Assessment of the Effects of Thiamine Deficiency on the Survival, Physiology, and Behavior of Early Life-Stage Winter-Run Chinook Salmon

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Assessment of the Effects of Thiamine Deficiency on the Survival, Physiology, and Behavior of Early Life-Stage Winter-Run Chinook Salmon

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Abstract

In late 2019, at multiple hatcheries in California's Central Valley (CCV), offspring of recently spawned fall-run Chinook salmon (*Oncorhynchus tshawytscha*) exhibited abnormalities in swimming, lethargy, and high early life-stage mortality; the combination of these symptoms is commonly referred to as thiamine deficiency complex (TDC). The cause of thiamine deficiency in Pacific salmonids is hypothesized to be due to a lack of diet heterogeneity and reliance on northern anchovies (*Engraulis mordax*), containing high levels of thiaminase, a thiamine-cleaving enzyme. Of CCV's four runs, the endangered Sacramento River winter-run Chinook salmon (WRCS) is perhaps the most vulnerable to thiamine deficiency as any additional stressor will exacerbate current threats to survival. The goal of this study was to evaluate the effect of thiamine supplementation in pre-spawn WRCS females on their offspring egg thiamine concentrations, survival, prevalence of TDC-related symptoms, and physiological performance and behavioral traits. Sixty female WRCS at Livingston Stone National Fish Hatchery were randomly administered either a thiamine injection (n=33; 500 mg/ml thiamine hydrochloride) at a dose of 50 mg/kg body weight or a sham injection (n=27; sterile saline solution) at a volume of 0.127 ml/kg body weight. After spawn, a subset of fertilized eggs from each female were analyzed for thiamine concentration. Eyed embryos were transferred to the University of California, Davis and kept for observations of TDC, survival, and to assess the effects of maternal thiamine treatments on offspring physiology and behavior. Upper thermal tolerance, routine metabolic rate, spontaneous swimming activity, boldness, and anti-predator responses were evaluated in swim-up fry. On average, total egg thiamine concentrations were 5.02 and 34.91 nmol/g for untreated (n=27) and thiamine treated (n=33) females, respectively. Mortality rate of offspring from untreated females averaged 32.97 ± 0.33 %, with rates ranging from 0 to 100%, while offspring from thiamine treated females had a mean mortality rate of less than 1%. Using a binomial logistic regression model, we predicted that a mean total egg thiamine concentration of ~5 nmol/g supports 95% survival within a family (EC95). There were no statistically significant effects of maternal treatment on any of the physiological or behavioral metrics assessed in this study. Overall, we recommend the administration of supplemental thiamine to increase thiamine concentrations past the threshold at which TDC symptoms are commonly observed; however, additional research should be conducted to further examine potential latent effects of thiamine deficiency in Pacific salmonids.

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Introduction

Chinook salmon (*Oncorhynchus tshawytscha*) are an ecologically, culturally, and commercially important species whose populations have declined due to a variety of anthropogenic factors such as habitat loss caused by mining, logging, dams, and water-diversions (Yoshiyama et al., 1998; Gresh et al., 2000), in addition to shifts in environmental conditions due to climate change (Crozier et al., 2008; Breitburg et al., 2018). As a result of growing threats to the species, two evolutionarily significant units (ESUs) have been listed as endangered and seven ESUs have been listed as threatened under the ESA as of 2017 (50 C.F.R. 17.11). Salmonid conservation efforts focused on the aforementioned challenges may be confounded by lesser known and inconspicuous threats such as deficiencies in nutrients, for example the water-soluble vitamin thiamine (B1). Thiamine diphosphate, or thiamine pyrophosphate (TPP), is the physiologically active form of thiamine and serves as a cofactor for enzymes required for carbohydrate and amino acid metabolism (Witt, 1985; Brown et al., 1998; Martin et al., 2003; Bettendorf et al., 2014); thus, a lack of thiamine may directly impact metabolism and have important consequences for organism fitness.

In fish, thiamine deficiency often presents as abnormalities in swimming (i.e. corkscrew swimming, losses of equilibrium, ataxia), lethargy, and high early life-stage mortality; the combination of these symptoms is commonly referred to as thiamine deficiency complex (TDC; Riley and Evans, 2008; Harder et al., 2018). The first documentation of thiamine deficiency in fish was made in 1968 after observations of high offspring mortality of salmonids (coho salmon [*O. kisutch*], Chinook salmon, steelhead [*O. mykiss*], brown trout [*Salmo trutta*], and lake trout [*Salvelinus namaycush*]) of the Great Lakes (Marcquenski and Brown, 1997; Honeyfield et al., 2005b). Additional observations of high mortality and behaviors indicative of thiamine deficiency have been observed in Atlantic salmon (*S. salar*) fry at Baltic Sea hatcheries since 1974 (Amcoff et al., 1999) and at the Finger Lakes in New York (Fisher et al., 1995). Thiamine deficiency has also been reported in adult Atlantic salmon returning to Baltic Sea hatcheries since the early 1990's (Amcoff et al., 1999).

The cause of thiamine deficiency in Great Lakes salmonids has been hypothesized to be due to a lack of diet heterogeneity and reliance on forage fish, such as alewife (*Alosa pseudoharengus*) (Fitzsimons, 1995, Brown et al., 2005a, Riley et al., 2011), containing high levels of a thiaminase, a thiamine-cleaving enzyme, (O'Gorman and Stewart, 1999; Wistbacka et al., 2002). Thiaminase has been linked with the onset of thiamine deficiency in adult salmonids (Amcoff et al., 1999; Brown et al., 2005a) and TDC in swim-up fry from thiamine deficient parents (Fitzsimons, 1995; Tillet et al., 2005; Honeyfield et al., 2005a). Chinook salmon are anadromous (reaching maturity in the ocean and spawning in freshwater), semelparous (reproducing once in their lifetime), and many adults return to their natal spawning grounds or hatcheries to reproduce. Successful operation of both production and conservation Chinook salmon hatcheries requires a pool of genetically fit and physiologically healthy individuals, thus, thiamine deficiency could pose risks to population recruitment.

Prevention of thiamine deficiency in returning adult salmonids may not be possible if the deficiency's cause is truly diet-linked. The nutritional state of a female salmon determines the amount of thiamine she can allocate to her eggs prior to spawning (Harder et al., 2018), thus, individual variation in female condition results in inter-clutch variation in thiamine concentrations. Modern methods of thiamine supplementation include the immersion of eggs and fry in a 1% thiamine hydrochloride (HCl) bath (Fisher et al., 1996; Wooster et al., 2000) and the injection of pre-spawn females with thiamine (Koski et al., 1999; Ketola et al., 2000; Fitzsimons et al., 2005; Futia et al., 2017). Koski et al. (1999) treated Baltic salmon females with intraperitoneal injections of thiamine HCl (ca 100 mg/kg) resulting in eggs with a mean total thiamine (TTH) concentration of 4.83 (range: 3.63 - 5.85) nmol/g; whereas eggs of untreated females averaged 0.23 (range: 0.18 - 0.37) nmol/g. Similarly, mature female steelhead trout injected with 50 mg/kg of thiamine mononitrate prior to spawn produced eggs with mean TTH of 20.4 ± 11.3 nmol/g while untreated females' eggs averaged 3.4 ± 0.6 nmol/g (Futia et al., 2017). Egg immersions have previously demonstrated the prevention of high offspring mortality (Fitzsimons et al., 2005) and fry immersions have physiologically rescued fish exhibiting TDC, reversing symptoms and decreasing mortality rates of early life-stages (Fitzsimons et al., 2001; Lee et al., 2009; Futia et al., 2017), however, egg and fry thiamine supplementation may not prevent latent effects of thiamine deficiency, including immune dysfunction (Ottinger et al., 2012; Ottinger et al., 2014; Futia et al., 2017). Thus, the most efficient way to prevent the manifestation of symptoms and latent effects of TDC could be to treat returning adults through thiamine supplementation prior to spawn.

CCV Chinook salmon are classified into four seasonal runs (fall, late-fall, winter, and spring-run), characterized by the time of year when they enter freshwater to begin their upstream spawning migration. In late 2019, abnormal swimming behavior and high early life-stage mortality were observed in the offspring of recently spawned fall-run Chinook salmon at multiple hatcheries within CCV. This was hypothesized to be due to TDC, and subsequent treatment of the fall-run Chinook salmon fry by thiamine bath restored the afflicted fish to a healthy condition with no obvious ailments (personal communication, Scott Foott, California-Nevada Fish Health Center). This was the first documented observation of TDC in Pacific salmonids on the West Coast of the United States (personal communication, Rachel Johnson, NOAA). The cause of thiamine deficiency in these Pacific coast salmonids is currently unknown, but is hypothesized to be due to a reliance on northern anchovies (*Engraulis mordax*) similar to what has been seen with alewife in the Great Lakes.

Of CCV's four runs of Chinook salmon, Sacramento River winter-run Chinook salmon (WRCS) are perhaps the most vulnerable to thiamine deficiency. This endangered run currently faces many challenges including 100% loss of historical spawning habitat (Yoshiyama et al., 1998; Bellido-Leiva et al., 2021), temperatures too warm for successful embryo development (Martin et al., 2017; Bellido-Leiva et al., 2021), and predation during early life-stages and outmigration (Lindley and Mohr, 2003; Sabal et al., 2016). Additional stressors can further complicate the battle for conservation of this run. As ectotherms, salmonids experience an increase in metabolic rate (Harbicht et al., 2018; Del Rio et al., 2019) and accelerated development (Cech and Myrick, 1999; Zillig et al., 2021) at increased acclimation temperatures. When developing from alevin to fry, the yolk-sac, containing a finite amount of nutrients, including thiamine, sustains a salmon until the digestive tract is fully developed and fish can begin exogenous feeding. Yolk utilization supports the metabolic costs associated with growth, tissue differentiation, maintenance, and activity; as fish grow and maintenance costs increase, the efficiency of yolk utilization decreases and may become negative nearing complete yolk absorption (Heming and Buddington, 1988). TPP is necessary for the production of adenosine triphosphate (ATP; Jankowska-Kulawy et al., 2010; Dhir et al., 2019), thus, limited thiamine may interfere with energy production and impact physiological performance directly through changes in metabolism or indirectly through changes in upper thermal tolerance. The physiological effects of thiamine deficiency may also contribute to abnormalities in early life-stage salmon behavior as thiamine deficient alevin and fry often elicit abnormal behaviors, such as lethargy and corkscrew swimming, which may decrease foraging success and increase risk of predation.

The goal of this study was to evaluate the effect of thiamine treatment in pre-spawn WRCS females on offspring thiamine concentrations and TDC-related survival. We also aimed to identify potential latent effects of thiamine deficiency on physiological performance and behavioral traits of early life-stage WRCS, prior to the manifestation of TDC. We measured WRCS swim-up fry upper thermal tolerance, basal metabolism, spontaneous swimming activity, boldness, and anti-predator responses. We predicted that i) offspring of thiamine treated females would have greater mean TTH concentration and higher survival than offspring of untreated females, ii) offspring of untreated females would show reductions in CTMax and RMR when compared to offspring of thiamine treated females, as a lack of bioavailable thiamine would decrease carbohydrate metabolism and production of ATP (Jankowska-Kulawy et al., 2010; Dhir et al., 2019), and iii) offspring of untreated females will be less likely to explore a novel environment (i.e. less bold) and more vulnerable to predation due to impaired movement and compromised anti-predator responses (Fitzsimons et al., 2009; Ivan et al., 2018).

Methods

Fish Acquisition and Care

Adult WRCS were collected via a fyke weir fish trap at the terminus of the fish ladder at Keswick Dam (Shasta County, CA, USA) on the Sacramento River and transported by truck to Livingston Stone National Fish Hatchery (US Fish and Wildlife Service, Shasta Lake, CA, USA) between December 2019 and June 2020. Upon arrival at the hatchery, salmon were phenotypically identified as winter-run Chinook salmon, fitted with two concurrently numbered floy tags, and genotyped to confirm run and sex identification. Once confirmed, female WRCS were anesthetized with buffered tricaine methanesulfonate (15 mg/L bath, MS-222, Syndel, Ferndale, WA, USA) and prophylactically treated with an intramuscular injection of tulathromycin (5 mg/kg, Draxxin, Zoetis, Parsippany, NJ, USA) for control of *Renibacterium salmoninarum* infection. To test effects of thiamine supplementation, 60 female WRCS were randomly given either a thiamine injection (n=33) or a sham injection (n=27). Thiamine treated females received an intraperitoneal injection of a buffered, sterile thiamine HCl solution (500 mg/ml thiamine HCl, Vet One, Sparhawk Laboratories, Inc. Lenexa, KS 66215. Distributed by MWI, Boise, ID 83705. www.vetone.net) at a dose of 50 mg/kg body weight. Draxxin and Thiamine HCl were administered via extra-label use prescription from Dr. Kevin Terra, DVM, Cottonwood Veterinary Clinic, Cottonwood, CA 96022. Sham treated females received a sham injection of a sterile saline solution at the volume of 0.127 ml/kg body weight. Beginning in May 2020, the maturation status of females was monitored weekly and ripe females were spawned and eggs were incubated in heath trays. A subset of fertilized eggs from each female were sent to Dr. Jacques Rinchard’s lab at The State University of New York, Brockport, USA for thiamine analysis. Upon reaching the eyed stage, 200 eggs from each female were counted by hand and transferred to the University of California, Davis (UC Davis). From this point onward, offspring of thiamine-injected females are referred to as “thiamine treated” and offspring of sham-injected females are referred to as “untreated.”

Upon arrival at the laboratory (June 25 to September 10, 2020), embryos were transferred to a flow-through tank (10.0 ± 1.0°C) specific to each female. Multiple tanks shared a temperature controlled water bath (10.0 ± 1.0°C). Embryos within a tank were kept separated in a mesh tray lined with plastic dividers. Once all eggs had hatched the trays were removed. To ensure that no dietary thiamine was introduced, fish were not fed prior to or during physiology or behavior assays. Fish instead relied on their endogenous yolk-sac reserves for nutrition (Kamler, 2008). After the completion of the assays, when fish had fully absorbed their yolk-sacs, fish were fed pelleted salmon food (3/64” semi-moist feed, Rangen, Inc., Buhl, Idaho, USA). This research project was conducted in accordance with animal welfare guidelines approved by the University of California Davis Institutional Animal Care and Use Committee (IACUC Protocol #22083).

Observations of TDC and Survival

Water temperatures were measured daily using a handheld digital thermometer (Model HH81A, Omega Engineering Inc., Norwalk, CT, USA) and mortalities were counted and removed. Observations of behaviors linked with TDC (e.g. corkscrew-like swimming, lethargy exhibited as low ventilation rates and resting on lateral sides unless prodded) were recorded.

Physiology and Behavior Assays

The physiological and behavioral assays were conducted at the swim-up developmental stage (mean age: 81 days post fertilization (dpf), range: 77 - 86 dpf), prior to the complete absorption of the yolk-sac, to best observe the potential latent effects of thiamine deficiency prior to signs of TDC. Our prediction was that there would be observable effects to physiological performance and behavior before the manifestation of severe symptoms of thiamine deficiency reflected in TDC.

Physiology Assays

Upper Thermal Tolerance

Acute upper thermal tolerance was estimated as the critical thermal maximum using methodology described by Becker and Genoway (1979). Twelve swim-up fry (mean age: 81 dpf, range 77 - 86 dpf) from each WRCS female (n=60) were individually placed into 185 ml chambers, contained in a water bath (10.0 ± 2.0°C) for a 30 min acclimation period. An airstone was placed in each chamber to ensure full oxygenation and mixing of water. After acclimation, the bath was heated by a titanium heater (TH-800, Finnex Aquarium Products, Countryside, IL, USA) at a rate of 0.3°C per min (Becker and Genoway, 1979). Water temperature was measured using a digital thermometer (HH81A, Omega Engineering Inc., Norwalk, CT, USA) every 5 min to ensure the proper heating rate was being achieved. The CTMax was recorded as the temperature at the observation of a loss of equilibrium (LOE), defined as the point at which a fish could no longer right itself and was unresponsive to a physical stimulus (Del Rio et al. 2019). At LOE, fish were removed from their chambers and placed in floating recovery baskets at their rearing temperature (10.0 ± 1.0°C) for 24 h. After recovery, fish were euthanized in tricaine methanesulfonate (MS-222, 0.5 g/L MS-222 buffered with 0.42 g/L sodium bicarbonate and 6 g/L salt) and weighed to the nearest 0.001 g, and measured for total length to the nearest 0.1 mm. CTMax values from fish that did not survive the 24-h recovery period were omitted from the analysis.

Routine Metabolic Rate

An intermittent-flow respirometry system, as outlined by Svendsen et al. (2015), was used to measure the routine oxygen consumption (MO_2) of swim-up fry (mean age: 81 dpf, range: 77 - 86 dpf). Fish (n=8) from each WRCS female (n=60) were individually placed in one of eight cylindrical 13 ml static respirometry chambers submerged in a water bath (10.0 ± 2.0°C). Each chamber was connected to two peristaltic pumps (Model BT100-1L, Longer Precision Pump Co., Ltd., Hebei, China). The first pump remained on throughout the trial to create a closed recirculating system and the second pump was activated periodically to replenish the chambers with oxygenated water from the water bath. An oxygen sensor spot (5 mm, Loligo Systems, Viborg, Denmark) was affixed to each chamber and a corresponding fiber optic probe (Loligo Systems) outside the chamber was connected to a four-channel oxygen instrument (Witrox-4, Loligo Systems) to measure O_2 every second. Information was transmitted to a data acquisition instrument (DAQ-M, Loligo Systems) and visualized in AutoResp (version 2.3.0, Loligo Systems). During the trials, each pump cycle consisted of a 300 s flush period (where both the recirculating and flush pumps were active), a 300 s wait period (where the flush pump was turned off), and a 1200 s measurement period (where only the recirculating pump was active). Once fish were placed in the chambers, O_2 was measured for six consecutive measurement periods (total duration) and upon completion of the trial, fish were euthanized in tricaine methanesulfonate (Syndel), weighed, and measured for total length (as stated above).

Oxygen consumption rates (MO_2) were calculated using the following equation (Schurmann and Steffensen, 1997):

$$MO_2 = s\beta O_2 V_{resp} M^{-1}$$

Where s is the slope of the linear decrease in percentage of O_2 of the closed chamber, βO_2 is the oxygen solubility at a given temperature, salinity, and atmospheric pressure, V_{resp} is the effective respirometer volume (total respirometer volume - fish volume), and M^{-1} is the mass of the fish (in kilograms).

The first two measurement periods were removed from analysis as oxygen consumption rates may have been influenced by the transfer of fish to the chamber (Del Rio et al., 2021). After daily experimental trials were completed (2 trials/day), a control trial was run without fish. Background respiration was estimated by subtracting the control trial's O_2 readings from the experimental trial's O_2 readings. The routine metabolic rate (RMR) for each trial was calculated by averaging the slopes of the lowest three oxygen consumption rates (MO_2) from the remaining measurement periods (n=4) and corrected for background respiration. Slopes were only used if r^2 was above 0.90.

Behavior Assays

Boldness and Spontaneous Activity

Boldness and spontaneous activity were assessed using modified methodology from Singer et al. (2019). Twelve swim-up fry (mean age: 81 dpf, range 77 - 86 dpf) from each WRCS female (n=60) were tested individually. Individual fish were randomly assigned and placed in the acclimation chamber (AC; Figure 1) in one of 12 replicate behavior arenas. A cover was placed over the AC and fish were allowed to acclimate for 15 minutes. At the start of the trial, the gate (G) was opened, allowing fish to swim into the main chamber (MC) of the arena of their own volition. The trial ended after 15 minutes and fish were euthanized, weighed, and measured for total length (as stated above).

Anti-Predator Response

Methods modified from Davis et al. (2018) were used to assess the behavioral responses of twelve swim-up fry (mean age: 81 dpf, range: 77 - 86 dpf) from each WRCS female (n=60) to a conspecific alarm cue. The conspecific alarm cue was prepared by rinsing 120 ml of water (10.0 ± 1.0°C) over the scored skin of a sacrificed, non-experimental Chinook salmon. The cue was separated into twelve 10 ml syringes. Individual fish were placed in the MC of each arena (Figure 1); the gate remained in place and the AC was not used in this experiment (Figure 1). Fish were allowed to acclimate in the MC for 15 minutes. At the start of the trial, a control cue of 20 ml of control water (10.0 ± 1.0°C) was injected into tubing affixed to the inside of the MC of the arena. After 10 minutes, 10 ml of a conspecific alarm cue was injected into the tubing followed by 10 ml of control water to clear the tube of any remaining cue; after an additional 10 minutes the trial ended and fish were euthanized, weighed, and measured for total length (as stated above).

Behavioral Analyses

The boldness, spontaneous activity, and anti-predator behavior assays were recorded by a video camera (Campark V30 Native 4k, Campark Electronics Co. Ltd, Shenzhen, Guangdong, China) mounted above each arena. Videos were analyzed using EthoVision XT (v.14, Noldus Information Technology, Wageningen, Netherlands) and total distance traveled, mean spontaneous swimming velocity, and percentage of time spent in the main chamber were calculated for each fish during the boldness assay. Anti-predator videos were analyzed for total distance traveled and mean spontaneous swimming velocity for each individual after both the control and predator cue injection.

Statistical Analysis

Statistical analyses were performed in R (version 4.0.2, R Development Core Team, 2020). Datasets were evaluated for assumptions of heteroskedasticity and normality of residuals. Using the “lme4” and “lmerTest” analysis packages and a stepwise model selection, we identified the best fitting linear mixed effects model (lowest AIC) for each response variable (CTMax, RMR, total distance traveled [boldness and anti-predator assays], spontaneous swimming velocity [boldness and anti-predator assays], and percentage of time spent in the main chamber of the arena [boldness assay only]). Thiamine treatment (i.e., thiamine treated and untreated) and fish mass were treated as fixed variables and WRCS female (i.e., mother) was included as a random effect in all models. Morphometrics, CTMax, MO_2, and behavior metrics are presented as means ± SD, with significance defined as $\alpha<0.05$. Experimental AutoResp files were analyzed in R (version 4.0.2, R Development Core Team, 2020).

Results

Observations of TDC and Survival

Thiamine supplementation of pre-spawn females resulted in eggs with a mean TTH concentration of 34.90 ± 7.34 nmol/g (range: 24.10 - 54.60 nmol/g, Table 2), while eggs from saline-injected, untreated females had a mean TTH concentration of 5.03 ± 4.30 nmol/g (range: 2.40 - 25.10 nmol/g, Table 1). From the eyed embryo stage to hatch and prior to the development of TDC at the swim-up fry stage, mortality was less than 0.1 % for both thiamine treated and untreated families. At the earliest age of 84 dpf and the latest age of 117 dpf, fish in untreated families began to exhibit symptoms of TDC, including lethargy and corkscrew swimming, while no thiamine treated families exhibited these symptoms. TDC-related mortality in untreated families (n=27) averaged 32.97 ± 0.33 %, with mortality ranging from 0 to 100 percent, while thiamine treated families (n=33) had a mean TDC-related mortality of less than 1 percent. The survival model (Figure 2) predicted that mean total egg thiamine concentrations of ~5 nmol/g or greater should support 95% survival within a family at this early life-stage. Forty-three percent of untreated families, all but one (5.3 nmol/g) with thiamine levels below the 5 nmol/g threshold, showed symptoms of TDC by 117 dpf.

Physiology and Behavior Assays

We predicted that thiamine deficient swim-up fry (mean age: 81 dpf, range: 77 - 86 dpf) would demonstrate observable effects to physiological performance and behavior prior to the physical manifestation of TDC, if in fact any of the effects of thiamine deficiency are mediated through metabolic mechanisms as hypothesized. No fish in the following assays exhibited TDC symptoms.

<u>Physiology Assays</u>

Upper Thermal Tolerance

There was no statistically significant effect of thiamine treatment (β=0.07, SE=0.11, df=56.81, p=0.54) on CTMax; however, there was a significant effect of mass (β=2.75, SE=0.89, df=466.85, p<0.05) on CTMax. Thiamine treated and untreated groups had mean CTMaxes of 27.3 ± 0.07°C and 27.5 ± 0.07°C, respectively. Mean fish masses were 0.26 ± 0.04 g (thiamine treated) and 0.25 ± 0.04 g (untreated). Fish that did not survive the 24 h recovery period were omitted from the final data set.

Routine Metabolic Rate

Oxygen consumption rates (Figure 4) of swim-up fry were not significantly affected by thiamine treatment (β=0.17, SE=0.38, df=39.89, p=0.66) or fish mass (β=-4.23, SE=5.01, df=150.34, p=0.40). Both treatment groups had a mean mass of 0.26 ± 0.03 g. On average, individuals in the thiamine treated group (n=117) had an RMR of 3.80 ± 2.04 mg O_2 kg^{-1} min^{-1} and those in the untreated group (n=91) had an RMR of 3.79 ± 2.05 mg O_2 kg^{-1} min^{-1}) (Figure 4).

Behavior Assays

Both spontaneous swimming activity and anti-predator behaviors were assessed through measures of total distance traveled and mean spontaneous swimming velocity (i.e. speed); in addition, boldness was also estimated by analyzing the percentage of time fish were present in the main chamber of the arena. For the two behavior assays, both treatment groups had a mean mass of 0.26 ± 0.03 g.

Boldness and Spontaneous Activity

Total distance traveled (Figure 5) was significantly affected by mass (β=1880.69, SE=888.14, df=495.61, p<0.05), but was not affected by thiamine treatment (β=79.94, SE=82.26, df=63.20, p=0.33). On average, the thiamine treated group (n=356) traveled 680.77 ± 563.47 cm and the untreated group (n=266) traveled 793.65 ± 592.47 cm. Mean spontaneous swimming velocity (Figure 6) was not significantly affected by treatment (β=0.16, SE=0.16, df=61.86, p=0.34); however, was significantly affected by mass (β=6.35, SE=1.93, df=416.29, p<0.01). The thiamine treated (n=336) and untreated (n=252) groups had average spontaneous swimming velocities of 2.34 ± 1.16 and 2.54 ± 1.33 cm/s, respectively. Percentage of time fish spent in the main chamber of the arena (Figure 7) was not significantly affected by treatment (β=3.02, SE=3.186, df=61.79, p=0.35) or mass (β=12.49, SE=41.57 df=354.88, p=0.76). Individuals in the thiamine treated group (n=358) were present in the main chamber, on average, 47.08 ± 27.51 percent of the time while individuals in the untreated group (n=268) were present 51.45 ± 26.89 percent of the time.

Anti-Predator Response

Treatment (β=30.65, SE=40.86, df=51.61, p=0.46) and mass (β=184.63, SE=607.61, df=187.56, p=0.76) had no significant effect on the difference in total distance traveled after exposure to a conspecific alarm cue (Figure 8). On average, thiamine treated (n=243) and untreated (n=228) individuals traveled 4.01 ± 363.65 and 31.74 ± 358.54 cm more after exposure to the cue, respectively. The change in mean spontaneous swimming velocity after exposure to the cue (Figure 9) was not significantly affected by treatment (β=0.15, SE=0.14, df=46.42, p=0.31) or mass (β=1.34, SE=2.20, df=151.92, p=0.55). The thiamine treated group (n=255) had a mean difference in spontaneous swimming velocities of 0.02 ± 1.39 cm/s while the untreated group (n=236) showed mean differences of 0.19 ± 1.28 cm/s.

Discussion

Thiamine Treatments & Thresholds

The present study supports the use of thiamine supplementation on pre-spawn thiamine deficient WRCS females to increase thiamine concentration in early-life stage offspring. Eggs of thiamine treated females had a 7-fold higher TTH concentration compared to untreated offspring. These results are consistent with previous studies assessing the use of thiamine injections to pre-spawn females where eggs of thiamine treated Baltic salmon and steelhead trout showed a 21 and 6-fold increase in TTH concentration over eggs of untreated females (Koski et al., 1999; Futia et al., 2017). In this study, TDC symptoms and related mortalities were observed in 43% of untreated groups of WRCS between 84 and 117 dpf, after the developmental swim-up stage; however, no thiamine treated groups exhibited symptoms of TDC. These results demonstrate the efficacy of thiamine treatments to mitigate negative impacts of TDC, similar to what has been observed by others (Fizsimons et al., 2005; Futia et al., 2017). Family survival and mean TTH concentration values were incorporated into a model (Figure 2), which predicted a mean total egg thiamine concentration threshold of 2.7 nmol/g to prevent 50% mortality (EC50) within a family; the predicted EC95 is 5.0 nmol/g. Studies on Great Lake salmonids evaluated EC50s of Chinook salmon, coho salmon, and lake trout as 1.03, 2.38, and 1.57 nmol/g, respectively (Brown et al., 2005a; Fitzsimons et al., 2007). Thiamine thresholds are likely species- and perhaps run-dependent, so threshold values may not be comparable between species. Additional experimentation should be conducted to determine the appropriate dose of thiamine that should be administered to pre-spawn adults, eggs, or alevin of salmonids, to alleviate the initial low egg thiamine levels and reduce prevalence of TDC.

Reaching the thiamine threshold to limit early life-stage mortality is important; however, there may be latent physiological or behavioral effects to individuals that survive initial TDC-related mortalities (Fitzsimons et al., 2009; Ottinger et al., 2012; Futia et al., 2017;

Ivan et al., 2018). Fifty percent reductions in foraging success and growth have been observed in larval lake trout with thiamine concentrations higher (2.9 & 5.1 nmol/g, respectively) than the proposed EC50 (1.57 nmol/g; Fitzsimons et al., 2009). Models incorporating these potential sublethal effects of TDC, including limited foraging success and increased incidences of predation, predict an EC50 of 7.4 - 10 nmol/g (Ivan et al., 2018). These findings suggest that latent, sublethal effects of thiamine deficiency may have significant impacts on physiology and behavior, influencing the survival and recruitment of salmonids (Fitzsimons et al., 2007). While our studies did not find evidence of any latent effects of thiamine deficiency, this could be because limited thiamine does not impact the physiology and behavior of early life-stage salmon in ways we predicted. Further experimentation should be conducted using different physiological and behavioral assays to capture any potential latent effects of thiamine deficiency.

Physiology Assays

Upper Thermal Tolerance

To date, there have been no studies of thermal tolerance of thiamine deficient fishes; thus, the present study is the first to demonstrate that thiamine supplementation does not affect CTMax. CTMax was measured prior to signs of TDC, thus, physiological performance of the fish may not have been thiamine-limited at this stage. There were no significant differences in CTMax between treatments and the untreated and thiamine treated groups had mean CTMaxes of 27.3 ± 0.07°C and 27.5 ± 0.07°C, respectively. Surprisingly, there have been few examinations of the upper thermal tolerance of CCV Chinook salmon (Myrick and Cech, 2004); however, Zillig et al. (2020) assessed the upper thermal tolerance of multiple Pacific Northwest

Chinook salmon populations. When fish (mean masses ranging from 17.21 to 26.44 g) were acclimated to 11.0°C, their CTMax values ranged from 27.9 to 28.1°C (Zillig et al., 2020). The differences in CTMax values between the present study (i.e. untreated: 27.3 ± 0.07°C and thiamine treated: 27.5 ± 0.07°C) and the study conducted by Zillig et al. (2020) are likely due to the differences in acclimation temperature, as increases in acclimation temperature is highly linearly correlated with increases in upper thermal tolerance (Beitinger et al., 2000). Another modified CTMax study used a slower heating rate (1.5°C/h vs. 0.3°C/min or 18°C/h) to replicate environmental temperatures seen in entrapment pools; it was determined that juvenile Snake River fall-run Chinook salmon (mean mass = 1.93 g) acclimated to 10°C lost equilibrium at approximately 26.8°C (Geist et al., 2010). At slower rates of ramping, fish may be able to acclimatize to the increasing temperature, which could lead to an overestimate of CTMax (Becker and Genoway, 1979).

Upper thermal tolerance measured by CTMax reflects a fish's capacity to tolerate an acute temperature change. It is possible that thiamine deficiency might impact physiological performance under more chronically elevated temperatures. In this study, fish acclimated to 10°C began exhibiting TDC symptoms at the tail end of the developmental transition from alevin to swim-up fry, perhaps signaling the yolk-sac's thiamine supply had been depleted. As salmonids reared at higher temperatures develop more quickly (Cech and Myrick, 1999; Zillig et al., 2018), the additional stressor of thiamine deficiency may cause juveniles to exhibit symptoms of TDC earlier with warm acclimation than individuals reared in colder environments. Further investigation of the interaction between temperature and TDC needs to be performed as climate change and increased river temperatures may further complicate the battle against thiamine deficiency in Pacific salmonid populations.

Routine Metabolic Rate

Routine metabolic rate describes the energy required for homeostatic maintenance plus random activity and is often measured indirectly through oxygen consumption (Schurmann and Steffensen, 1997). Previous mammalian studies have shown that experimentally-induced thiamine deficiency decreased oxygen consumption and increased blood lactate (Prasannan et al., 1976; Gioda et al., 2010); similarly, the administration of TPP prior to aerobic activity increased oxygen consumption and decreased serum lactate levels (Bautista-Hernandez et al., 2008). Taken together, we hypothesized that thiamine deficiency would impact energy metabolism of the offspring of untreated females. In contrast, however, routine metabolic rates (RMRs) were not significantly affected by maternal treatment. Offspring (mean mass: 0.26 ± 0.03 g) of untreated and thiamine treated females had RMRs of 3.79 ± 2.05 mg O_2 kg^{-1} min^{-1} and 3.80 ± 2.04 mg O_2 kg^{-1} min^{-1}, respectively. There have been few examinations of the RMR of juvenile Chinook salmon; however, the data presented here are consistent with the late-fall Chinook salmon fry (approximate mean mass: 0.28 g) RMR values reported by Del Rio et al. (2021). Zillig et al. (2020) also evaluated the RMRs of Sacramento River WRCS acclimated to 11.0°C at test temperatures ranging from 8 to 24°C; WRCS juveniles (mean mass: 21.6 ± 2.2 g) had an estimated RMR of 1.62 mg O_2 kg^{-1} min^{-1}(regression equation: RMR [mg O_2 kg^{-1} min^{-1}] = $0.00924x^2 - 0.07493x + 1.44794$ where x = temperature [°C]; Zillig et al., 2020). The mass-adjusted RMR values for swim-up fry in this study are comparatively greater than those estimated by Zillig et al. (2020). A higher metabolic rate is likely observed in younger fish, especially those actively absorbing their yolk-sac, as development, growth, and tissue-building are energetically expensive processes (Biro and Stamps, 2010). As the assessment of RMRs were performed prior to the observation of symptoms of TDC, it is possible that experiments were performed too early to capture any physiological differences between the treatments. Our findings showed no effect of maternal thiamine treatment on RMR, perhaps indicating that thiamine deficiency does not directly impact metabolism, warranting the need for additional studies investigating the metabolic role of thiamine in salmonids.

Behavior Assays

Boldness and Spontaneous Activity

The boldness-shyness axis describes the behavioral variation of individuals in a population to engage in high-risk behaviors. More bold individuals will be more likely to explore unfamiliar environments in search of food, mates, or desirable habitats, while more shy individuals will be less likely to take risks for rewards (Ward et al., 2004; White et al., 2013). In this study, boldness was assessed through measures of total distance traveled and the percentage of time fish were present in the main chamber of the arena. Though maternal treatment had no significant effect on the measured behaviors, on average, untreated fish traveled a greater distance and spent more time in the main chamber of the arena when compared to thiamine treated fish. In addition, spontaneous swimming activity was not significantly affected by maternal thiamine treatment, yet despite our predictions, untreated fish traveled at a greater mean velocity than thiamine treated fish. Behavioral studies in other San Francisco Estuary fishes have shown increases in environmental exploration and swimming speeds as a result of hypoxic conditions and elevated temperatures (Davis et al., 2019; Davis et al., 2018). Furthermore, Hansen et al. (2015) demonstrated that food-deprived mosquitofish (*Gambusia holbrooki*) traveled at greater mean speeds and explored more of an experimental arena than satiated fish. When compared to thiamine replete fish, thiamine deficient fry may have a greater propensity to explore novel environments in the search for food as they would benefit more from increased foraging opportunities, nutrient intake, and resulting growth (Mikheev et al., 1994). In contrast, if fry are unable to feed after yolk-sac absorption and TDC progresses through the development of symptoms, fish may be unable to effectively forage due to impaired movements. After the completion of experiments and during the observational period of TDC and survival, we fed the remaining fish of each family ad libitum. During this period, fish that began exhibiting symptoms of TDC, especially those which laid at the bottom of the tank, did not transition onto feed, became anorexic, and died.

Anti-Predator Response

Thiamine deficiency may impact anti-predator defenses including hiding, shoaling, and interindividual communication through chemical alarm systems. Berejikian et al. (1999) determined that Chinook salmon communicate through chemical alarm signaling after conducting a study in which juveniles increased time spent lower in the water column, increased time spent motionless, and reduced feeding after exposure to the combination of a conspecific tissue extract and a predator odor compared to the tissue extract of an unrelated species. In this study, to evaluate the anti-predator responses of swim-up fry, we calculated the difference in total distance traveled and mean swimming velocity before and after exposure to a conspecific alarm cue. Maternal treatment had no significant effect on these behaviors; however, untreated fish traveled further and swam more quickly than thiamine treated fish after exposure to the alarm cue. Previous studies have determined that after exposure to a conspecific alarm cue, juvenile salmon decrease activity and reduce foraging (Mirza and Chivers, 2001; Berejikian et al., 1999), and that this reduces detection by predators (Lima and Dill, 1990; Brown and Smith, 1997). In this study, both thiamine treated and untreated fish increased activity after exposure to the alarm cue. Using the framework set by Valentincic and Caprio (1994), who described alarm responses as biphasic, Volpato and Giaquinto (2001) observed reactions of juvenile pintado (*Pseudoplatystoma coruscans*) to a conspecific alarm cue and determined that panic responses were influenced by feeding condition; feed-deprived fish responded to the alarm cue only by dashing and not by freezing, the second of the biphasic panic responses. Thus, the increase in activity seen after exposure to the conspecific alarm cue in our study may have been modulated by the feeding condition of the swim-up fry; the fish tested neared complete yolk-sac absorption and were ready to begin exogenous feeding. Overall, thiamine deficient fish in the untreated group were more thiamine-limited than those of the thiamine treated group, which may explain why they dashed further and more quickly. Freezing is not necessarily an optimal response for food-deprived fish as there is a trade-off between predator avoidance and foraging opportunities (Ryer and Olla, 1998).

Fish exhibiting symptoms of TDC may be more susceptible to incidences of predation due to their impaired movements and potentially impacted neurological function. Fitzsimons et al. (2009) assessed the threat of predation by round goby (*Neogobius melanostomus*) on emergent lake trout fry and found a significant decline in fry emergence in the presence of a single round goby. Predation of fry was not correlated with the egg thiamine concentration; however, if thiamine deficient fry were actively exhibiting symptoms of TDC, such as lethargy, corkscrew swimming, and ataxia, chances of survival would likely decrease in the presence of a predator. In the CCV, Chinook salmon threats of predation are exacerbated by their small size and spatial overlap of critical habitat with predators (Rubenson, 2020); high losses of juveniles would likely have negative impacts on population recruitment (Fitzsimons et al., 2009).

Conclusions

Endangered Sacramento River winter-run Chinook salmon currently face challenges due to loss of historical spawning habitat (Yoshiyama et al., 1998; Bellido-Leiva et al., 2021), shifts in environmental conditions due to climate change (Crozier et al., 2008; Breitburg et al., 2018), and predation during early life-stages and outmigration (Lindley and Mohr, 2003; Sabal et al., 2016). Additional stressors impacting performance of this run could lead to drastic reductions in population abundance.

This study demonstrated the efficacy of thiamine injections to pre-spawn Chinook salmon females on the reduction of TDC symptoms and related mortalities observed in early life-stage offspring. Currently, we recommend the administration of supplemental thiamine to increase thiamine concentrations past the threshold at which TDC symptoms are commonly observed. There were limited latent effects on physiological and behavioral performance in thiamine deficient fish; however, the prevalence of TDC in offspring of thiamine deficient returning females demonstrates the severity of this issue and justifies the need for additional research to understand: i) the lack of diet heterogeneity of CCV Chinook salmon, ii) dose-dependent responses of thiamine supplementation, iii) interactions between thiamine and abiotic and biotic factors (e.g., temperature, dissolved oxygen, foraging success, predation, etc.), and iv) TDC-related pathological changes, as experimentally-induced thiamine deficiency has been shown to produce brain lesions (Krampitz and Woolley, 1944; Blank et al., 1975; Witt and Goldman-Rakic, 1983a,b) causing tremors, fatigue, lethargy, and ataxia (Swank and Prados, 1942; Rinehart et al., 1948; Dreyfus and Victor, 1961; Witt, 1985).

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Tables and Figures

Tables

Table 1: Mean egg thiamine pyrophosphate (TPP), thiamine monophosphate (TMP), free thiamine (TH) and their sum, total thiamine (TTH), for each untreated, saline-injected winter-run Chinook salmon female.

Female ID	Treatment	Spawn Date	TPP (nmol/g)	TMP (nmol/g)	TH (nmol/g)	TTH (nmol/g)	TTH (mean ± SD)
3	Untreated - Sham Injection	5/22/2020	2.8	0.5	2.1	5.4	5.03 ± 4.30
6		5/28/2020	2	0.8	22.2	25.1	
9		6/8/2020	2.2	0.6	2.8	5.6	
10		6/8/2020	1.4	0.5	1.1	3	
14		6/15/2020	1.8	0.5	2.2	4.5	
15		6/15/2020	3	1	6.7	10.6	
18		6/22/2020	2.1	0.5	0.8	3.4	
19		6/22/2020	1.6	0.6	1.5	3.8	
20		6/22/2020	2.1	0.5	1.5	4.1	
25		6/29/2020	1.6	0.6	1.1	3.4	
26		6/29/2020	2.5	0.8	2	5.3	
30		7/6/2020	2.2	1	1.5	4.7	
32		7/6/2020	2.4	0.7	1	4.1	
33		7/6/2020	1.1	0.4	1.2	2.7	
36		7/6/2020	1.6	0.7	1.2	3.5	
38		7/7/2020	1.7	0.6	1.3	3.6	
39		7/7/2020	1.9	0.6	2.7	5.2	
43		7/13/2020	2	0.5	1.8	4.3	
45		7/13/2020	2.3	0.4	1.3	4	
49		7/14/2020	1.3	0.4	1.4	3.1	
50		7/14/2020	2.2	0.5	2.2	4.9	
55		7/21/2020	2	0.6	2.7	5.3	

56		7/21/2020	1.4	0.4	1	2.8	
57		7/21/2020	1.9	0.4	1.2	3.5	
58		7/21/2020	2.2	0.5	1.1	3.7	
59		7/27/2020	1.5	0.3	0.6	2.4	
60		8/3/2020	1.7	0.4	1.7	3.9	

Table 2: Mean egg thiamine pyrophosphate (TPP), thiamine monophosphate (TMP), free thiamine (TH) and their sum, total thiamine (TTH), for each treated, thiamine-injected winter-run Chinook salmon female.

Female ID	Treatment	Spawn Date	TPP (nmol/g)	TMP (nmol/g)	TH (nmol/g)	TTH (nmol/g)	TTH (mean ± SD)
1	Treated - Thiamine Injection	5/22/2020	2.1	0.7	35.5	38.3	34.90 ± 7.34
2		5/22/2020	3	1	35.6	39.6	
4		5/26/2020	2.1	0.9	24.5	27.5	
5		5/26/2020	1.3	0.5	33.5	35.3	
7		6/1/2020	2.2	0.9	27.3	30.4	
8		6/8/2020	2.4	0.9	33	36.3	
11		6/15/2020	1.9	0.8	27.8	30.5	
12		6/15/2020	3.1	0.7	27.8	31.5	
13		6/15/2020	2.1	0.7	38.5	41.3	
16		6/16/2020	1.6	0.7	21.8	24.1	
17		6/22/2020	2.3	0.8	29	32.1	
21		6/23/2020	2	0.7	31.6	34.4	
22		6/23/2020	2.3	0.8	28	31.1	
23		6/23/2020	2.4	0.8	32.3	35.5	
24		6/29/2020	2.5	0.7	42.2	45.4	
27		6/30/2020	2.3	0.7	50.7	53.8	
28		6/30/2020	1.9	0.7	32.1	34.7	
29		7/6/2020	2.2	1.3	30.7	34.2	
31		7/6/2020	2.5	0.9	33.1	36.4	

34		7/6/2020	1.8	0.7	28.2	30.7	
35		7/6/2020	1.6	0.9	30.9	33.4	
37		7/6/2020	2.1	0.6	30.2	32.9	
40		7/7/2020	2.1	1	37	40	
41		7/7/2020	2.1	0.8	26.6	29.5	
42		7/7/2020	1.6	0.7	23.9	26.2	
44		7/13/2020	1.5	0.5	25	27.1	
46		7/13/2020	2.1	1	51.5	54.6	
47		7/14/2020	1.5	0.5	23.6	25.6	
48		7/14/2020	1.6	0.7	37.5	39.8	
51		7/14/2020	N/A				
52		7/14/2020					
53		7/14/2020					
54		7/14/2020					

Figure Legends

Figure 1. The arena used in both the boldness and anti-predator assays consists of an acclimation chamber (AC), main chamber (MC), and removable gate (G). (A.) The acclimation chamber and main chamber of the arena are separated while the gate is in place. (B.) The acclimation chamber and the main chamber of the arena are connected when the gate is removed.

Figure 2. Proportion of untreated fish surviving from 80 to 120 dpf across egg total thiamine (TTH) concentration (nmol/g). Each point represents one untreated family; thiamine treated families not included as they had 99.5% survival from 80-120 dpf. The line represents expected survival, as predicted by a binomial logistic regression model with a logit link function ($p<0.001$) and using mean total egg thiamine concentration as an independent variable. The shaded area surrounding the line represents the 95% confidence interval of the model. The model predicts

the total egg thiamine concentration that supports 95% survival within a family (EC95) is 4.97 nmol/g (vertical line).

Figure 3. Critical thermal maxima (CTMax; °C) of thiamine treated (n=391) and untreated (n=315) winter-run Chinook salmon juveniles. The centerline of the boxplots represents the median, the box represents the interquartile range (IQR), the whiskers extend 1.5 times the IQR, and the points represent the values outside 1.5 times the IQR. Letters indicate a significant ($p < 0.05$) difference between treatments.

Figure 4. Routine metabolic rate ($\mathit{M}O_2$; mg O_2 kg^{-1} min^{-1}) of thiamine treated (n=117) and untreated (n=91) winter-run Chinook salmon juveniles. The centerline of the boxplots represent the median, the box represents the interquartile range (IQR), the whiskers extend 1.5 times the IQR, and the black points represent the values outside 1.5 times the IQR. The mean value for each boxplot is represented by a white point.

Figure 5. Total distance traveled (cm) of thiamine treated (n=356) and untreated (n=266) winter-run Chinook salmon juveniles. The centerline of the boxplots represent the median, the box represents the interquartile range (IQR), the whiskers extend 1.5 times the IQR, and the black points represent the values outside 1.5 times the IQR. The mean value for each boxplot is represented by a white point.

Figure 6. Mean spontaneous swimming velocity (cm/s) of thiamine treated (n=336) and untreated (n=252) winter-run Chinook salmon juveniles. The centerline of the boxplots represent the median, the box represents the interquartile range (IQR), the whiskers extend 1.5 times the IQR, and the black points represent the values outside 1.5 times the IQR. The mean value for each boxplot is represented by a white point.

Figure 7. Percentage of time fish were present in the main chamber of the arena of thiamine treated (n=358) and untreated (n=268) winter-run Chinook salmon juveniles. The centerline of the boxplots represent the median, the box represents the interquartile range (IQR), the whiskers extend 1.5 times the IQR, and the black points represent the values outside 1.5 times the IQR. The mean value for each boxplot is represented by a white point.

Figure 8. Difference in total distance traveled (cm) by thiamine treated (n=257) and untreated (n=240) winter-run Chinook salmon juveniles following exposure to a conspecific alarm cue. The centerline of the boxplots represent the median, the box represents the interquartile range (IQR), the whiskers extend 1.5 times the IQR, and the black points represent the values outside 1.5 times the IQR. The mean value for each boxplot is represented by a white point.

Figure 9. Difference in mean velocity (cm/s) attained by thiamine treated (n=257) and untreated (n=239) winter-run Chinook salmon juveniles following exposure to a conspecific alarm cue. The centerline of the boxplots represent the median, the box represents the interquartile range (IQR), the whiskers extend 1.5 times the IQR, and the black points represent the values outside 1.5 times the IQR. The mean value for each boxplot is represented by a white point.

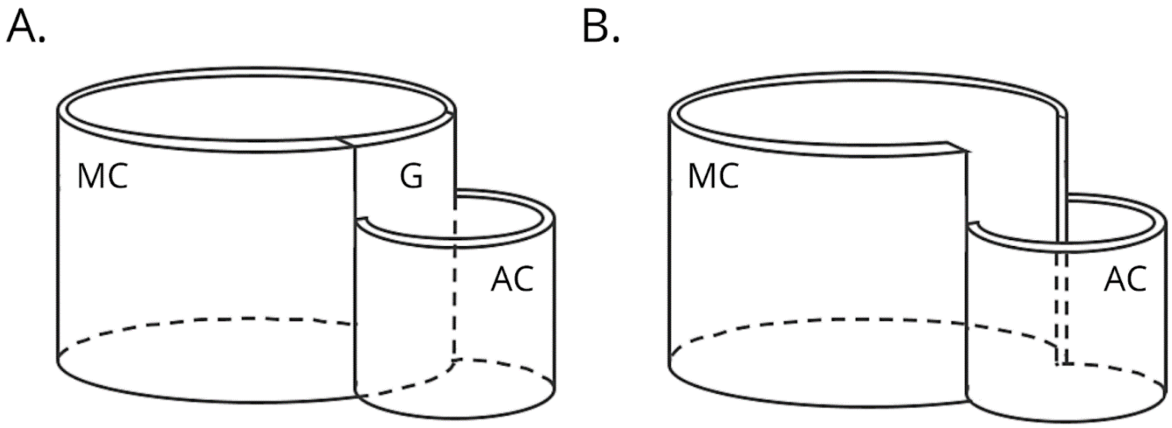
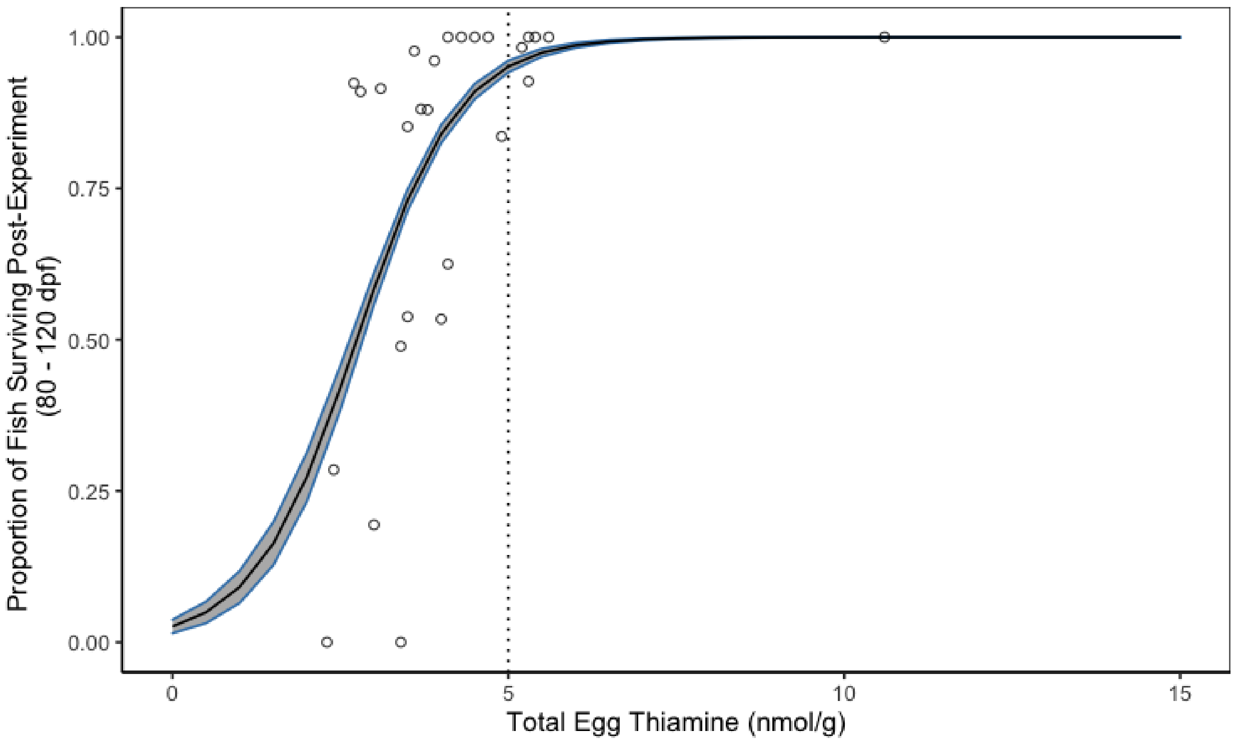


Figure 1



Proportion of Fish Surviving Post-Experiment
(80 - 120 dpf)
1.00
0.75
0.50
0.25
0.00
0
5
10
15
Total Egg Thiamine (nmol/g)

Figure 2

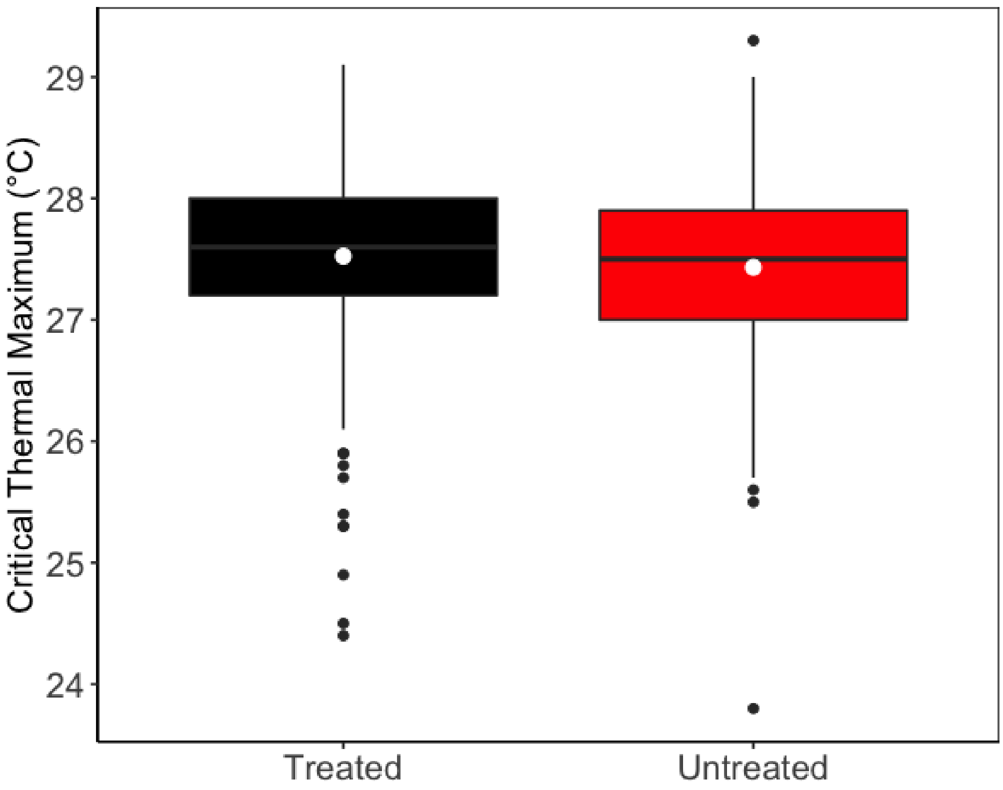

Critical Thermal Maximum (°C)
29
28
27
26
25
24
Treated
Untreated

Figure 3

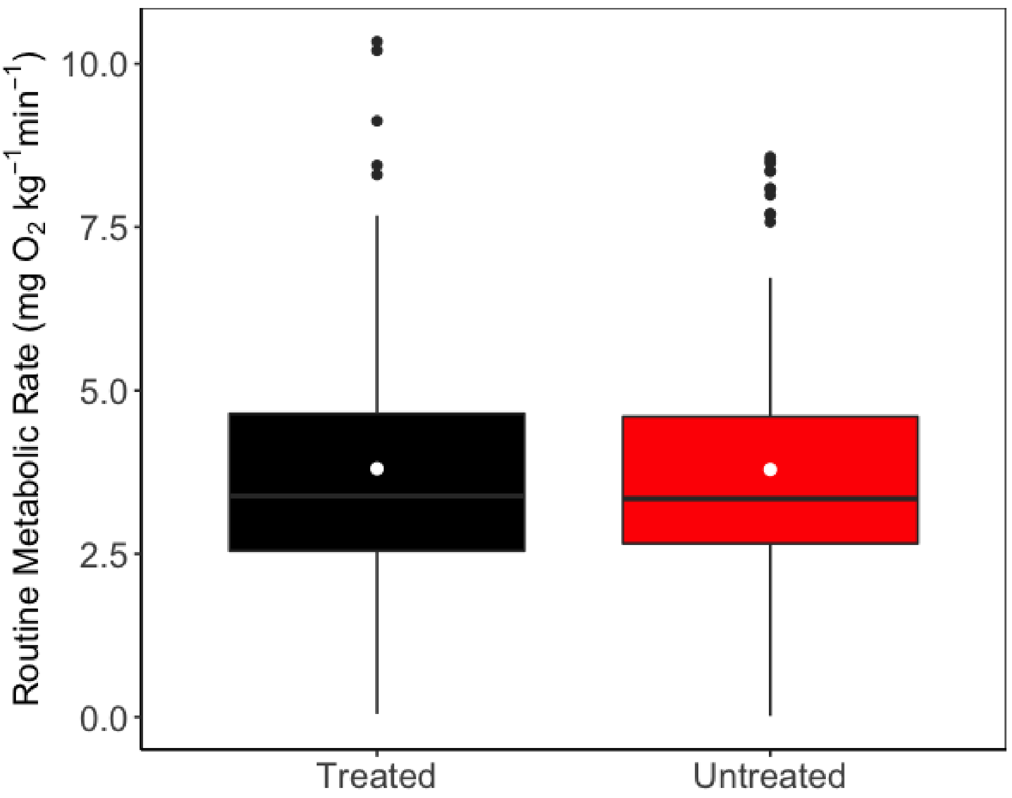

Routine Metabolic Rate (mg O_2 kg^{-1} min^{-1})
10.0
7.5
5.0
2.5
0.0
Treated
Untreated

Figure 4

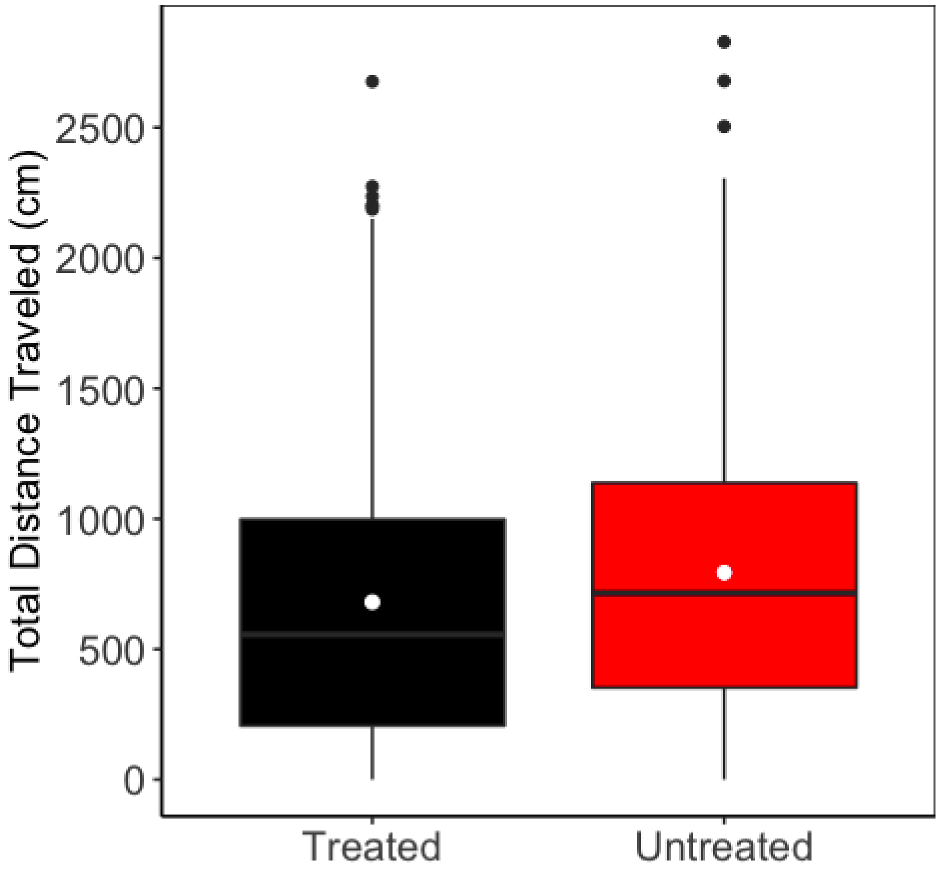

Total Distance Traveled (cm)
2500
2000
1500
1000
500
0
Treated
Untreated

Figure 5

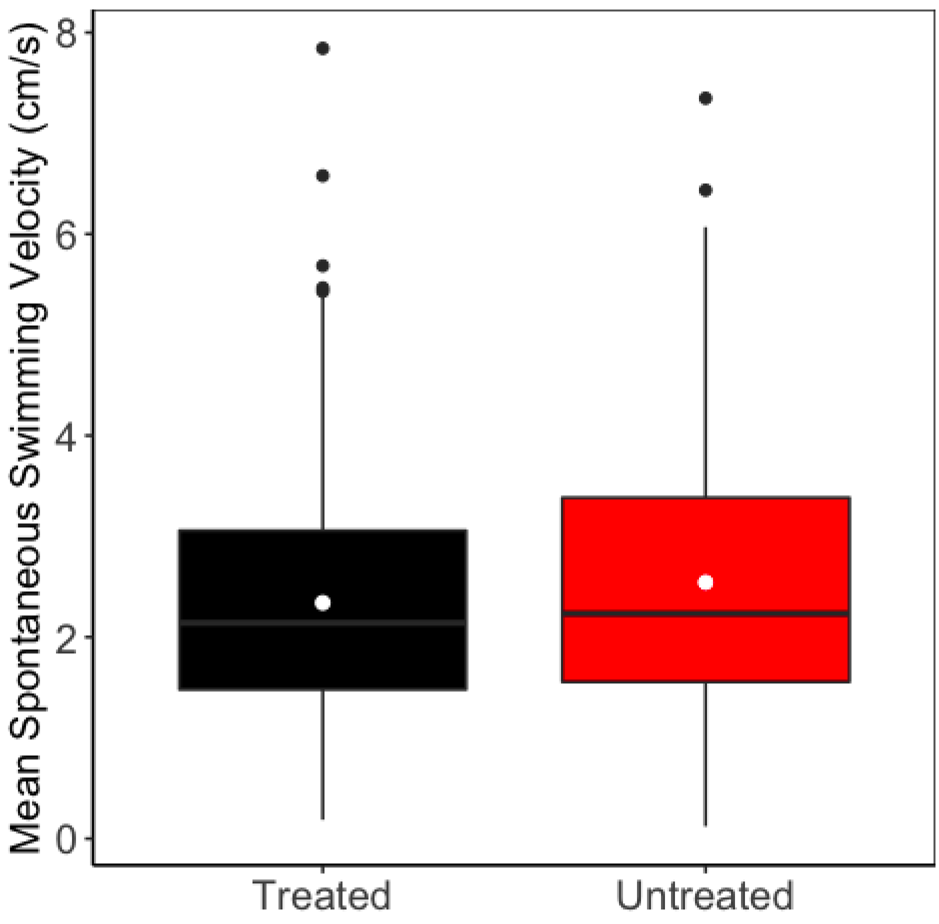

Mean Spontaneous Swimming Velocity (cm/s)
0
2
4
6
8
Treated
Untreated

Figure 6

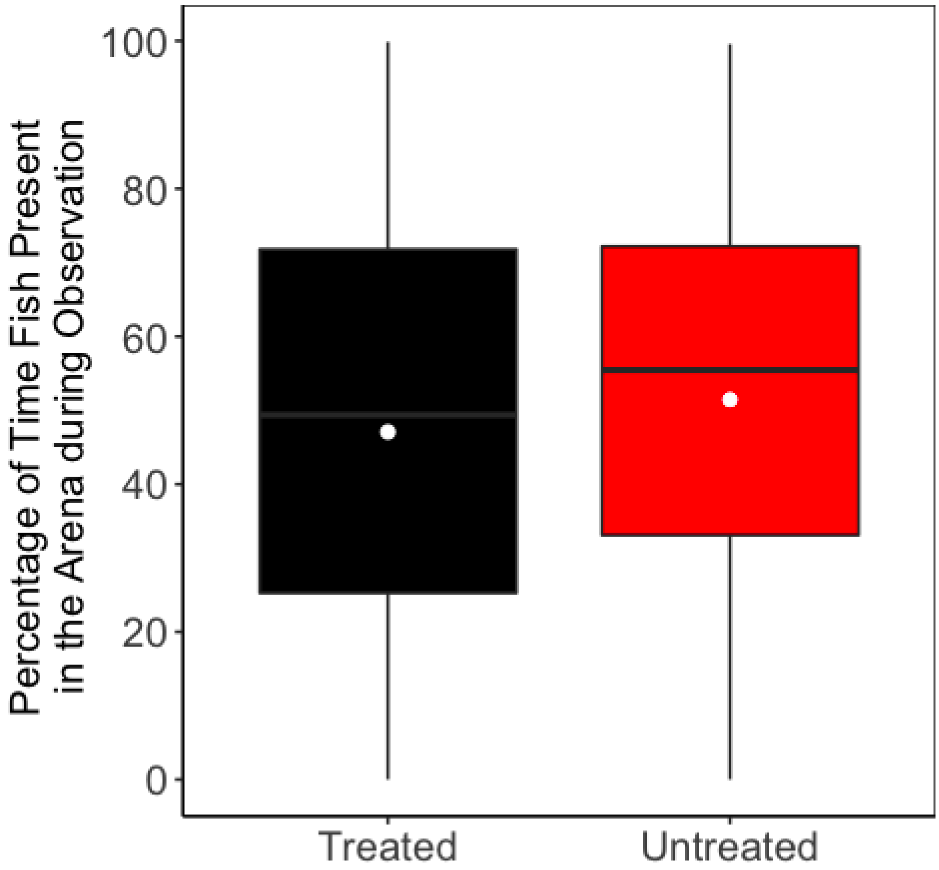

Percentage of Time Fish Present
in the Arena during Observation
100
80
60
40
20
0
Treated
Untreated

Figure 7

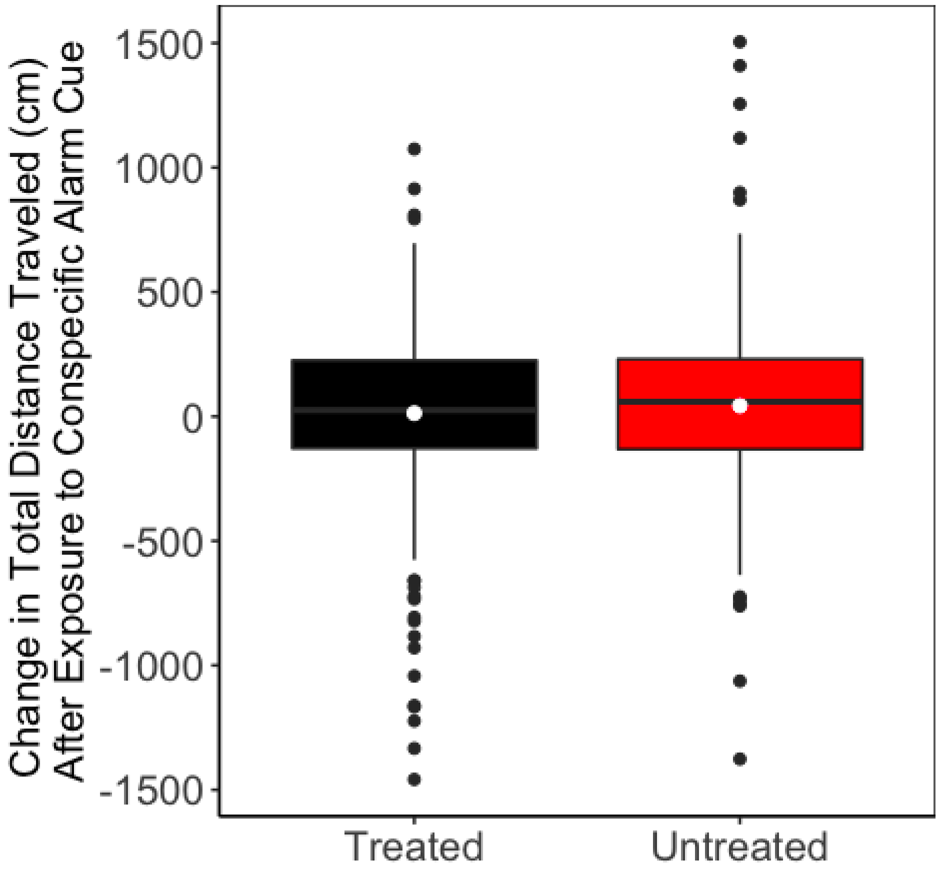

Change in Total Distance Traveled (cm)
After Exposure to Conspecific Alarm Cue
1500
1000
500
0
-500
-1000
-1500
Treated
Untreated

Figure 8

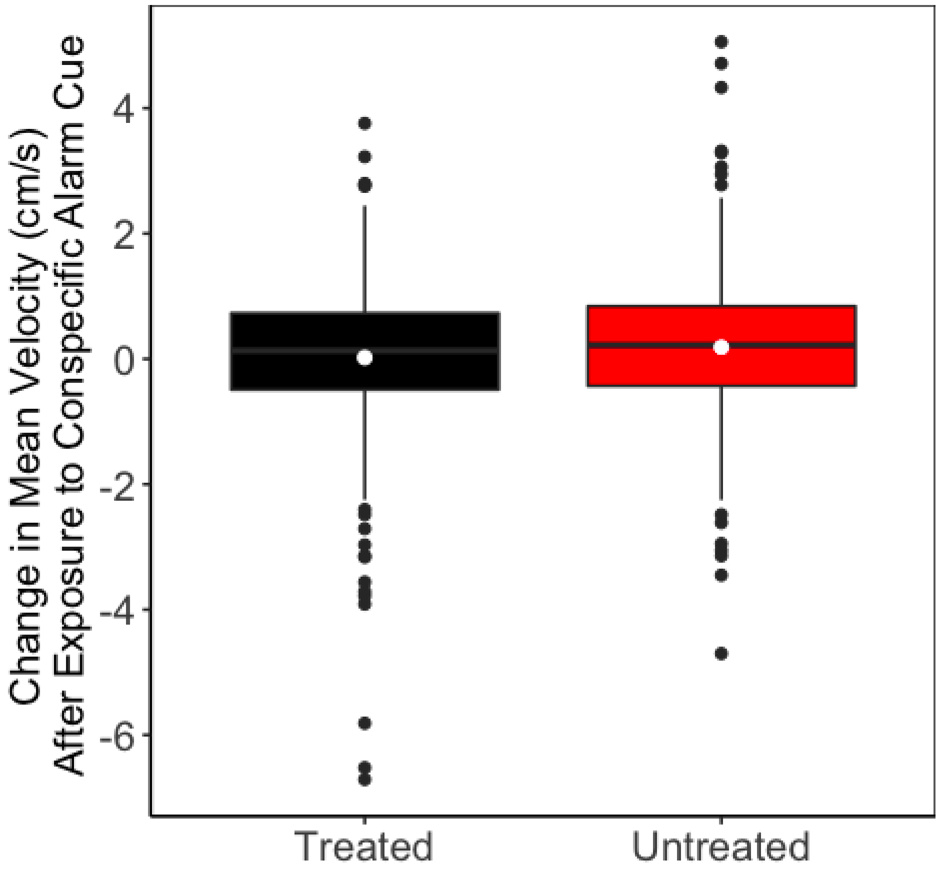

Change in Mean Velocity (cm/s)
After Exposure to Conspecific Alarm Cue
4
2
0
-2
-4
-6
Treated
Untreated

Figure 9