**Articles**

Effects of Stocking Transport Duration on Age-0 Walleye

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**Abstract**

Fish hatcheries are generally not adjacent to stocking locations. Thus, hatchery fish undergo transportation processes for variable durations that can result in changes in water quality, fish physiology (e.g., whole blood glucose and plasma cortisol), and reduced survival. Walleye *Sander vitreus* are commonly stocked throughout North America with variable stocking success, possibly due to altered physiological responses associated with changes in water quality parameters during transportation. We hypothesized increased transport duration would be associated with increases in water temperature, carbon dioxide, and total ammonia nitrogen and decreases in pH and total alkalinity. We also hypothesized that increases in carbon dioxide, water temperature, un-ionized ammonia, and total ammonia nitrogen would be positively related with Walleye whole blood glucose and plasma cortisol concentrations. Walleye were transported for either 0, 0.5, 3, or 5 h and whole blood glucose and plasma cortisol concentrations and mortality were evaluated for 48 h posttransport. Total ammonia nitrogen concentrations, carbon dioxide, pH, and water temperature increased with transportation duration while total alkalinity decreased. Plasma cortisol and whole blood glucose concentrations of Walleye transported for longer durations took longer to decline relative to those not transported. Water quality parameters were not associated with changes in Walleye whole blood glucose and plasma cortisol concentrations, but they were negatively related with time since transport \( (P < 0.05) \). Despite increases in stress, mortality was low (2.5%). Overall, we found evidence to support our hypotheses regarding reduced water quality associated with increasing transport duration. Finally, whole blood glucose and plasma cortisol concentration of Walleye that were transported were similar to those not transported, suggesting handling procedures before transportation could play a significant role in physiological responses measured after transportation. Further, evaluation of stocking procedures aimed at decreasing handling during the loading process could enhance stocking protocols. Elevated concentrations of whole blood glucose and plasma cortisol following transport could make recently stocked Walleye more susceptible to the effects of other environmental factors such as starvation, predation, and disease, all of which could result in increased mortality rates after stocking.

Keywords: cortisol; glucose; mortality; transportation; Walleye

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**Introduction**

Fisheries managers stock hatchery-reared fishes for many purposes, such as human consumption (put-take; Naylor et al. 2000), supplemental stocking programs aimed at enhancing current fish populations, and establishing new populations (Wedemeyer 2001). However, a challenge is exposure to numerous handling and transport-related stressors (e.g., changes in water chemistry, transport duration, and crowding) that negatively influence health and survival of fish that can ultimately limit stocking success (Huntingford et al. 2006). Thus,
evaluating the effects of handling and transport stressors on physiological changes may improve poststocking survival. However, assessing the influence of transportation and handling practices on physiological stress indicators (e.g., glucose and cortisol) in the field is challenging because of fish exposure to simultaneous transportation-related stressors and species-specific physiological responses (Barton 2000). Thus, species-specific evaluations of stocking stress that control for multiple stressors are necessary.

The effects of hatchery handling techniques (e.g., dry versus wet transfer and fish density) and transportation processes (e.g., handling, fish density, and changes in water quality) on water quality and fish physiology (e.g., glucose and cortisol concentrations) have been evaluated extensively (e.g., Wedemeyer 1976, 2001; Barton et al. 1985; Gomes et al. 2003; Bosisio et al. 2017). Additionally, prior experimental evaluations have assessed the effects of fish density in nets and holding containers and their association with increases in glucose and cortisol concentrations (Wedemeyer 1976; Gomes et al. 2003). Changes in water quality parameters during transportation, such as dissolved oxygen (Caldwell and Hinshaw 1994; Evans et al. 2003), ammonia (Randall and Tsui 2002; Wicks and Randall 2002; Barbieri and Bondioli 2015), carbon dioxide (Ross et al. 2001), and salinity (Bosisio et al. 2017) can lead to increased fish glucose and cortisol concentrations, and in some instances, mortality. However, to understand the effects of transportation practices on fish, simultaneous monitoring of changes in water chemistry, fish physiology, and handling techniques are necessary (Barton et al. 1985; Evans et al. 2003; Gomes et al. 2003). Therefore, developing an experimental methodology for simultaneously evaluating multiple components related to transportation has the potential to further our understanding of observations made in field evaluations regarding the behavior, health, and physiology of transported fish.

One of the most stressful periods for hatchery fish is during the transportation process (Wedemeyer 2001). Field evaluations assessing the effects of transportation practices with physiological changes and mortality rates have received considerable attention (Urbinati et al. 2004; Lima and Oliveira 2018; Ball et al. 2020). Transportation duration has been associated with increased Common Carp Cyprinus carpio metamyelocytes and neutrophils (Dobšíková et al. 2006) and negatively related with fish glucose and cortisol concentrations (Sampaio and Freire 2016). However, challenges associated with field evaluations are unavoidable and include random effects such as multiple transportation dates (Carmichael 1984; Forsberg et al. 2001; Ball et al. 2020), stocking fish into different waterbodies with variable water quality parameters (Barton et al. 2003; Dobšíkova et al. 2006), and simultaneous changes of water quality parameters during transportation (e.g., pH, dissolved oxygen, carbon dioxide, and ammonia; Sampaio and Freire 2016). Consequently, experimental designs that eliminate as many confounding factors as possible (e.g., changes in water quality parameters, stocking water characteristics, and multiple transportation days) will further our understanding of the effects of transportation duration on stress-related physiological changes and posttransportation mortality.

Walleye Sander vitreus is a commonly cultured sport fish in the United States (Lutz 1995; Barton 2011). Walleye stocking success is highly variable spatially and temporally but can often result in weak year-classes (Forney 1976) in part due to stress and mortality associated with transportation and stocking (Mitzner 1992). In addition to direct stocking mortality (McWilliams and Larscheid 1992; Ball et al. 2020), age-0 Walleye (150–299 mm; hereafter referred to as Walleye) exhibit lethargic behaviors following extended transportation (5–6 h) with elevated plasma cortisol concentrations relative to individuals transported for shorter distances (20–360 min; Forsberg et al. 2001), which may result in behavioral changes (Weber and Weber 2020) and indirectly contribute to mortality through increased starvation and predation (Fayram et al. 2005; Freedman et al. 2012; Grausgruber and Weber, in press). However, the relationship between transportation duration and changes in physiological parameters indicative of stress and mortality is unclear because of confounding factors, such as multiple transportation events across multiple days and systems (Carmichael 1984; Forsberg et al. 1999; Ball et al. 2020).

Our objectives were to evaluate the relationships between transport duration, water quality, and Walleye whole blood glucose, plasma cortisol concentrations, and mortality. Specifically, we wanted to assess whether Walleye whole blood glucose and plasma cortisol concentrations and mortality were negatively related to increased transportation duration and changes in water quality parameters. We hypothesized increased transport duration would be associated with adverse changes in water quality parameters, such as increases in water temperature, carbon dioxide, and total ammonia nitrogen and decreases in pH and total alkalinity. We also hypothesized increases in Walleye whole blood glucose and plasma cortisol concentrations would increase with increases in carbon dioxide, pH, un-ionized ammonia, total ammonia nitrogen, and transport duration as well as decreases in dissolved oxygen and total alkalinity. Finally, we hypothesized Walleye mortality would be positively related to transport duration. Unlike prior research, our evaluation removes confounding parameters common in field evaluations (multiple stocking days and systems) as well as evaluates the effects of water quality parameters and transport duration on whole blood glucose and plasma cortisol concentrations of Walleye. Collectively, these results provide insight into the effects of transportation duration on Walleye. Understanding the tolerance of Walleye to transportation-induced stress has the potential to improve transportation practices and Walleye stocking success.

Methods

Transportation protocol

We collected 1,268 Walleye (301 lbs [~137 kg]; 175–266 mm) on 14 November 2017, from a single pond
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(pond #1) at Rathbun Fish Hatchery, Moravia, Iowa. We exposed Walleye to one of four transportation durations (0, 0.5, 3, or 5 h) and stocked them into a new hatchery pond (pond #2). To decrease waste (e.g., ammonia and carbon dioxide) in transport water, we did not feed Walleye for 48 h before transportation. The transportation truck had three 1,260-L compartments that were all equipped with a ram-air ventilation (RAV) system (Forsberg et al. 1999), supplemental oxygen (0.2–0.4 L/min), and OxyGuard Pacific water temperature probes (Forsberg et al. 1999), and a ram-air ventilation (RAV) system. Every transportation truck had three 1,260-L compartments that were all equipped with a ram-air ventilation (RAV) system (Forsberg et al. 1999), supplemental oxygen (0.2–0.4 L/min), and OxyGuard Pacific water temperature probes (Forsberg et al. 1999), and a ram-air ventilation (RAV) system.

Hatchery personnel filled each of the three tanks with freshwater that originated from the hatchery’s flow-through system that filters water from Rathbun Lake through a 10-micron filtration system. Exposure to multiple, sequential, or simultaneous stressors can accumulate increases in fish plasma cortisol concentrations (Barton 2002). Thus, we attempted to decrease the number of stressors not typical of transportation processes, such as multiple seining events (Barton et al. 1986). Before loading Walleye into the transportation truck, we divided pond #1 in half with a seine that allowed us to minimize Walleye stress responses related to density (e.g., crowding; Gomes et al. 2003), reduce the number of disturbances (e.g., multiple seining events), and make it easier for hatchery personnel to net Walleye. Walleye that not were transported remained loosely confined in the seine for 5 h and Walleye that were transported for 30 min were in the seine for 4.5 h. Our experimental design allowed us to ensure that we only included Walleye from a single pond, avoiding cross pond variability. However, the experimental design did not allow us to evaluate whether the holding duration in a seine influenced the physiological responses.

We used a staggered loading protocol to ensure transportation of all Walleye occurred on the same truck on the same day. Additionally, the staggered loading protocol allowed us to keep fish densities in each of the three transportation tank compartments consistent throughout the experiment. The staggered loading protocol also allowed us to unload fish from each transportation truck tank simultaneously, exposing Walleye to the same posttransport conditions (Specker and Schreck 1980). The procedure for loading the transportation truck began with netting every Walleye and pushing them across a wet metal sort table and placing them into a metal sieve connected to a scale. We then placed Walleye into one of three transportation truck tanks and transported them different amounts of time (0.5, 3, or 5 h) or held them in the low-density seine for 5 h (no transportation). The staggered loading protocol started with loading the rear tank with 33.7 kg of Walleye and then driving the truck for 2 h. Next, we loaded the middle transportation tank with 33.7 kg of Walleye and drove the hatchery truck for 2.5 h. Finally, we loaded the front transportation tank with 33.7 kg of Walleye and we drove the transportation truck for half an hour with Walleye in the front, middle, and rear tanks. The amount of time it took to fill each of the three transportation tanks with water and 33.7 kg Walleye was roughly 15 min. The amount of time the transportation truck was not moving during the loading process was similar to the amount of time transportation trucks stop when on long transportation trips (e.g., stops for refueling).

After transportation, the truck returned to the hatchery, and all Walleye received a fin clip to identify different transportation durations (e.g., transported 5 h right pelvic fin; transported 3 h left pelvic fin; 0.5 h transported right pectoral fin; not transported-on transportation truck = left pectoral fin; Table S2, Supplemental Material). Clipping fins to identify Walleye exposed to different treatments does not influence mortality rates (Pratt and Fox 2002). Combinations of Walleye from each of the four transportation durations (0, 0.5, 3, and 5 h) were either put into one of four identical rectangular cages (1.2 m x 1.2 m x 2.4 m) or one of twelve-cylinder cages (0.9 m x 0.9 m x 1.2 m) that were in a hatchery pond (pond #2). The density of Walleye in both cage types was 52 individuals/m³. We used Walleye in the rectangular cages for the blood analysis portion of the experiment. Walleye in each cage were used for different blood draw times (0, 2, 24, and 48 h posttransportation), limiting the number of posttransportation disturbances and ensuring no Walleye had multiple blood draws. We monitored lethargic behaviors (i.e., a lack of flight or fight responses) and mortality rates during the 0, 2, 24, and 48-h intervals of Walleye in cylindrical cages. Similar to field transportation evaluations (e.g., Carmichael 1984; Iversen et al. 1998; Forsberg et al. 2001; Ball et al. 2020), each transportation event occurred once as a result of logistical constraints.

Blood collection and analysis

We drew blood samples the morning of the experiment at 0700 hours from 20 Walleye in pond #1 to establish reference values before transportation (hereafter referred to as ‘reference Walleye’; Table S2, Supplemental Material). After we loaded all cages in pond #2, we collected posttransportation blood sampled from 20 Walleye/transportation duration. We removed 400 µL of whole blood by inserting a 23-gauge needle with a vacutainer containing 143 units of sodium heparin ventrally anterior of the anal fin (Houston 1990). To mix the sodium heparin with the whole blood, we inverted each vacutainer 10 times and placed it into an ice slurry. After collecting all the blood samples, we removed 0.3 µL of whole blood from each vacutainer and placed directly onto a FreeStyle Lite test strip (FreeStyle Lite Meter, Abbott Diabetes Care, Inc., Alameda, CA) to measure whole blood glucose concentrations ranging from 0 to 500 mg/dL (Ball and Weber 2017; Table S2, Supplemental Material). The remaining sample underwent centrifugation in a 14-mL centrifuge tube and spun at 3,500 revolutions per minute for 10 min to separate plasma.
from red blood cells (Gomes et al. 2005). We removed plasma from the top of each sample using a clean disposable pipette and placed it in a microfuge tube, and froze it at −80°C for future plasma cortisol processing in the laboratory at Iowa State University.

We determined plasma cortisol concentrations via Cortisol ELISA Kit (Enzo Life Sciences Inc., Farmingdale, NY), a competitive immunoassay for the quantitative determination of plasma cortisol in biological fluids. The kit uses a monoclonal antibody that competitively binds to plasma cortisol. After a 2-h incubation time, we stopped the binding reaction, and the yellow color generated was read on a microplate reader at 405 nm. We developed a standard curve of a series of known cortisol concentrations ranging from 156 to 10,000 pg/mL (10,000, 5,000, 2,500, 1,250, 625, 313, and 156 pg/mL). We developed standard curve concentrations in conjunction with the samples collected from Walleye. We then based plasma cortisol concentrations on the developed standard curve (Chard 1995; Table S2, Supplemental Material).

**Water quality parameters**

We measured water quality parameters (dissolved oxygen, pH, water temperature, total ammonia nitrogen, and total alkalinity) in pond #1 before the loading of each transportation tank, in each compartment before loading, and each time the truck returned to the hatchery. Additionally, we measured water quality parameters in pond #2 with the holding cages at 24 and 48 h after transportation. We used a HACH Multi HQ 40d (HACH Company, Loveland, CO) to measure dissolved oxygen (mg/L), pH, and water temperature (°C). We used a LaMotte Carbon Dioxide DRT Kit (LaMotte Company, Chestertown, MD) to quantify carbon dioxide concentrations (mg/L). The LaMotte Carbon Dioxide DRT Kit uses a titration method. First, we mixed two drops of Phenolphthalein Indicator 1% with 20 mL of sample water. Then we added a series of 1-mL drops of carbon dioxide reagent B (4253DR) to the solution and gently swirled until a faint pink color was produced and persisted for 30 s. The amount of carbon dioxide reagent B used in the titration represented the carbon dioxide concentration in parts-per-million (mg/L). We used a HACH TNT 830 Plus test tubes (HACH Company) in a HACH DR2800 spectrophotometer (HACH Company) to measure total ammonia nitrogen (TAN; mg/L) concentrations. We added a 5-mL water sample to each HACH TNT 830 Plus test tube solution and inverted each tube three times, followed by a 15-min reaction time. After 15 min, we put the test tube into the HACH DR2800 spectrophotometer at 694 nm. We calculated un-ionized ammonia (mg/L; UIA) concentrations as (Wedemeyer 2001):

\[
\text{UIA} = \frac{(\text{TAN} \times \text{percent un-ionized ammonia at pH and temperature})}{100}
\]

where TAN is total ammonia nitrogen (mg/L), pH is of the source water at the same time in which the TAN sample was collected, and temperature was of the source water at the same time in which the TAN sample was collected. We calculated percent un-ionized ammonia concentrations at a given pH and water temperature from a standardized table (Emerson et al. 1975). Finally, we quantified total alkalinity (mg/L CaCO₃) by swirling one phenolphthalein powder pill (HACH Permachem Reagent 94299) with a 100-mL water sample followed by the addition of one bromcresol green-methyl red powder pill (HACH Permachem Reagent 943-99). The solution was titrated with 1.6N sulfuric acid (HACH Reagent 14389-01). Last, we used an OxyGuard Pacific probe system to record water temperature and dissolved oxygen concentrations every 18 min in each of the three compartments during Walleye transportation (Table S1, Supplemental Material).

**Statistical analysis**

We used R-Studio to develop a set of candidate linear models (R Core Team 2017) to evaluate relationships between whole blood glucose and plasma cortisol concentrations relative to water quality parameters (i.e., carbon dioxide, dissolved oxygen, water temperature, pH, total ammonia nitrogen, un-ionized ammonia, total alkalinity, and transportation duration) and time since transport. Temporal changes in whole blood glucose and plasma cortisol concentrations following exposure to a stressor generally increase (Forberg et al. 2001). However, the magnitude of change and duration of time associated with elevated whole blood glucose and plasma cortisol concentrations are a function of stressor type, intensity, and duration (Barton et al. 1986). Increases of whole blood glucose and plasma cortisol concentrations, as well as mortality, have been associated with exposure to increasing carbon dioxide concentrations (Ball et al. 2020), transport duration (Robertson et al. 1987; Mitzner 1992), and water temperature (Clapp et al. 1997). Thus, we expected a positive relationship between whole blood glucose and plasma cortisol concentrations with increasing transport time. For Walleye transported from pond #1 to pond #2 (no transportation), we based water quality parameters on average values from pond #1. Candidate models consisted of one of two continuous response variables (i.e., whole blood glucose or plasma cortisol concentrations), a singular continuous water quality variable (carbon dioxide, dissolved oxygen, water temperature, pH, total ammonia nitrogen, un-ionized ammonia, total alkalinity, or transportation duration), and a continuous time since transport (0, 2, 24, and 48 h) predictor variables (Table S3, Supplemental Material). Response variables in the candidate models were an average of whole blood glucose or plasma cortisol concentration at a given blood draw time (0, 2, 24, or 48 h; Table S3, Supplemental Material). We used averages for response variables because Walleye within a transport tank were...
not independent and therefore were pseudoreplicates (Riley and Edwards 1998). All candidate models had the following structure:

\[ Y_i = \beta_0 + \beta_1 x_{1,i} + \beta_2 x_{2,i} + \epsilon_i \]

where \( \beta_0 \) represents the y-intercept, \( \beta_1 \) is an estimate of the slope for a singular independent water-quality variable (\( x_{1,i} \)—carbon dioxide, dissolved oxygen, water temperature, pH, total ammonia nitrogen, un-ionized ammonia, total alkalinity, or transportation duration), \( \beta_2 \) is the slope estimate for the second continuous time since transport variable (\( x_{2,i} \)—0, 2, 24, 48 h), and \( \epsilon_i \) accounts for random error in the model (Mendenhall and Sincich 2012). We used post hoc residual analysis to assess normality and homoscedasticity of the residuals for each candidate model (Mendenhall and Sincich 2012). All residuals for all candidate models were normally distributed and had a mean probability distribution of zero (Mendenhall and Sincich 2012). Thus, transformations were not necessary, and regressions were performed on raw values (Table S3, Supplemental Material).

In each model, we only included one water quality parameter because of our lack of observations per predictor variable in the model (Peduzzi et al. 1996). Specifically, each candidate model consisted of 16 observations: 4 observations were associated with 4 different times since stocking (0, 2, 24, and 48 h) and 4 observations were associated with different transport durations (0, 0.5, 3, and 5 h). Random effects associated with different transport tanks and holding Walleye in an uncrowded seine could have influenced our observations. However, including these random effects was beyond the scope of data with only one transportation event. For each candidate model, random error was accounted for in the \( \epsilon_i \) parameter (Mendenhall and Sincich 2012). We used the “AICmodavg” package to rank and compare model performance across models with the same response variables (Mazerolle 2019). We had a small sample size, so we used corrected Akaike Information Criterion (AICc) and AICc model weights to compare model performance across models with the same response variables.

**Results**

**Water quality parameters**

During and after transportation, dissolved oxygen concentrations ranged from 10.2 to 13.6 mg/L and water temperatures ranged between 7.9°C to 9.5°C (Figure 1). Dissolved oxygen concentrations in the ponds ranged between 0.05 and 2.00 mg/L before and after transportation, whereas concentrations ranged between 5.5 and 6.8 mg/L in all three transport tanks during transportation (Figure 2). Carbon dioxide concentrations in the rear and middle tanks markedly increased at the time when the front tank was loaded with Walleye and remained elevated until the end of transport (Figure 2). Similarly, pH in the ponds (6.99–7.91) was lower than in the transport tanks after 2 h of transportation (9.1–9.5; Figure 2). Total ammonia nitrogen and un-ionized ammonia concentrations in both
Figure 2. Changes in water quality parameters on 14 November 2017 when Walleye *Sander vitreus* were transported to and from the Rathbun Fish Hatchery in southeastern Iowa. Changes in dissolved oxygen (mg/L), water temperature (°C), carbon dioxide (mg/L), pH, total ammonia nitrogen (mg/L), and total alkalinity (mg/L CaCO₃) prior to (Pond #1), during (loading rear tank, loading middle tank, loading front tank, and end of transport), and 2 d after transportation (Pond #2: 24 h and Pond 2: 48 h). Calculated un-ionized ammonia concentrations (mg/L) are denoted above bars on the total ammonia nitrogen panel. Each bar represents a different water stock transport duration on Age-0 Walleye.
ponds were low, with levels ranging between 0.026 and 0.083 mg/L and <0.01 mg/L, respectively (Figure 2). However, total ammonia nitrogen and un-ionized ammonia concentrations increased with transport duration (Figure 2). Total ammonia nitrogen and un-ionized ammonia concentrations in the rear and middle tanks rapidly increased at the time when the front tank was loaded with Walleye and remained elevated until the end of transportation. Total alkalinity concentrations in the ponds and transport tanks varied between 66.5 and 93.0 mg/L CaCO₃ and tended to increase with transportation duration (Figure 2).

Physiological parameters

Transported Walleye had whole blood glucose concentrations higher than reference Walleye at 0, 2, 24, and 48 h posttransportation; however, Walleye transported for 3 h had whole blood glucose concentrations lower than reference Walleye 48 h posttransport (Figure 3). At 0, 2, 24, and 48 h posttransportation, Walleye transported for 0, 0.5, 3, and 5 h had variable whole blood glucose concentrations (Figure 3). Specifically, at 0 h posttransport, whole blood glucose concentrations of Walleye transported for 3 h were higher than those transported for 0.5 and 5 h (Figure 3). At 2 h posttransport, whole blood glucose of Walleye transported for 3 h were higher than those transported for 0, 0.5, and 5 h (Figure 3). At 24 h posttransport, whole blood glucose concentrations of Walleye that were not transported were less than those transported for 0.5, 3, and 5 h; but during 0, 2, and 48 h posttransport, Walleye that were not transported had whole blood glucose concentrations similar to Walleye transported for 0.5 and 5 h (Figure 3). At 48 h posttransport, Walleye transported for 3 h had whole blood glucose concentrations lower than those transported for 0, 0.5, and 5 h (Figure 3).

In addition to variation of whole blood glucose concentrations within specific posttransport monitoring times (0, 2, 24, and 48 h), Walleye transported for different durations of time (0, 0.5, 3, or 5 h) exhibited temporal changes in whole blood glucose concentrations. Specifically, all transported Walleye had whole blood glucose concentrations that increased immediately after transportation and then decreased 2 h after transportation (Figure 3). However, between 2 and 24 h posttransport, Walleye transported for 0, 3, and 5 h had whole blood glucose concentrations that continued to decrease. In contrast, Walleye transported for 0.5 h had whole blood glucose concentrations that remained elevated (Figure 3). Between 24 to 48 h posttransport, whole blood glucose concentrations of Walleye transported for 0.5, 3, and 5 h continued to decline, whereas whole blood glucose concentrations of Walleye that were not transported remained unchanged (Figure 3).

Five whole blood glucose candidate linear models had ΔAIC < 2.0 and AIC weight ≥ 0.12, suggesting various levels of support. Candidate models with some support included those with pH, un-ionized ammonia, time since transport, total ammonia nitrogen, and transport duration; however, only time since transport had a P-value that was significantly < 0.05 (Table 1). Whole blood glucose concentrations of Walleye type (e.g., black = ponds 1 and 2, white = rear transportation truck tank, white with horizontal black lines = front transportation truck tank, and grey = middle transportation truck tank). Walleye in the rear tank were transported for a total of 5 h, Walleye in the middle tank were transported for 3 h, and Walleye in the front tank were transported for 0.5 h. The duration of transportation can influence changes in water quality, which can affect physiological effects exhibited by transported Walleye.
Table 1. Linear models with different Walleye Sander vitreus average whole blood glucose and plasma cortisol response variables and various transport parameters. On 14 November 2017, Walleye were reared and transported from and returned to Rathbun Fish Hatchery, Moravia, Iowa. Transport parameters assessed included average dissolved oxygen (DO; mg/L), carbon dioxide (CO$_2$; mg/L), water temperature during transportation (WT; °C), transport water pH, un-ionized ammonia (mg/L; UIA [un-ionized ammonia]), total ammonia nitrogen (mg/L; TAN), total alkalinity (mg/L CaCO$_3$; TA), and transport duration (TD; hours). Additionally, each model included a time since stocking effect (TST; hours). Summary statistics for each candidate model included beta estimates (β), standard error (SE) estimates for beta estimate, F-statistic based on 1 and 13 degrees of freedom for all models except for the TST model with 1 and 14 degrees of freedom, P-values, corrected Akaike Information Criterion (AIC$_c$), delta corrected Akaike Information Criterion (∆AIC$_c$), and corrected Akaike Information Criterion model weights (AIC$_c$ weights). Models are ranked according to their AIC$_c$ values for each response variable. The most supported whole blood glucose candidate model included pH, while the most supported plasma cortisol candidate model included dissolved oxygen concentrations, suggesting that these two water quality parameters are affecting Walleye stress responses during transportation.

| Model parameters | Intercept | Transport parameter |
|------------------|-----------|---------------------|
|                  | β         | SE      | β         | SE      | F       | P       |
| Glucose          |           |         |           |         |         |         |
| pH + TST         | −557.46   | 55.47   | 117.62    | 55.47   | 3.86    | 0.054   |
| UIA + TST        | 272.14    | 17.70   | 115.72    | 55.60   | 4.33    | 0.058   |
| A + TST          | 271.79    | 18.39   | 29.64     | 15.10   | 3.86    | 0.071   |
| TD + TST         | 275.90    | 17.71   | 9.26      | 5.07    | 3.34    | 0.090   |
| TST              | 295.56    | 15.19   | 3.65      | 0.57    | 41.53   | 0.0001  |
| TA + TST         | 247.08    | 36.05   | 0.51      | 0.35    | 2.17    | 0.165   |
| DO + TST         | 538.10    | 182.56  | −20.07    | 15.06   | 1.78    | 0.0001  |
| WT + TST         | −1,202.26 | 1,127.45| 173.36    | 130.48  | 1.81    | 0.206   |
| CO$_2$ + TST     | 257.52    | 32.82   | 7.27      | 5.60    | 1.69    | 0.217   |
| Cortisol         |           |         |           |         |         |         |
| DO + TST         | 631.32    | 170.74  | −27.79    | 14.09   | 3.89    | 0.070   |
| TST              | 295.57    | 15.19   | 3.65      | 0.57    | 41.53   | 0.0001  |
| CO$_2$ + TST     | 254.91    | 32.52   | 7.78      | 5.55    | 1.97    | 0.183   |
| TA + TST         | 258.80    | 37.30   | 0.39      | 0.36    | 1.16    | 0.0001  |
| pH + TST         | −166.48   | 449.02  | 63.71     | 61.88   | 1.06    | 0.322   |
| WT + TST         | −839.74   | 1,159.54| 131.40    | 134.19  | 0.96    | 0.345   |
| UIA + TST        | 287.09    | 20.09   | 41.91     | 63.14   | 0.44    | 0.519   |
| A + TST          | 290.04    | 20.81   | 6.89      | 17.08   | 0.16    | 0.693   |
| TD + TST         | 292.64    | 19.81   | 1.38      | 5.67    | 0.06    | 0.812   |

Plasma cortisol concentrations were positively related (β ± 95% CI) to pH (117.62 ± 108.72), un-ionized ammonia (115.72 ± 108.98), total ammonia nitrogen (29.64 ± 29.57), and transport duration (9.26 ± 9.93), and negatively related to time since transport (−3.65 ± 1.11; Table 1). Plasma cortisol concentrations were higher than reference Walleye values for all transport durations during 0 and 2 h postransport (Figure 3). However, at 24 h postransport, Walleye that were not transported had plasma cortisol concentrations similar to reference Walleye concentrations (Figure 3). Similarly, at 48 h postransport, Walleye transported for 3 h had plasma cortisol concentrations similar to reference Walleye concentrations (Figure 3). At 0 h postransport, plasma cortisol concentrations of Walleye transported for 3 h were higher than those transported for 0.5 and 5 h (Figure 3). At 2 h postransport, plasma cortisol concentrations of Walleye transported for 3 h were higher than those transported for 0, 0.5, and 5 h (Figure 3). At 24 h postransport, plasma cortisol concentrations of Walleye transported for 0 h were lower than those transported for 0 and 24 h postransport (Figure 3). Finally, at 48 h postransport, plasma cortisol concentrations of Walleye transported for 3 h were lower than Walleye transported for 0, 0.5, and 5 h (Figure 3).

Similar to whole blood glucose concentrations, plasma cortisol concentrations also exhibited temporal changes. Plasma cortisol concentrations of Walleye transported for 3 h continually decreased after transport, whereas Walleye transported for 0.5 and 5 h had plasma cortisol concentrations that initially decreased but then remained constant. Walleye that were not transported had plasma cortisol concentrations that increased through time (Figure 3).

There were three candidate plasma cortisol linear models with ∆AIC$_c$ < 2.0 and AIC$_c$ weight ≥0.12, indicating various levels of support. The beta (±95% CI) of the top three candidate models suggested plasma cortisol concentrations are positively related to carbon dioxide (7.78 ± 10.88) and negatively related to dissolved oxygen (−27.79 ± 27.61) and time since transport (−3.65 ± 1.11; Table 1). Only time since transport had a P-value that was significantly <0.05 (Table 1).

Mortality

Following transportation, Walleye did not appear lethargic nor demonstrate a lack of flight or fight response when we raised the cages to count and remove dead Walleye. Mortality was low throughout
the 48 h since transportation monitoring time, with no mortalities occurring 2 h after transport. The only mortality was a Walleye transported 0.5 h that died within 2 h after transport (2.5% of that treatment).

### Discussion

During transportation, we observed fluctuations in all water quality parameters (e.g., carbon dioxide, dissolved oxygen, water temperature, pH, ammonia, and total alkalinity) as well as Walleye physiological blood parameters (e.g., whole blood glucose and plasma cortisol), all of which are commonly reported in transportation studies (see Forsberg et al. 1999, 2001; Barton et al. 2003; Ball et al. 2020). During transportation, water temperature, carbon dioxide, and total ammonia nitrogen increased with transport duration; however, we found limited support for the hypothesis regarding pH and total alkalinity. There was some support for a positive relationship between whole blood glucose and plasma cortisol concentrations and changes in pH, un-ionized total ammonia, total ammonia nitrogen, transport duration, water temperature, and carbon dioxide concentrations; however, these relationships were not statistically significant. Whole blood glucose and plasma cortisol concentrations tended to increase with transport duration, with Walleye transported for longer durations having whole blood glucose and plasma cortisol concentrations that remained elevated for longer durations. Furthermore, we observed low mortality rates (2.5%) and no lethargic behavior after transportation. Collectively, our results suggest transportation of Walleye does elicit physiological changes indicative of stress; however, these physiological changes were not associated with various water quality parameters nor resulted in significant mortality.

As we hypothesized, water temperature, carbon dioxide, and total ammonia nitrogen increased with transport duration; however, we found limited support for the hypothesis that pH and total alkalinity would decrease with transportation duration. Typically, increased transportation duration is associated with increased ammonia (Dobšíkova et al. 2006; Nomura et al. 2009), carbon dioxide concentrations (Nomura et al. 2009), water temperatures (Forsberg et al. 1999), and decreases in pH (Dobšíkova et al. 2006). Carbon dioxide concentrations in each transport tank were higher than concentrations in pond #1; however, changes in these parameters were not associated with transport duration. For reasons unknown, we did observe increased carbon dioxide concentrations in all three transport tanks when Walleye were loaded into the front tank and at the end of transportation. However, carbon dioxide concentrations in each transport tank did not approach levels associated with anesthetizing or euthanizing fish (150–250 mg/L carbon dioxide; Post 1979), suggesting these changes were likely not biologically meaningful. When transporting fish, it is crucial to consider the type of circulation system that can influence changes in water chemistry parameters. Ram-air ventilation systems, like the one on the transportation truck we used, regulate carbon dioxide concentrations (Forsberg et al. 1999) and explain the lack of a negative relationship between carbon dioxide concentrations and transport duration as well as elevated carbon dioxide concentrations. However, transport duration was associated with changes in water temperature, pH, ammonia, and alkalinity. Total alkalinity, pH, water temperature, and total ammonia nitrogen concentrations are interrelated and collectively influence the percentage of total ammonia nitrogen in the un-ionized form (Wedemeyer 2001). Thus, decreases in total alkalinity would allow pH to increase, resulting in increases in the percentage of un-ionized ammonia that could partially explain why we did not observe a negative relationship between carbon dioxide concentrations and pH (Wedemeyer 2001). Additionally, we observed a rapid increase in total ammonia concentrations in the middle transport tank. Increasing water temperatures throughout the day could partially explain this observation. We observed increases in total ammonia nitrogen and un-ionized ammonia concentrations in the rear and middle transport tanks after Walleye had been transported for either 4.5 or 2.5 h, respectively. These changes in ammonia suggest Walleye produce a significant amount of ammonia during transport even when they are held off feed prior to transport. The highest observed un-ionized ammonia concentration during our study was 0.723 mg/L, which is near the 1.06 median lethal level for Walleye (Mayes et al. 1986). An increase in un-ionized ammonia with transportation duration to levels approaching lethal doses could affect Walleye physiological responses and mortality.

Changes in whole blood glucose and plasma cortisol concentrations is partially dependent on the intensity of a stressor (Barton et al. 1986). Thus, Walleye exposed to degraded water quality parameters should exhibit increased concentrations of whole blood glucose and plasma cortisol. We initially hypothesized that increases in whole blood glucose and plasma cortisol concentrations would be associated with changes in water quality parameters (dissolved oxygen, carbon dioxide, water temperature during transportation, transport water pH, un-ionized ammonia, total ammonia nitrogen, and total alkalinity). However, there was little evidence supporting this hypothesis. Instead, candidate models suggested time since transport was an important predictor of Walleye whole blood glucose and plasma cortisol concentrations, suggesting prolonged transportation is an important stressor because other factors beyond those we were able to quantify in this study. Similarly, changes in water temperature, carbon dioxide, and dissolved oxygen were not related to changes in plasma cortisol concentrations or cumulative survival rates of transported Walleye (Ball et al. 2020). Thus, stress responses observed posttransport may be due to loading procedures rather than water quality changes occurring during transportation.

The duration of time associated with whole blood glucose and plasma cortisol concentrations returning to reference levels is partially dependent on the duration of exposure to a stressor (Barton et al. 1986). We initially hypothesized a positive relationship between transport...
duration and concentrations of Walleye whole blood glucose and cortisol. However, we found little evidence supporting our hypothesis. Similarly, changes in whole blood glucose and plasma cortisol of Walleye transported for between 3.5 and 6.2 h were not strongly related to transport duration (Ball et al. 2020). The lack of relationship between transport duration and changes in whole blood glucose and plasma cortisol could partially be due to extended exposure to a stressor (e.g., transportation duration) and subsequent acclimation (Sampaio and Freire 2016). This could partially explain why Walleye transported 5 h had lower whole blood glucose and plasma cortisol concentrations than those transported 3 h. The duration of time required for physiological parameters to return to reference concentrations (concentrations before handling and transport) is variable (Barton 2000; Forsberg et al. 2001; Gomes et al. 2003; Acerete et al. 2004). We observed that Walleye exposed to different transportation durations exhibited elevated plasma cortisol and whole blood glucose concentrations relative to the reference pretransportation concentration. However, Walleye that were not transported recovered from handling within 24 h; whereas, those transported either 0.5, 3, or 5 h had elevated whole blood glucose and plasma cortisol concentration. Exposing fish to multiple stressors (e.g., handling and transportation) can have cumulative effects including increases in whole blood glucose and plasma cortisol concentrations that remain elevated for longer durations (Barton et al. 1986). Additionally, elevated whole blood glucose and plasma cortisol concentrations of Walleye that were not transported occurred after handling procedures, suggesting the physical handling of Walleye or some other stressor unique to each transportation event (e.g., the act of being in an uncrowded seine for various amounts of time, or multiple instances of hatchery personnel entering pond #1) could partially explain elevated physiological parameters. Collectively, our results suggest stocking programs should consider the number of stressors and stressor type and duration because the cumulative effects of stressors have the potential to influence Walleye behavior (Weber and Weber 2020; Weber et al. 2020), making them susceptible to predation (Freedman et al. 2012; Grausgruber and Weber, in press) and starvation (Santucci and Wahl 1993), which can negatively influence the success of stocking initiatives and management goals.

Exposure to multiple stressors produces a cumulative stress response that may be more than the sum of an individual stressor and, in some instances, result in mortality (Power 1997). We initially hypothesized that with increased transportation, there would also be an increase in mortality; however, our data did not support this hypothesis. We observed low mortality rates overall, with only one Walleye transported 0.5 h dying within 2 h after transportation. Similarly, Black Bass Micropterus spp. (Carmichael 1984), Matrinxá Brycon cephalus (Urbinati et al. 2004), and Striped Bass Morone saxatilis (Mazik et al. 1991) all experienced low mortality rates following transportation. In some instances, Walleye transported for shorter durations (<5 h) exhibited no observable changes in fight-and-flight responses but variable cumulative survival rates (0–60% 1–2 d poststocking; Ball et al. 2020). Walleye transported for ≥5 h can exhibit lethargic behaviors, but only a few mortalities (0.4–1.8%; Forsberg et al. 1999; Ball et al. 2020). Altered behaviors associated with transportation may not directly affect survival, but it could make stocked Walleye more vulnerable to predation, which can be high poststocking (Grausgruber and Weber, in press). Therefore, across-study variability in mortality rates among studies could, in part, be due to differences in field and laboratory conditions and confounding factors associated with field evaluations (i.e., variability associated with multiple stocking locations and dates; Forsberg et al. 1999; Ball et al. 2020). Additionally, the lack of observed lethargic behavior and few mortalities in the present study could partially be due to low water temperatures (8.0–9.5°C) at time of transportation. Lower water temperatures can reduce physiological responses in fish, resulting in decreased mortality ( Louison et al. 2017). Thus, transporting Walleye at low temperatures (8.0–9.5°C) could have decreased metabolism and result in reduced changes in water quality, physiological responses, and mortality rates.

No matter the fisheries-management stocking objective, the initial success of a stocking initiative is highly dependent on poststocking survival. Exposure to reduced water quality during transportation paired with increased stress can result in disease and, in some instances, increases in poststocking mortality (Bernoth and Crane 1995). We found some evidence to support our hypotheses regarding the relationships between increased transportation durations and increases in water temperature, carbon dioxide, and total ammonia nitrogen. However, we did not have evidence to support our hypothesis regarding increases in Walleye whole blood glucose and plasma cortisol concentrations associated with reductions in water quality parameter; however, whole blood glucose and plasma cortisol concentrations did increase following transportation. Similar to field evaluations, we observed that in a more controlled environment, handling and transporting Walleye elicits physiological responses indicative of stress. Overall, Walleye transported different durations had high survival rates, indicating transportation may play a limited role in the success of stocking initiatives. However, handling procedures before transportation could play a significant role in physiological responses measured after transportation. Thus, a future direction for fish transportation research is evaluating stocking procedures to decrease handling during the loading process.

Supplemental Material

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Stocking Transport Duration on Age-0 Walleye
E.E. Grausgruber and M.J. Weber

Table S1. Summary of dissolved oxygen concentrations (mg/L) and water temperature (°C) in each tank on the transport truck (rear, middle, and front) in 15-min increments during transportation (hours:minutes) to and from Rathbun Fish Hatchery, Moravia, Iowa on 14 November 2017. Transportation duration for each tank is relative to the entire duration time of 5 h and 45 min. NA denotes times when Walleye Sander vitreus were not being transported in a specific transport truck tank.

| Time (h:min) | Rear | Middle | Front |
|-------------|------|--------|-------|
| 0:00        | 8.2  | 8.3    | 8.4   |
| 0:15        | 7.9  | 7.7    | 7.8   |
| 0:30        | 7.6  | 7.4    | 7.6   |
| 0:45        | 7.3  | 7.2    | 7.3   |

Table S2. Summary of whole blood glucose (mg/L) and plasma cortisol (ng/mL) concentrations collected from individual fin-clipped (NA = no fin clip; R = right pectoral; L = left pectoral; RP = right pelvic; LP = left pelvic) Walleye Sander vitreus transported 0 (no transport), 0.5, 3, or 5 h. On 14 November 2017, blood samples were collected from 20 Walleye (reference Walleye) reared at Rathbun Fish Hatchery, Moravia, Iowa, 1 h before transportation to determine resting concentrations of whole blood glucose and plasma cortisol. Blood samples were collected from individual Walleye immediately after transportation (0 h) and then at 2, 24, and 48 h after transport. No Walleye had its blood drawn more than one time. NA denotes instances when Walleye were not being transported, when fin clipping did not occur, or when blood samples were not large enough to perform plasma cortisol analysis. Increases in whole blood glucose and plasma cortisol suggest that individual fish are perceiving some type of stressor, such as water quality parameters, or being handled, chased, crowded, or agitated.

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Table S3. Summary of linear model response (average whole blood glucose and plasma cortisol concentrations) and predictor variables (dissolved oxygen [mg/L], water temperature [°C], carbon dioxide [mg/L], pH, un-ionized ammonia [mg/L], transportation duration [hours], and total alkalinity [mg/L CaCO₃]) in each transport truck tank (rear, middle, and front) or pond #1 (no transportation). Data were collected on 14 November 2017 while Walleye Sander vitreus were transported to and from Rathbun Fish Hatchery, Moravia, Iowa. Changes in water quality parameters during transportation can influence whole blood glucose and plasma cortisol concentrations.

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Any use of trade, product, website, or firm names in this publication is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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