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[Article]

# Physiological Stress Responses of Rio Grande Silvery Minnow: Effects of Individual and Multiple Physical Stressors of Handling, Confinement, and Transport

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*Abstract.*—In an attempt to reestablish populations of Rio Grande silvery minnow *Hybognathus amarus* within its former range, initiatives were set forth to propagate the species in hatcheries, augment wild populations through repatriation, and rescue the species from isolated and receding pools. Physical stressors associated with these recovery efforts, however, result in stress that increases the vulnerability of the minnow to opportunistic pathogens and predation, possibly decreasing poststocking survival. We assessed the physiological stress response to standard management practices by characterizing the changes in plasma cortisol, glucose, and osmolality. When subjected to the individual stressors of 30 s of handling, 3 h of confinement (density, 100 kg/m$^3$), or 3 h of transport (density, 40 kg/m$^3$), moderate changes in plasma glucose and plasma osmolality were observed during recovery whereas changes in plasma cortisol were not detectable. When fish were subjected to consecutive stressors (30 s of handling only, 30 s of handling plus 3 h of confinement, or 30 s handling plus 3 h of confinement and 3 h of transport), plasma glucose exhibited a cumulative increase that was not observed for plasma cortisol. This increase in plasma glucose was observed within 3 h poststress when fish were subjected to a single stressor (22 mg/dL), two consecutive stressors (28 mg/dL), and three consecutive stressors (63 mg/dL). Plasma osmolality decreased from 282 to 269 mosmol/kg (compared with unstressed levels of 279 mosmol/kg) when minnows were subjected to three consecutive stressors, indicating moderate osmoregulatory dysfunction. Plasma glucose and osmolality returned to unstressed levels within 48 h, indicating that the species can regain its physiological homeostasis within a relatively short time as long as the stressors are reasonable in duration and intensity.

The Rio Grande silvery minnow *Hybognathus amarus* was once the most widespread and predominant species in the Rio Grande basin (Bestgen and Platania 1991). Its original distribution ranged throughout the larger order streams of the Rio Grande basin, including the Rio Chama, Pecos River, and from the middle Rio Grande to the Gulf of Mexico. Habitat alterations from water diversion for irrigation of agricultural lands adjacent to the Rio Grande may be the most substantive factor in the species' decline. A low-flow conveyance canal was constructed during the 1950s adjacent to the river beginning at San Acacia and flowing downstream to Elephant Butte Reservoir. The canal resulted in habitat modification and fragmentation by reducing water flow, drying large reaches of the river. Within 40 years, the species inhabited less than 7% of its historical range from Cochiti Reservoir downstream to Elephant Butte Reservoir (Bestgen and Platania 1991). In 1994 the minnow was listed as federally endangered by the U.S. Fish and Wildlife Service (USFWS 1994).

The Rio Grande silvery minnow recovery plan (USFWS 1999, 2007) was developed to reestablish, stabilize, and enhance populations within its former range. The recovery plan resulted in a series of initiatives designed to guide the species' captive propagation and augmentation and to salvage it from river intermittency, where "propagation" focuses on the establishment and management of broodstock in fish culture systems, "augmentation" contributes to self-sustaining minnow populations in the middle Rio

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Grande, and "salvage" is the emergency translocation of the minnow from isolated and drying pools to flowing reaches in an effort to reduce fish loss.

In accordance with each portion of the recovery initiatives, the minnow is inevitably subjected to various physical stressors without recovery between disturbances (e.g., netting, handling, confinement, transport). Cumulative effects of multiple stressors can be lethal to fish even though the effect of each stressor may only be sublethal (Barton and Iwama 1991). The intensity and duration of stressors, as well as preexisting conditions, probably have cumulative effects and ultimately result in increased susceptibility to disease. Thus, characterization of the physiological response to individual and cumulative effects of stressors associated with conservation efforts was undertaken to assist with species recovery.

Our objective was to characterize the minnow's physiological stress response and time to recovery when subjected to a series of stressors, both individually or consecutively without time for recovery between each stressor. Although the intent of conservation and recovery efforts is to improve a species' chances for survival, the stress associated with management efforts is inevitable. It is our intent that the results of this research would be used to modify management practices and thereby minimize stress effects on the Rio Grande silvery minnow and improve its chances for survival.

## Methods

*Experimental fish.*—Adult Rio Grande silvery minnow (2004 brood year from wild broodstock, reared in captivity for 2 years, mean total length and weight, 8.56 mm and 5.70 g) were obtained from the A-Mountain Fish Culture and Research facility at New Mexico State University. Before each experiment, the fish were transferred to a water recirculating system (total volume, 3,300 L) containing 48 glass aquaria (38 L). Well water was fed into a 950-L sump tank. A 1/8-hp pump moved the water through a bubble bead filter, then into a Rainbow Lifegard UV97 ultraviolet-light sterilizer (both by Aquatic Eco-Systems, Inc., Apopka, Florida), and on to a 378-L head tank aerated with a 122-cm bioweave diffuser hose.

Fish were acclimated to the recirculating system at a density of 5 kg/m$^3$ for 4 weeks before each experiment. Fish were maintained on a photoperiod of 12 h light and 12 h dark and fed three times each day (0900, 1300, and 1700 hours) at 1.5% body weight/d. Fish were offered a diet formulated for the species (Caldwell et al. 2005). Water quality was monitored daily. Nitrite (mg/L) and ammonia (total N; mg/L) were monitored from the sump tank and aquaria using a HACH (DR/2010) spectrophotometer (Hach Chemical, Loveland, Colorado). Dissolved oxygen (mg/L) and temperature (°C) were monitored daily using a dissolved oxygen meter (Yellow Springs, Ohio).

*Sample collections.*—Before assessing effects of acute stress in Rio Grande silvery minnow, the diurnal rhythm of plasma cortisol was characterized to assess its daily variations associated with normal endocrine function (S. J. Cho, unpublished data). There were no detectable changes in the circadian pattern of the minnow's plasma cortisol throughout 28 consecutive hours of sampling at 4-h intervals (i.e., 0800, 1200, 1600, 2000, 2400, 0400, 0800, and 1200 hours).

To avoid altering endogenous plasma cortisol concentrations, feeding was withheld 24 h before each experiment. A series of pilot studies revealed elevated stress responses in fish when repeatedly disturbed in the same aquarium during sampling. Thus, to prevent increase in plasma cortisol by repeated sampling from the same aquarium, five fish were sampled only once from each aquarium in all experiments. These five fish were pooled to form one replication in replicates of three aquaria ($N = 3$) for each recovery time. Concurrent with the treatment groups, three replicates of five fish were pooled per replicate to characterize plasma cortisol, glucose, and osmolality for each control (unstressed fish) in all experiments. Fish were quickly captured by a dip net and anesthetized in a solution of tricaine methanesulfonate (MS-222; 200 mg/L of water) to avoid additional sampling-related stressors. Fish were completely anesthetized within 30 s. The anesthetic not only immobilized the fish but also prevented additional elevation of plasma cortisol associated with sampling procedures (Wedemeyer et al. 1990; Barton and Iwama 1991). The caudal peduncle of the fish was severed by a Number-22 surgical blade, and blood from the severed hemal arch was collected using a heparinized microhematocrit capillary tube. Total time to complete blood collection after initial capture of fish was less than 5 min. After each fish was bled, it was euthanatized in a lethal dose of MS-222. Hematocrit tubes containing the pooled blood samples from five fish were centrifuged at 14,000 × gravity for 5 min at 12°C. The composited plasma was separated from the packed red cells and stored at −80°C until analyses were performed.

*Experiment 1 (30-s handling).*—Two weeks before the experiment, 30 fish placed in each of three aquaria composed the treatment group. Within each of the three aquaria, fish were captured and held in a dip net exposed to air for 30 s. Fish were immediately apportioned equally into six aquaria (five fish/aquarium) to assess their recovery. These five fish were

sampled and pooled at 0.5, 1, 3, 6, 12, and 24 h posthandling. Two weeks before the experiment, the control group was represented by five fish released into each of 18 aquaria for sample collections at 0.5, 1, 3, 6, 12, and 24 h in replicates of three. Fish representing the control group were sampled concurrently with the treatment group in the same manner as the handled fish. Final density posthandling was 2 kg/m$^3$ for both treatment and control groups.

*Experiment 2 (3-h confinement).*—For each replicate, 35 fish in each of three aquaria ($3.5 \times 14.5 \times 24.5$ cm) were confined at a density of 100 kg/m$^3$ for 3 h by crowding them via a screen (5-cm bar wire mesh) into a reduced portion of each aquarium. Confinement was conducted in the same aquaria to eliminate the stress effects of moving fish among aquaria. During confinement, dissolved oxygen concentration was maintained at saturation. After 3 h confinement, five fish were immediately sampled from each of the three replicates to reflect time 0; the 90 remaining fish (30/treatment aquarium) were released into 18 recovery aquaria (5 fish/aquarium, density of 2 kg/m$^3$). Five fish were sampled and pooled at 1, 3, 6, 12, 24, and 48 h postconfinement in replicates of three. Unstressed fish (5 fish/aquarium) representing the control group were sampled concurrently with the treatment group in replicates of three for a total of 105 untreated fish. Plasma from each sample of five fish in both treatment and control groups was pooled for analyses.

*Experiment 3 (3-h transport).*—For each replicate, 35 fish were dip-netted from an aquarium and confined at a density of 40 kg/m$^3$ within a plastic bag saturated with dissolved oxygen concentrations (7.6 mg/L) and containing 0.5% of NaCl; this mimicked transport conditions used by federal personnel. Each plastic bag containing fish was placed within its own ice chest to maintain independence of each experimental unit. After 3 h of transport, posttreatment processing followed that used in experiment 2.

*Experiment 4 (multiple stressors).*—Three groups of fish were subjected to one of three different combinations of physical stressors: single stressor (30-s handling), double stressor (30-s handling and 3-h confinement without recovery between stressors), and (3) triple stressor (30-s handling, 3-h confinement, and 3-h transport without recovery between stressors). Three replicates were used for each treatment group as well as for the control group at each poststress time. Each replicate used plasma samples pooled from five fish within each aquarium. For the single stressor, 30 fish were held in a dip net and exposed to the air for 30 s. For the double stressor, 30 fish were held in a dip net for 30 s and then immediately confined for 3 h at a density of 100 kg/m$^3$. For the triple stressor, 30 fish were held in a dip net for 30 s, confined for 3 h at a density of 100 kg/m$^3$, and then transported for 3 h at a density of 40 kg/m$^3$. At the end of each stressor combination, fish were released into aquaria (five fish/aquarium, density of 2 kg/m$^3$). Blood samples were taken immediately upon release from the combinations of stressors (time 0), and at 3, 6, 12, and 48 h poststress. For the single stressor, the first blood sample was taken at 0.5 h poststress in replicates of three. Because of limited time, control fish were sampled at different times than treatment groups. Fish representing controls were sampled at 1.5, 8.5, 15.5, and 25.5 h throughout recovery in replicates of three because the time required to collect, anesthetize, and bleed the treated fish precluded concurrent analysis of the control group.

*Analyses of plasma constituents.*—Plasma cortisol was measured using the DPC Coat-A-Count radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, California). The assay relies on competition between nonradioactive cortisol (unlabeled cortisol) and radioactive cortisol ($^{125}$I-labeled cortisol) for the binding sites, which are cortisol-specific antibodies coated in a polypropylene tube. Nonradioactive cortisol from samples and radioactive cortisol labeled with radioactive iodine ($^{125}$I) were added to the antibody-coated tubes. Binding of nonradioactive cortisol to the binding sites in the tube prevents the binding of $^{125}$I-labeled cortisol. Endogenous cortisol concentration is inversely proportional to $^{125}$I-labeled cortisol remaining in the tubes. The radioactivity of $^{125}$I-labeled cortisol remaining in the tube was measured by a gamma counter (Cobra Π Auto-Gamma, Packard Bioscience Company, Connecticut). Analytical sensitivity of the assay was 2 ng/mL. Intraassay and interassay coefficients of variation (CV = 100 × standard deviation/mean) were measured to validate precision of the cortisol assay. Intraassay CV was 12.7% ($N = 15$) for experiments 1, 2, and 3 and 13.5% ($N = 6$) for experiment 4. Interassay CV for all experiments was 9.6% ($N = 4$). Plasma glucose was measured colorimetrically (Stanbio Glucose Liqui-Color, Procedure 1070, Boerne, Texas) based on glucose oxidase and peroxidase, with phenol and 4-aminoantipyrine to form a red-violet quinone complex (Keilin and Hartree 1948). Plasma osmolality was measured using a vapor pressure osmometer (5520 VAPRO, Wescor, Inc. Logan, Utah) calibrated with manufacturer's standard solutions before each use.

*Data analysis.*—Two-way analysis of variance (ANOVA) was conducted in SAS (version 9.1) for each single-stress experiment to compare response variables (cortisol, glucose, osmolality) among treatments (including the control group) and to compare

each response variable over time. Unless otherwise indicated, the time–treatment interaction was nondetectable. A detectable time–treatment interaction would be expected to occur if initial effects of stress are apparent, yet recovery occurs within the time frame studied. Tukey posthoc analysis was used to identify where differences occurred. A two-way ANOVA was used in experiment 4 to compare each response variable among the three treatments and to compare each response variable across time. As previously described, control fish in the multiple stress experiment were collected at different times from the experimental treatments. Once no detectable differences over time were confirmed, a 95% confidence interval for the control was presented for comparison with treatments. Statistical detectability was determined at $\alpha = 0.05$ for all tests. Normality and equal variance assumptions were checked by examining residuals. For the cases in which unequal variances were evident, Welch's (1951) variance-weighted $F$-statistics were reported.

## Results

*Experiment 1 (30-s Handling)*

Detectable differences were not observed for plasma cortisol between treatment and control groups throughout the 24-h recovery period ($F_{1,24} = 1.9$, $P = 0.1819$). Plasma cortisol concentrations for the control group were similar to those obtained for the treatment group within 0.5 h of the handling stress (Figure 1). We suspect the fish were disturbed by our activity around the experimental aquaria in preparation of the sample collection and modified subsequent sampling protocol to minimized activities in proximity to the fish in the remaining confinement, transport, and multiple stress experiments. Within 3 h of recovery, there was a detectable time effect ($F_{5,24} = 8.4$, $P = 0.0001$), plasma cortisol concentrations declining by nearly 50% to 75.1 ng/mL (SE, 11.42) in the treatment group and 58.9 ng/mL (SE, 11.36) in the control group.

Detectable differences were observed for plasma glucose between the treatment and control groups ($F_{1,24} = 13.7$, $P = 0.0011$) but not over time ($F_{5,24} = 0.6$, $P = 0.7026$). Within 0.5 h of a 30-s handling stressor, concentration of plasma glucose increased to its highest value, 60.4 mg/dL (SE = 9.89) compared with the control group (36.0 mg/dL, SE = 1.52; Figure 1). Detectable differences were also observed for plasma osmolality between the treatment and control groups ($F_{1,24} = 10.4$, $P = 0.0036$). No time effect or time–treatment interaction was identified ($P > 0.10$ for both). Within 1 h posthandling stress, plasma osmolality was lower in stressed fish than control fish and remained that way throughout the 24-h recovery (Figure 1).

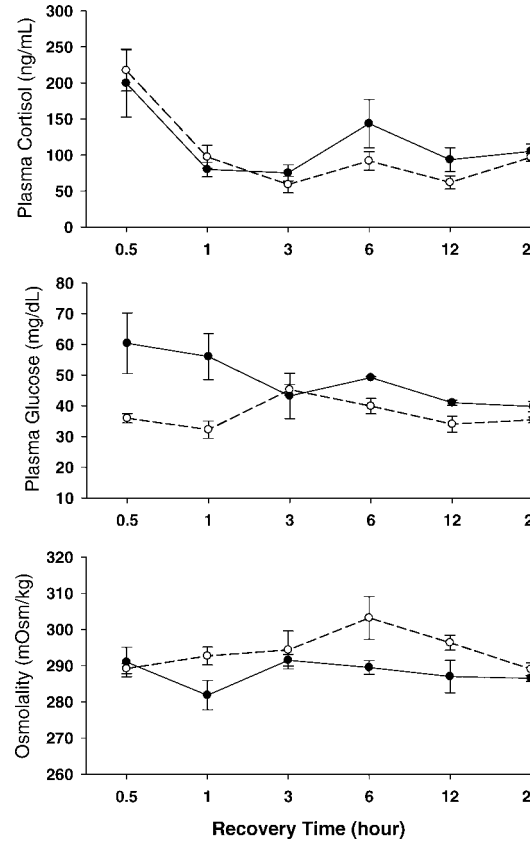


FIGURE 1.—Plasma cortisol and glucose concentrations and osmolality of Rio Grande silvery minnow after 30 s of handling. Treatment (solid circles) and control (open circles) groups are represented by means and SE bars based on three replicates and five fish per replicate.

*Experiment 2 (3-h Confinement)*

Despite modifications in the sampling protocol to reduce disturbances around the experimental aquaria, concentrations of plasma cortisol were not detectably different between treatment and control groups ($F_{1,28} = 3.05$, $P = 0.0916$). A detectable time effect was observed (3 h versus 48 h poststress; Tukey posthoc analysis; Figure 2). Detectable differences were observed for plasma glucose between the treatment and control groups ($F_{1,33.1} = 10.4$, $P = 0.0029$). Plasma glucose concentrations for fish subjected to 3 h of confinement were nearly double those of control fish after 1 h of recovery. Plasma osmolality did not differ between treatment and control groups throughout the 48-h recovery ($F_{1,28} = 1.4$, $P = 0.2442$) nor did they differ over time ($F_{6,28} = 0.9$, $P = 0.4851$).

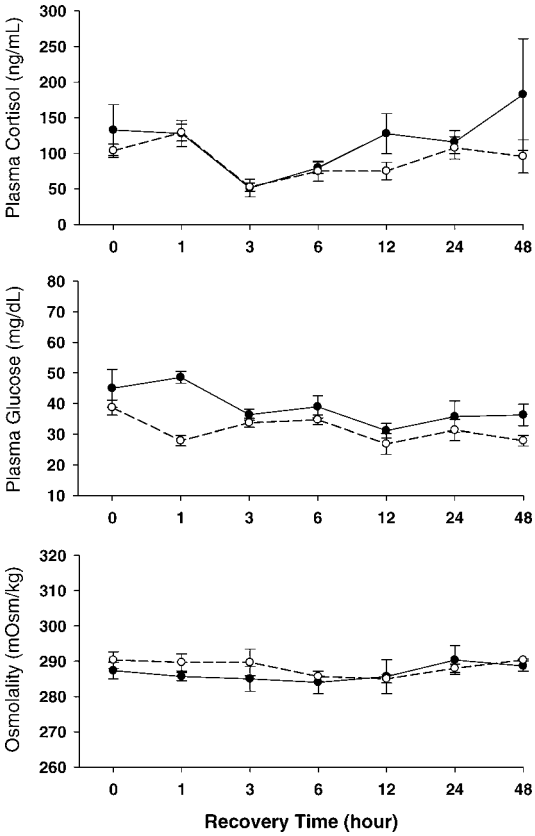


FIGURE 2.—Plasma cortisol and glucose concentrations and osmolality in Rio Grande silvery minnow after 3 h of confinement. Treatment (solid circles) and control (open circles) groups are represented by means and SE bars based on three replicates and five fish pooled per replicate.

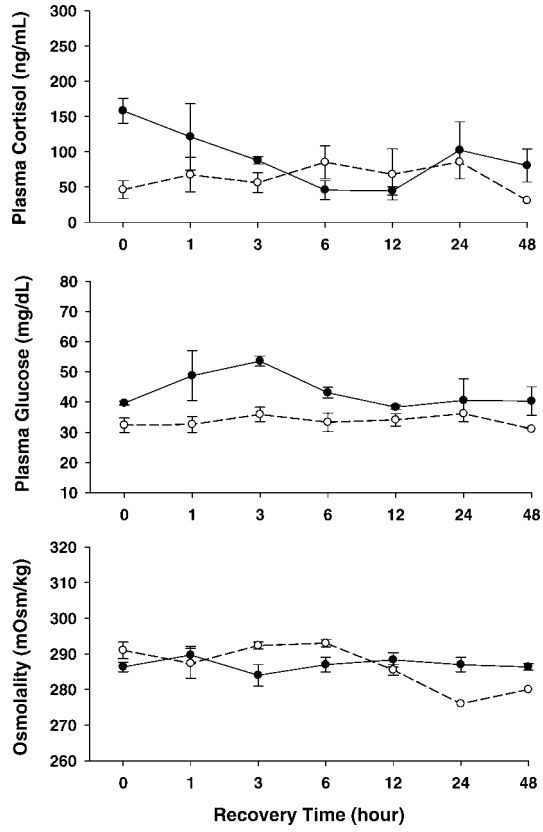


FIGURE 3.—Plasma cortisol and glucose concentrations and osmolality in Rio Grande silvery minnow after 3 h of transport. Treatment (solid circles) and control (open circles) groups are represented by means and SE bars based on three replicates and five fish pooled per replicate.

*Experiment 3 (3-h Transport)*

There were no detectable treatment ($F_{1,17} = 3.5$, $P = 0.0798$) or time effects ($F_{4,17} = 1.2$, $P = 0.3540$) for plasma cortisol concentration. A notable interactive effect, however, was evident between treatment and time ($F_{4,17} = 3.1$, $P = 0.0447$) immediately after transport (time 0), the treatment group (158.2 ng/mL, SD = 17.70) being 3.4 times control group (46.1 ng/mL, SE = 12.74; Figure 3). Detectable differences were observed for plasma glucose between the treatment and control groups ($F_{1,17.8} = 21.3$, $P = 0.0002$); however, no time or time–treatment interaction ($P > 0.10$) was observed. Plasma glucose concentration peaked at 53.5 ng/mL (SE, 1.67) in fish subjected to 3 h transport within 3 h of recovery and was followed by a gradual decrease throughout the remainder of the 48 recovery period. Plasma osmolality did not differ between treatment and control groups or over time throughout the 48-h recovery.

*Experiment 4 (Multiple Stressors)*

Despite overall differences in the severity of the three treatments (single versus two versus three consecutive stressors), concentrations of plasma cortisol were not detectably different among treatments throughout the 48-h recovery period ($F_{2,37} = 0.2$, $P = 0.7814$). There was a time effect, however, as seen by the initial increase and subsequent decrease in plasma cortisol concentrations among treatments ($F_{8,37} = 7.7$, $P < 0.0001$). Within 6 h of recovery, plasma cortisol concentrations had declined in all three treatments to levels observed for plasma cortisol concentrations in the control fish (mean = 31.9 ng/mL, 95% confidence interval = 11.19–52.61, $N = 12$; Figure 4). Detectable differences were observed for plasma glucose among the three treatments ($F_{2,38} = 14.4$, $P < 0.0001$), as well as a time effect ($F_{8,38} = 2.3$, $P = 0.0388$). A time–treatment interaction occurred at 3 h of recovery, whereby plasma glucose peaked at 62.6 mg/dL (SE,

12.66) in fish subjected to three stressors compared with 28.0 mg/dL (SE, 4.32) for those subjected to two stressors and 22.0 mg/dL (SE, 2.16) for fish subjected to the single handling stressor. The order of increasing concentrations with increasing severity of stressor for plasma glucose among treatment groups was maintained through 12 h of recovery. Within 48 h, plasma glucose concentrations for all treatments returned to baseline conditions (20.1 mg/dL, SE =1.74, $N = 12$). Plasma osmolality did not differ among the three treatments throughout the 48 h recovery ($F_{2,37} = 0.6$, $P = 0.5794$); however, there was a time effect ($F_{7,37} = 2.7$, $P = 0.0201$) and time–treatment interaction ($F_{7,37} = 2.8$, $P = 0.0180$) for the triple stressor group at 3 h and 6 h (Tukey posthoc analysis). Plasma osmolality had decreased from 282.2 mosmol/kg (SE = 6.74) to 269.2 mosmol/kg (SE = 5.06) by 6 h recovery, well below the overall average of the control fish (279.1 mosmol/kg, SE = 2.46, $N = 12$). Within 48 h of recovery, however, plasma osmolality concentrations in all treatments had returned to concentrations within the 95% confidence limits of the control fish.

## Discussion

The timing of sample collection with regards to the application and assessment of physiological stressors in fish presents one of the most difficult challenges to fisheries biologists. If stress effects in fish are to be adequately characterized, investigators must anticipate the timing in activation of the hypothalamus-pituitary-interrenal (HPI) axis and the subsequent release of cortisol into circulation. Although handling stress resulted in higher plasma cortisol concentrations in the Rio Grande silvery minnow compared with either 3-h confinement or 3-h transport, this does not necessarily reflect that handling is more stressful to this cyprinid. The HPI axis is considered a closed-loop system that controls cortisol release into circulation through negative feedback at the pituitary and other points in the axis. Thus, lower values of plasma cortisol in the minnow subjected to either 3 h confinement or 3 h transport can be attributed to either an attenuated response by cortisol due to a previous release into the blood stream (Rotllant et al. 2000; Barton et al. 2005) or a longer duration of time between induction of the stressor and the sample collection point, during which negative feedback would have occurred to metabolize circulating cortisol (Mommsen et al. 1999).

Despite our efforts to minimize disturbance before initiating the handling and confinement treatments, the subject minnow possibly perceived some unforeseen stressor that resulted in elevated plasma cortisol concentrations in the control fish. Our attempts to minimize disturbances before each experiment were

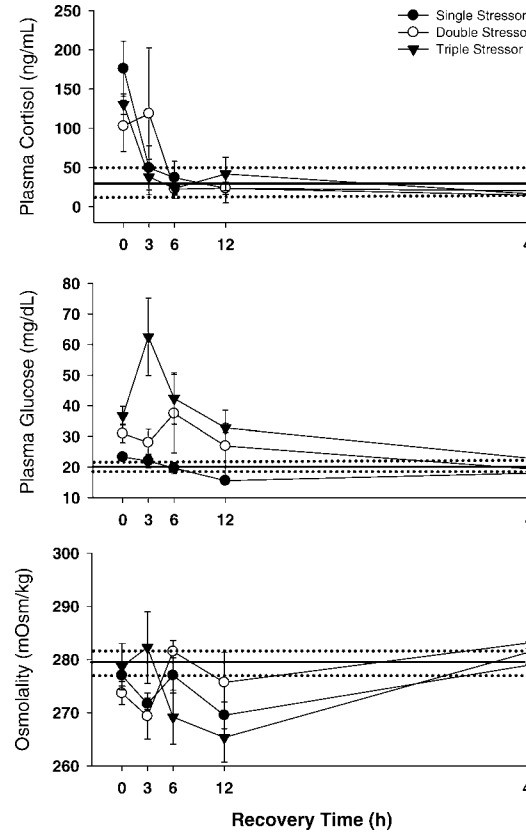


FIGURE 4.— Plasma cortisol and glucose concentrations and osmolality in Rio Grande silvery minnow through 48 h of recovery after fish were subjected to a single stressor (30 s of handling), two stressors (30 s of handling and 3 h of confinement), or three stressors (30 s of handling, 3 h of confinement, and 3 h of transport). Symbols denote averages, error bars SEs. Time 0 refers to the time immediately after release from the stressors. The solid horizontal lines represent average control values over the recovery period, the dotted lines the corresponding 95% confidence intervals based on three replicates and five fish per replicate.

met with lower plasma cortisol values in control fish. Despite our efforts, these uncontrolled disturbances diminished our ability to detect differences between the experimental groups. Although differences were not detected between treatment and control groups, the minnow exhibited the classic primary stress response seen in other teleosts subjected to similar stressors (Barton 2002). When subjected to a 30-s handling stress, the range of cortisol concentrations in the minnow (188–200 ng/mL) was comparable to elevated plasma cortisol observed in pearl dace *Margariscus margarita* (50–230 ng/mL) by Rehnberg et al. (1987), juvenile Chinook salmon *Oncorhynchus tshawytscha* (10–225 ng/mL) by Barton and Schreck (1987), and

gilthead seabream (also known as gilthead bream) *Sparus auratus* (37–139 ng/mL) by Barton et al. (2005). The range of plasma cortisol in the minnow subjected to either the 3-h confinement or 3-h transport (42–158 ng/mL) was comparable to that observed in largemouth bass *Micropterus salmoides* subjected to a 3-h confinement (20–70 ng/mL) by Carmichael et al. (1984a) and coho salmon *O. kisutch* subjected to a 4-h transport (10–160 ng/mL) by Specker and Schreck (1980).

Effects of a consecutive increase in the number of physical stressors applied to the minnow were not manifested by an additive increase in plasma cortisol concentrations. Regardless of the severity of the stressor, the response by the Rio Grande silvery minnow to either individual or consecutive stressors was similar. This was in contrast to a stepwise increase in concentrations of plasma cortisol in olive flounder *Paralichthys olivaceus* subjected to a continuous series of handling and transport stressors (Hur et al. 2007). Although Barton et al. (1986) reported a stepwise increase in plasma cortisol in juvenile Chinook salmon subjected to repeated handling at 3-h intervals, the authors acknowledged negative feedback to the HPI may operate less effectively when fish are provided a brief time for recovery between stressors. We chose to characterize a series of continuous stressors (handling followed immediately by confinement and then by transport) without recovery between each stressor to best mimic typical management practices in recovery efforts.

When subjected to 30 s of handling, 3 h of confinement, or 3 h of transport, the minnow exhibited elevated plasma glucose concentrations that were similar in magnitude among the three stressors (50–70 mg/dL), indicating the severity of each stressor was comparable to one another. Although the magnitude and duration of elevated plasma glucose would be expected to vary depending on the type and severity of stressor, the levels observed here were generally in the range of values reported for a variety of other fish species, including largemouth bass (Carmichael et al. 1984a), gilthead sea bream (Barton et al. 2005), Pacific halibut *Hippoglossus stenolepis* (Haukenes and Buck 2006), and lingcod *Ophiodon elongatus* (Milston et al. 2006). The minnow recovered relatively quickly in response to each stressor as reflected by plasma glucose returning to control or prestress levels within 3 or 6 h.

Cumulative effects of multiple stressors on the secondary stress response of the minnow were observed with increasing concentrations of plasma glucose as additional stressors were applied. Similar to the results reported here, others have also demonstrated that a fish's response to multiple stressors without recovery between disturbances results in a concomitant increase in plasma glucose concentrations (Carmichael et al. 1983; Hur et al. 2007; Minchew et al. 2007). The magnitude of change in plasma glucose was highest when the Rio Grande silvery minnow was subjected to three consecutive stressors followed by two stressors and lastly a single stressor, which presumably indicates additional metabolic costs were incurred as stressors accumulated.

Changes in plasma osmolality are often related to the severity of the stressor (Carmichael et al. 1984b; Barton et al. 2005; Hur et al. 2007). However, sublethal stressors of 30 s of handling, 3 h of confinement, or 3 h of transport were not severe enough to result in osmoregulatory disturbances in the Rio Grande silvery minnow. It was not until stress effects began to accumulate from one to three consecutive stressors that osmoregulatory changes were manifested. A reduction in plasma osmolality was observed in the minnow throughout 12 h of recovery that was comparable with the effect of angling and 5 h of transport in white bass *Morone chrysops* (Allyn et al. 2001) and to osmoregulatory changes observed in largemouth bass handled and transported for 4 h (Carmichael et al. 1984b).

Osmoregulatory changes often provide the most meaningful information regarding the long-term effects of stress in fish. Depending on the severity of the stressor, it may take days or even weeks for recovery of the hydromineral balance in fish (Carmichael et al. 1984b). In this study, we observed plasma osmolality had returned to prestress levels within 48 h poststress. Thus, the severity of multiple sublethal stressors applied without recovery were not sufficient to result in deleterious effects to osmoregulatory processes in the Rio Grande silvery minnow.

Interestingly, we noted a temporary elevation in plasma osmolality in the minnow subjected to three consecutive stressors. In addition to elevated glucose concentrations, the severity of the physical stressors would have presumably resulted in lactic acid build up in tissues, which would have favored the movement of water from the blood compartment to tissues (Okimoto et al. 1994; Wendelaar Bonga 1997). Although we did not measure lactic acid, the shift in solutes among compartments would have contributed to the temporary increase in osmotic pressure at the time of the sample collection.

In summary, a series of single sublethal stressors were not severe enough to result in prolonged primary or secondary stress responses in this imperiled cyprinid. When subjected to a series of consecutive stressors, however, cumulative effects were manifested by elevated plasma glucose and reduced plasma

osmolality levels. Despite the disruptive influence of multiple stressors, the minnow was able to restore circulating glucose and osmoregulatory balance within 48 h.

*Management Implications*

We demonstrated that hatchery-reared Rio Grande silvery minnow recovered relatively quickly (i.e., 6–12 h) from the effects of individual physical stressors, albeit a longer timeframe was needed when recovering from the cumulative effects of the same stressors (i.e., 24–48 h). Although stress associated with management practices of propagation and augmentation efforts for the minnow are simply unavoidable, we recommend reducing the severity and duration of the individual stressors and release the fish only when environmental conditions are deemed favorable to their survival (i.e., establish and adhere to optimal water quality criteria). The Rio Grande silvery minnow exhibited a sensitive and responsive HPI axis, as indicated by elevated concentrations in plasma cortisol in response to unforeseen disturbances before the experimental treatments. These disturbances were not manifested in either plasma glucose or osmolality; thus, throughout propagation and augmentation efforts these response variables may provide a more accurate representation than that of plasma cortisol for the time course of the stress response and subsequent recovery.

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