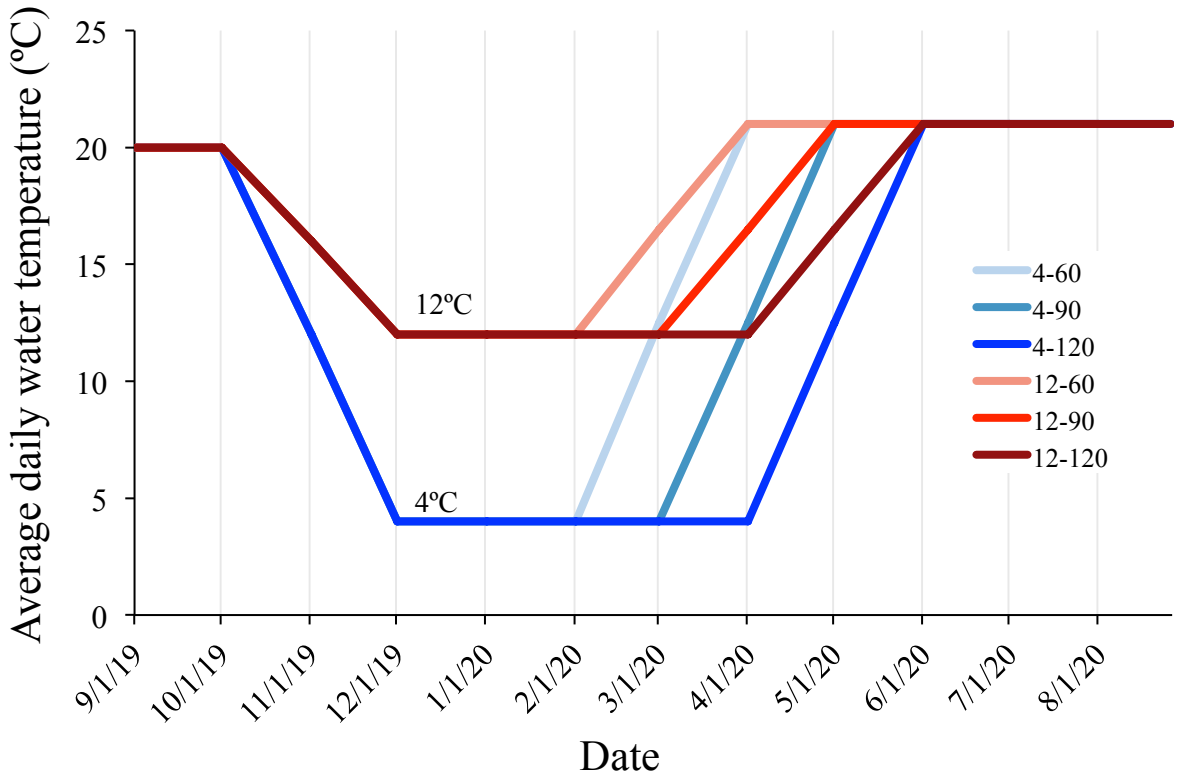

Figure 3. Timeline of winter water temperature and winter duration treatments for Johnny Darter at 4ºC and 12ºC in Experiment 2 (2019-2020). Fish collection occurred from September 22-24th, 2019 and thereafter fish in all treatments experienced temperatures in the laboratory.

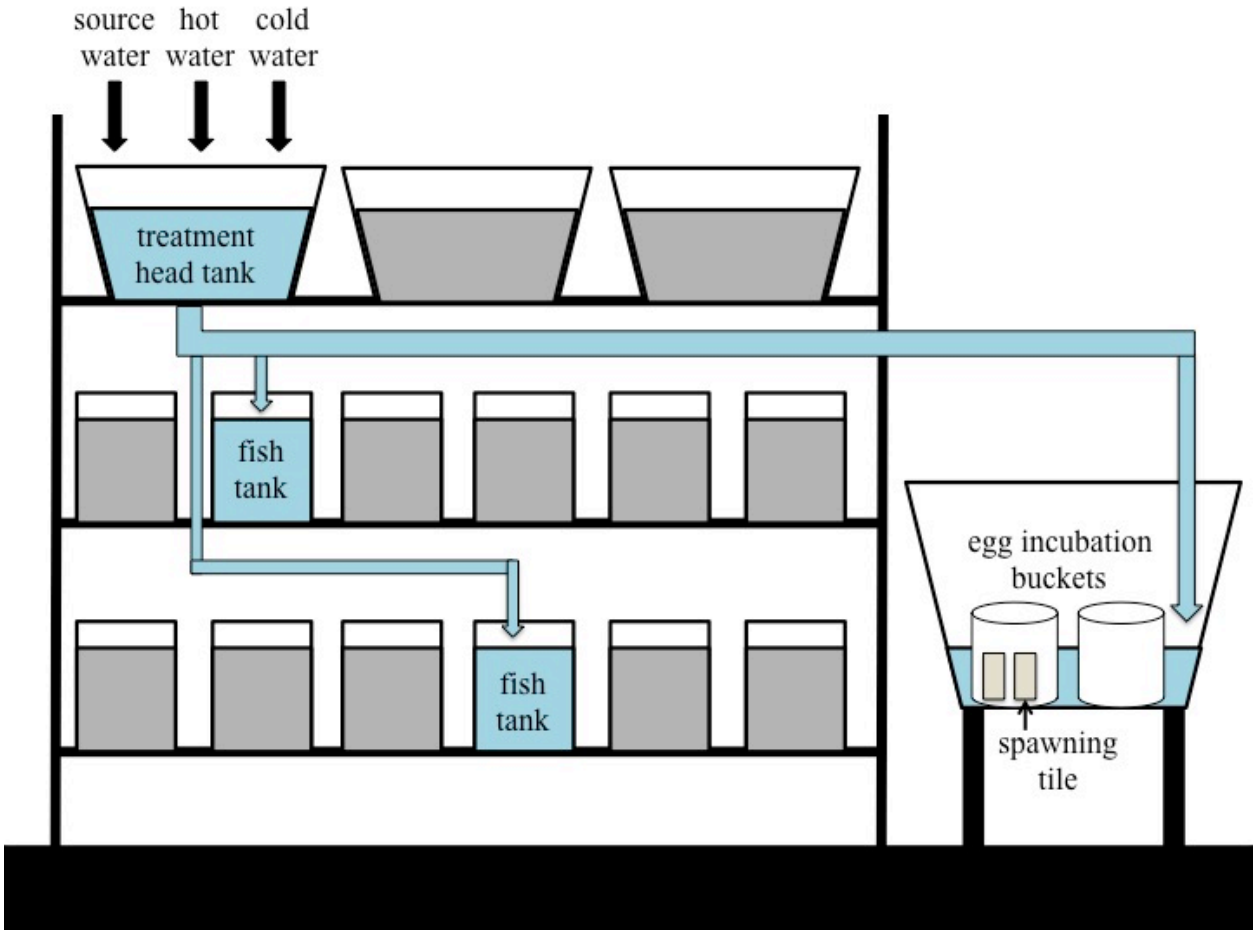

Figure 4. Aquaculture system used for Experiment 1 and 2 (one aquaculture rack shown for illustration). Each insulated treatment head tank gravity-fed water to six 37.9-L replicate fish tanks (two are shown for illustration). Each rack held eighteen tanks (six additional fish tanks are located behind middle shelf tanks). Each treatment head tank also fed an incubating water bath to maintain appropriate water temperature for spawning tiles after they were removed from the tank. Air was supplied through a 6.35-mm diameter perforated irrigation hose at the bucket bottom to create a gentle flow of oxygenated water over the eggs.

Figure 5. Photograph of a spawning tile with eggs. Tile number, date, treatment, and replicate tank were recorded, occasionally with notes about the eggs on the tile.

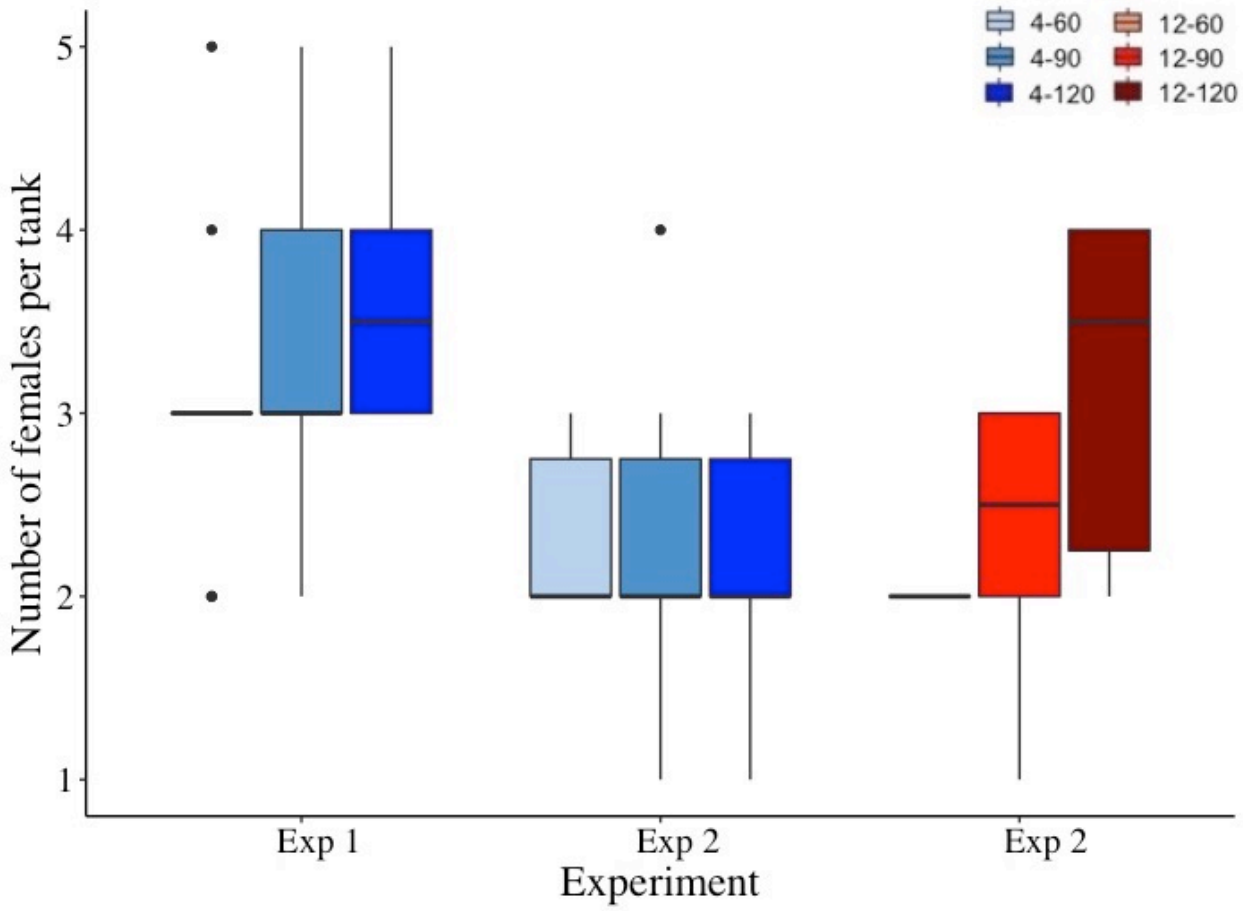

Figure 6. Box and whisker plot of number of females per tank of Experiment 1 and 2. The color of the boxes refers to winter temperature treatment: blue are the 4ºC treatments and red are the 12ºC treatments. Shade of the color refers to the winter duration treatment: lightest shade is the 60-day winter duration and the darkest shade is the 120-day winter duration. The horizontal bars represent the median, the box is the interquartile range, and the vertical bars are the minimum and maximum. Dots represent outliers.

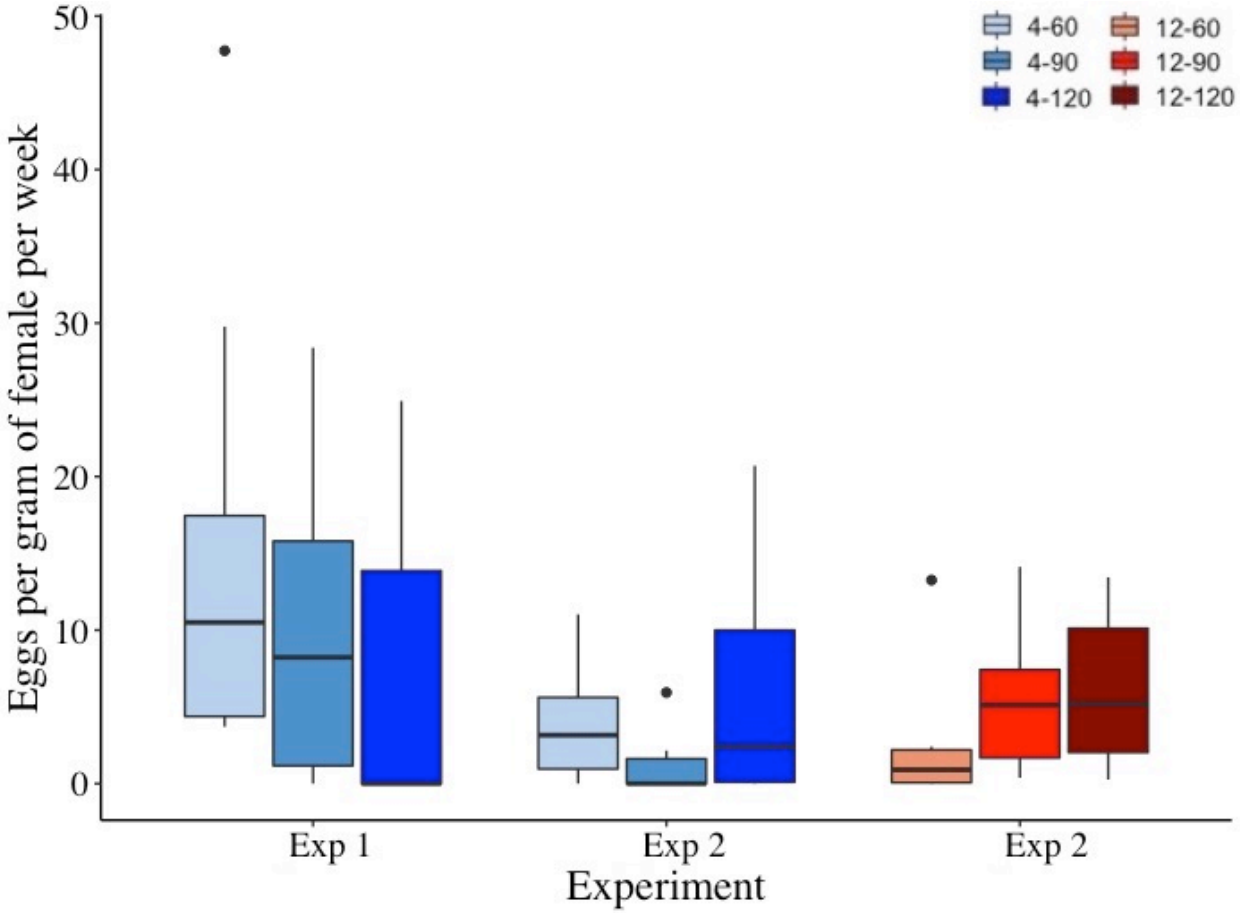

Figure 7. Box and whisker plot of number of eggs per gram of female per week in each tank of Experiment 1 and 2. The color of the boxes refers to winter temperature treatment: blue are the 4ºC treatments and red are the 12ºC treatments. Shade of the color refers to the winter duration treatment: lightest shade is the 60-day winter duration and the darkest shade is the 120-day winter duration. The horizontal bars represent the median, the box is the interquartile range, and the vertical bars are the minimum and maximum. Dots represent outliers.

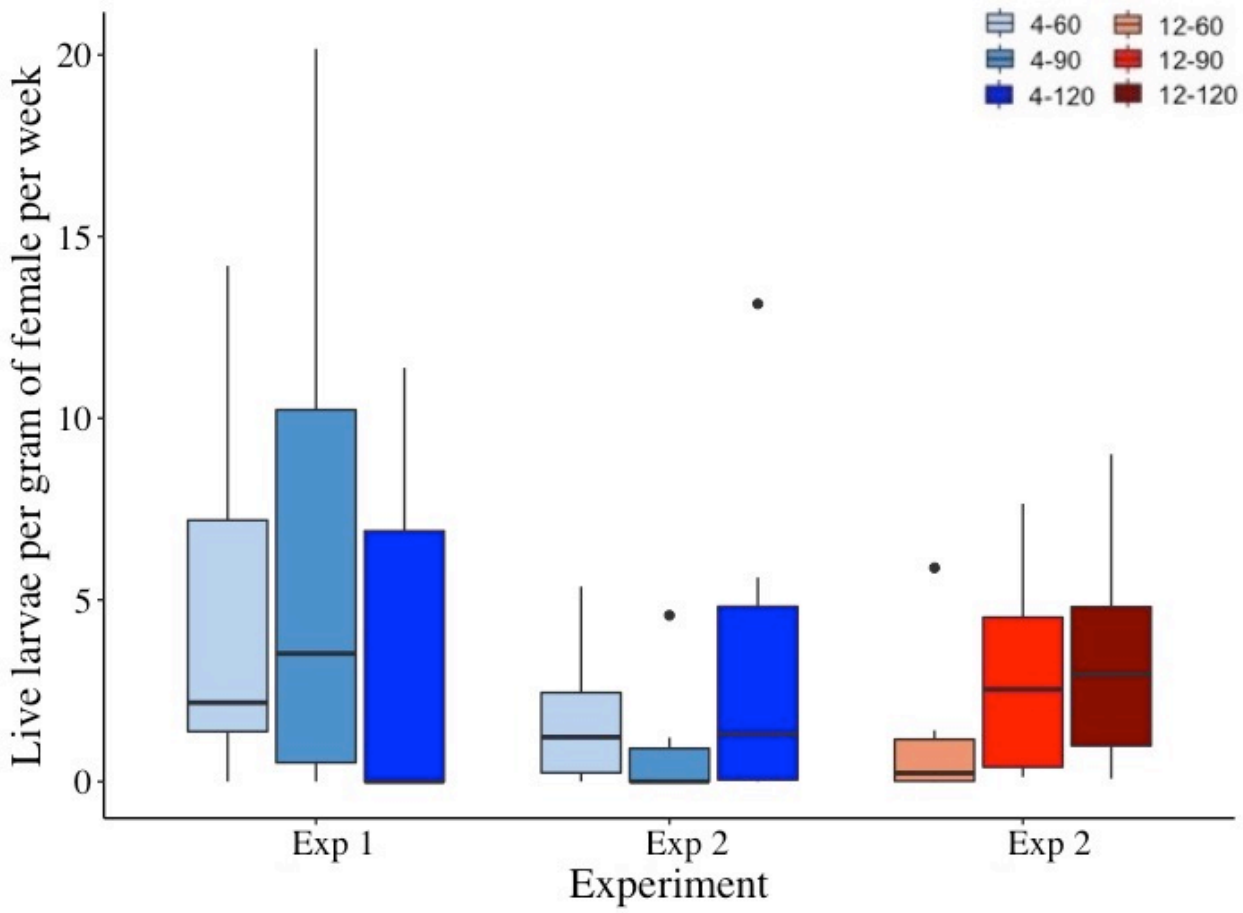

Figure 8. Box and whisker plot of number of live larvae per gram of female per week in each tank of Experiment 1 and 2. The color of the boxes refers to winter temperature treatment: blue are the 4ºC treatments and red are the 12ºC treatments. Shade of the color refers to the winter duration treatment: lightest shade is the 60-day winter duration and the darkest shade is the 120-day winter duration. The horizontal bars represent the median, the box is the interquartile range, and the vertical bars are the minimum and maximum. Dots represent outliers.

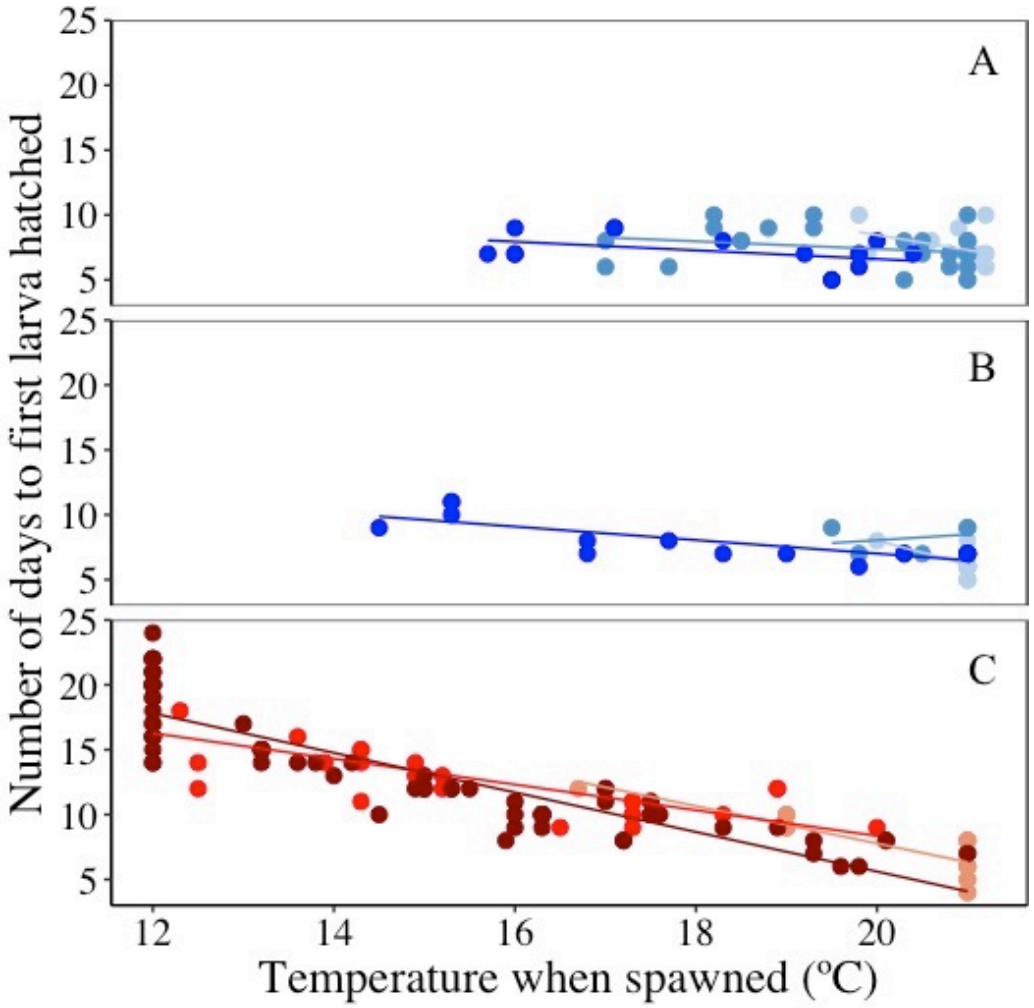

Figure 9. Relationship between the number of days to hatch and spawn water temperature (ºC). Each data point represents a tile that had eggs. The color of the points refers to winter temperature treatment: blue are the 4ºC treatments and red are the 12ºC treatments. Shade of the color refers to the winter duration treatment: lightest shade is the 60-day winter duration and the darkest shade is the 120-day winter duration. A) 4ºC treatments of Experiment 1 at 60, 90, and 120 days; B) 4ºC treatments of Experiment 2 at 60, 90, and 120 days; and C) 12ºC treatments of Experiment 2 at 60, 90, and 120 days.

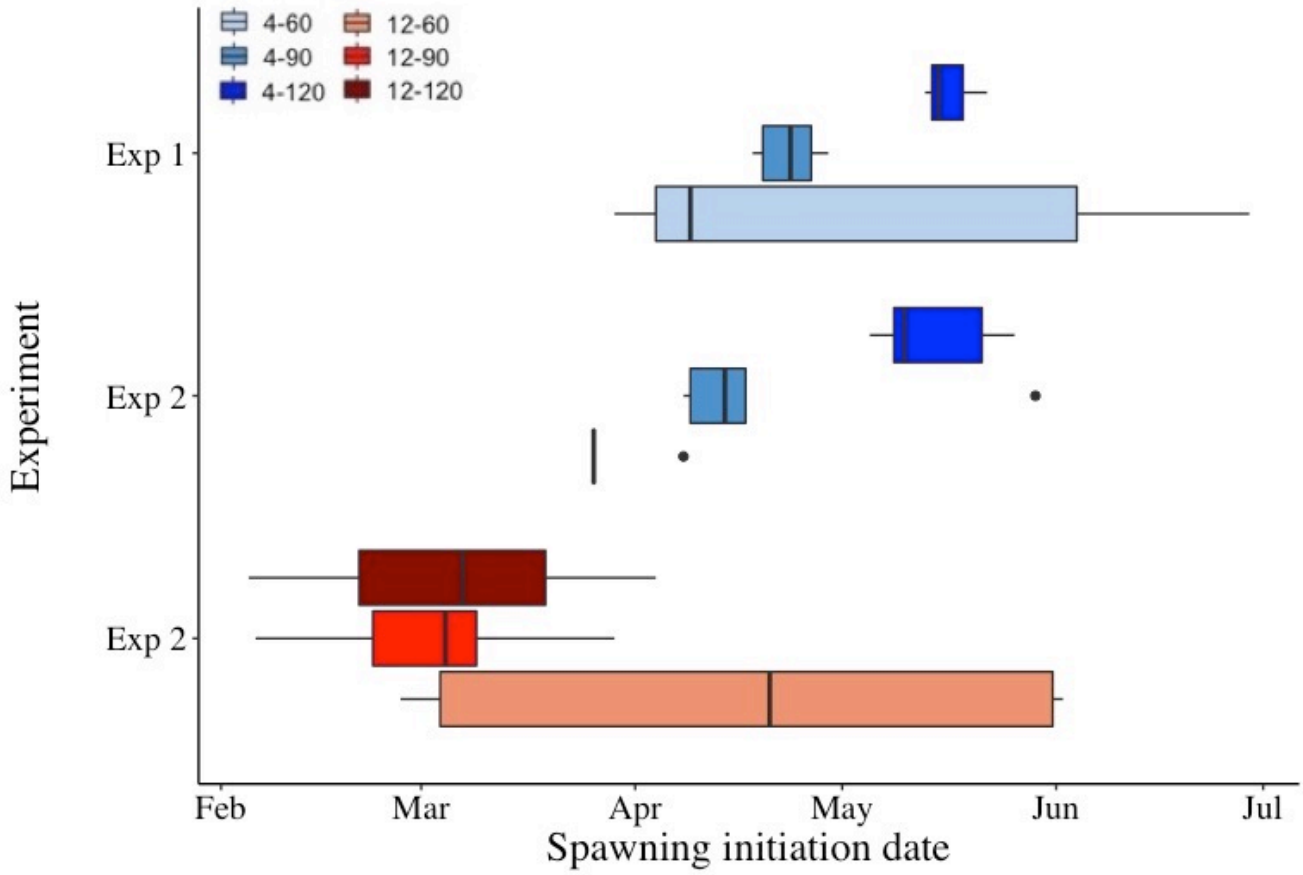

Figure 10. Box and whisker plot of spawning initiation date in each tank of Experiment 1 and 2. The color of the boxes refers to winter temperature treatment: blue are the 4ºC treatments and red are the 12ºC treatments. Shade of the color refers to the winter duration treatment: lightest shade is the 60-day winter duration and the darkest shade is the 120-day winter duration The vertical bars represent the median, the box is the interquartile range, and the horizontal bars are the minimum and maximum. Dots represent outliers.

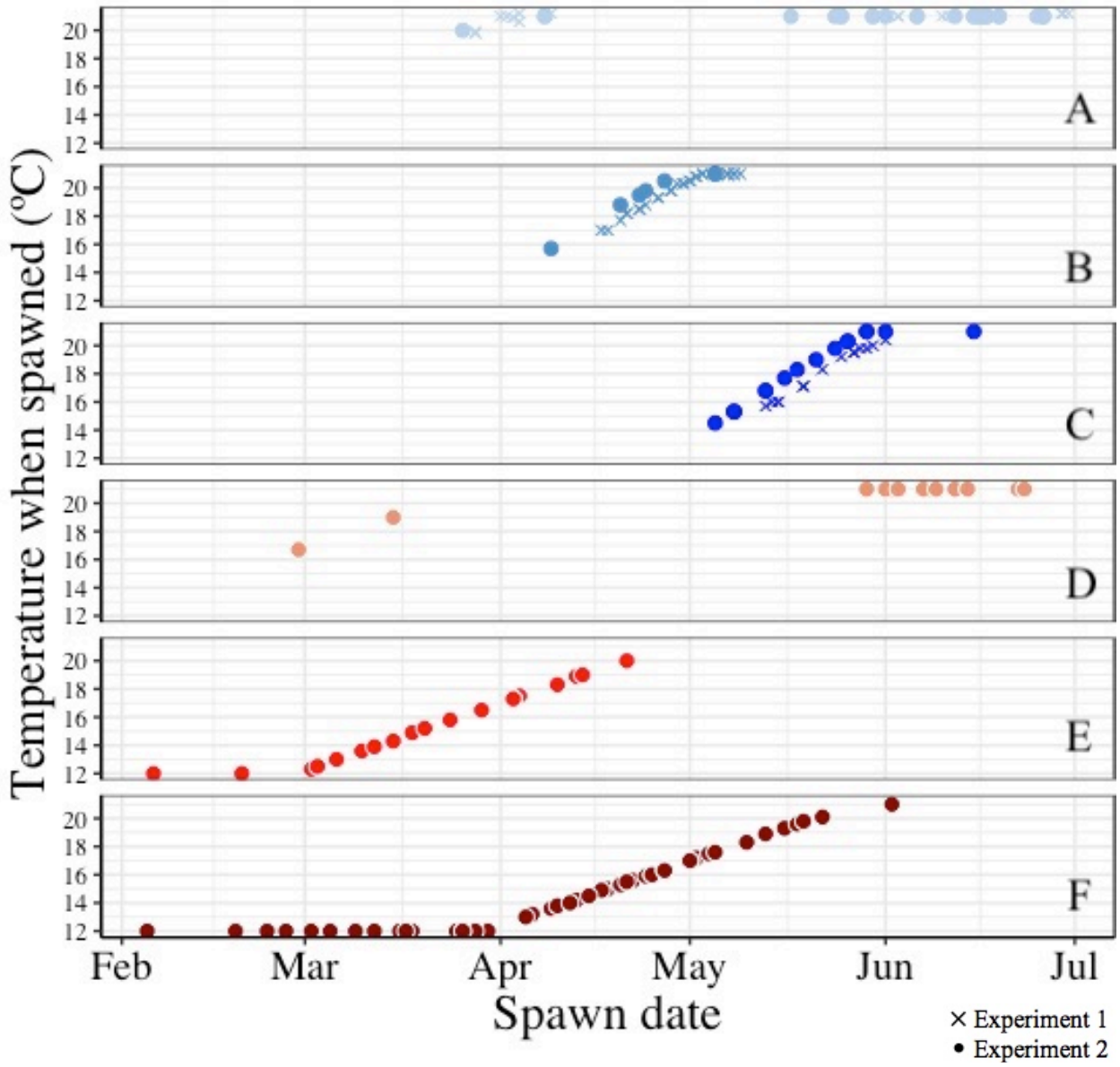

Figure 11. Faceted scatterplot of the spawning timing of all treatments in Experiment 1 and 2. Each data point represents a tile that had eggs. The color of the points refers to winter temperature treatment: blue are the 4ºC treatments and red are the 12ºC treatments. Shade of the color refers to the winter duration treatment: lightest shade is the 60-day winter duration and the darkest shade is the 120-day winter duration. A) treatment 4_{60} of Experiment 1 and 2; B) treatment 4_{90} of Experiment 1 and 2; C) treatment 4_{120} of Experiment 1 and 2; D) treatment 12_{60} of Experiment 2; E) treatment 12_{90} of Experiment 2; and F) treatment 12_{120} of Experiment 2. All data from both experiments are shown: Experiment 1 treatments are represented by the X's and are from the 2019 spawning season, whereas Experiment 2 treatments are represented by the circles and are from the 2020 spawning season.

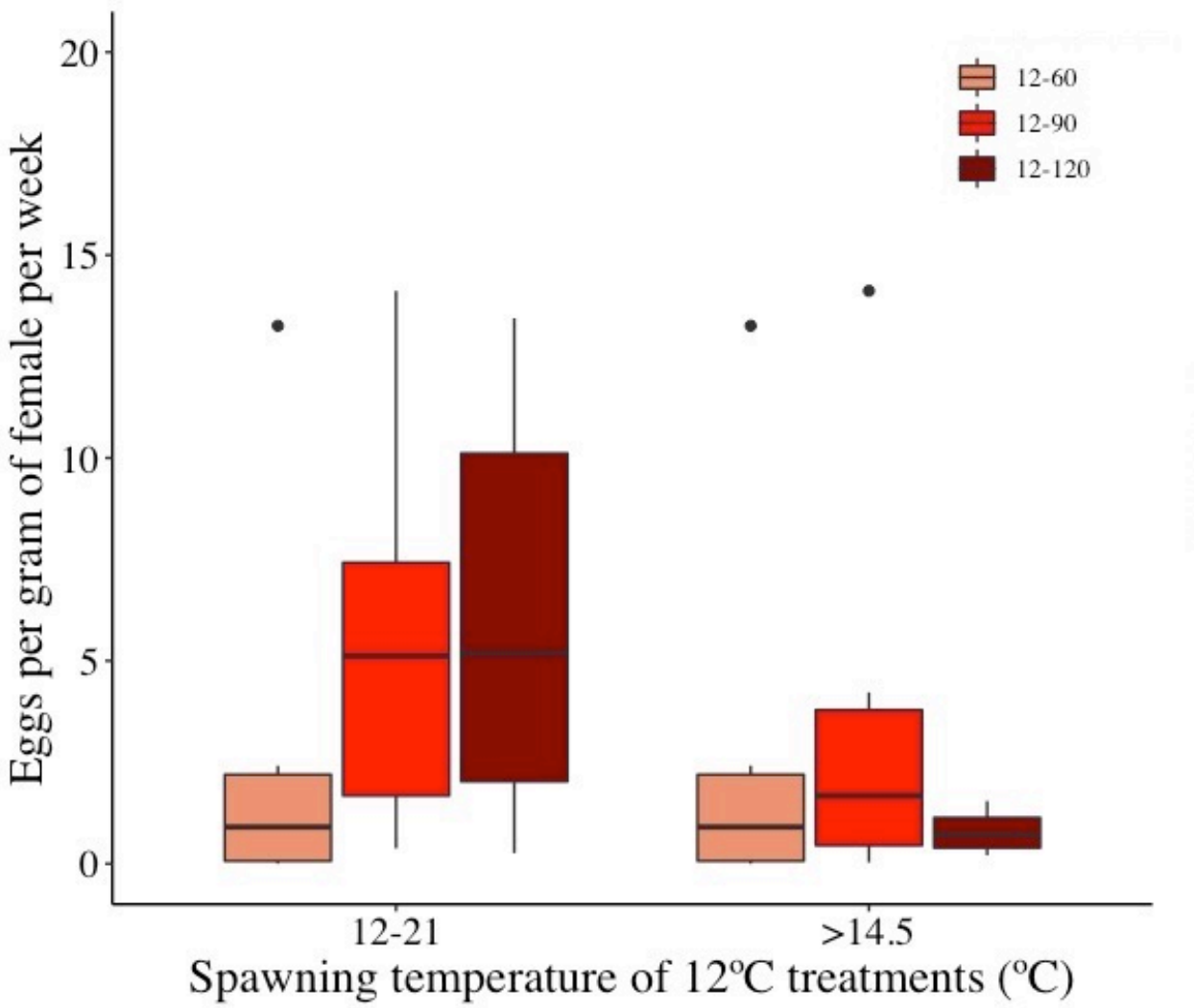

Figure 12. Box and whisker plot comparing eggs per gram of female per week of the 12ºC treatments of Experiment 2, omitting eggs that were spawned below 14.5ºC. The three boxes grouped on the left represent the number of eggs per gram of female per week laid at all temperatures (12-21ºC) in the three 12ºC treatments. The three boxes grouped on the right represent the number of eggs per gram of female per week laid at temperatures above 14.5ºC in the three 12ºC treatments. The horizontal bars represent the median, the box is the interquartile range, and the vertical bars are the minimum and maximum. Dots represent outliers.

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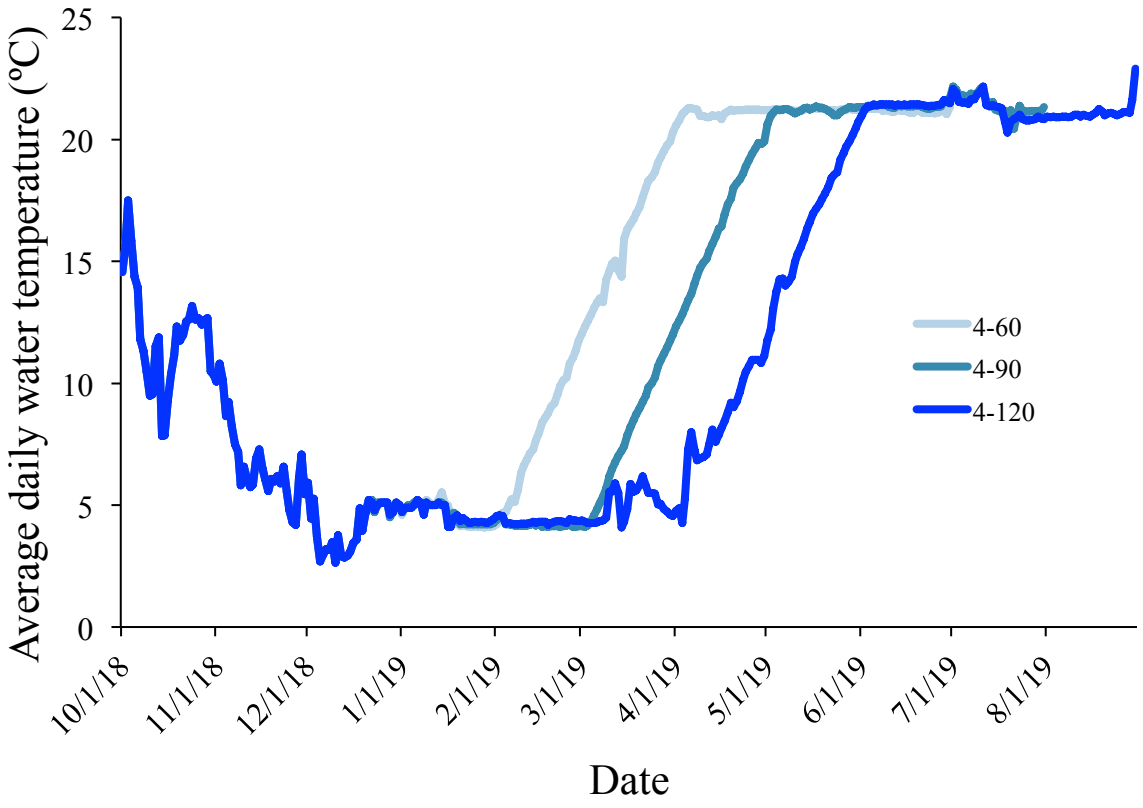
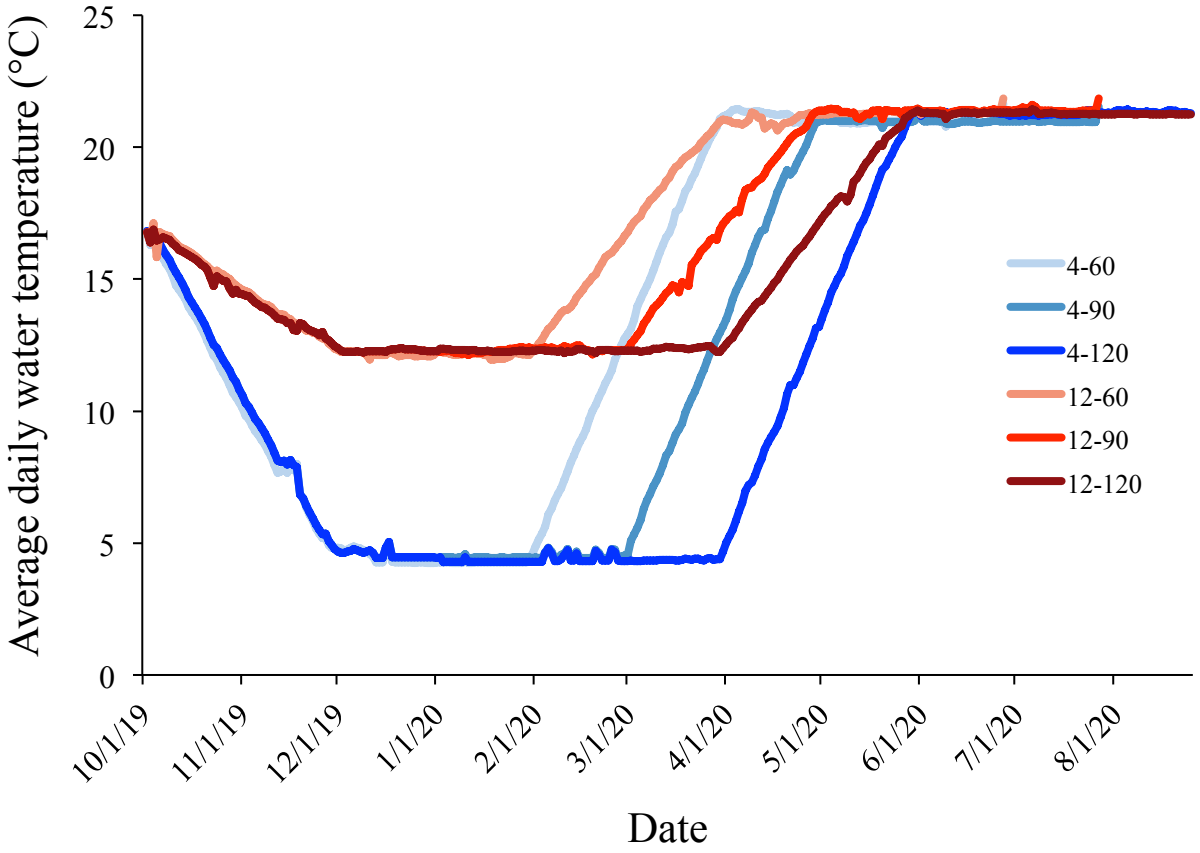
APPENDIX



Appendix 1-1. Water temperature data of Experiment 1 from collection stream site (October 1st, 2018 – December 19th, 2018) and laboratory treatment head tanks (December 20th, 2018 – August 30th, 2019).



Appendix 1-2. Water temperature data of Experiment 2 from laboratory treatment head tanks from October 1st, 2019 – August 26th, 2020.