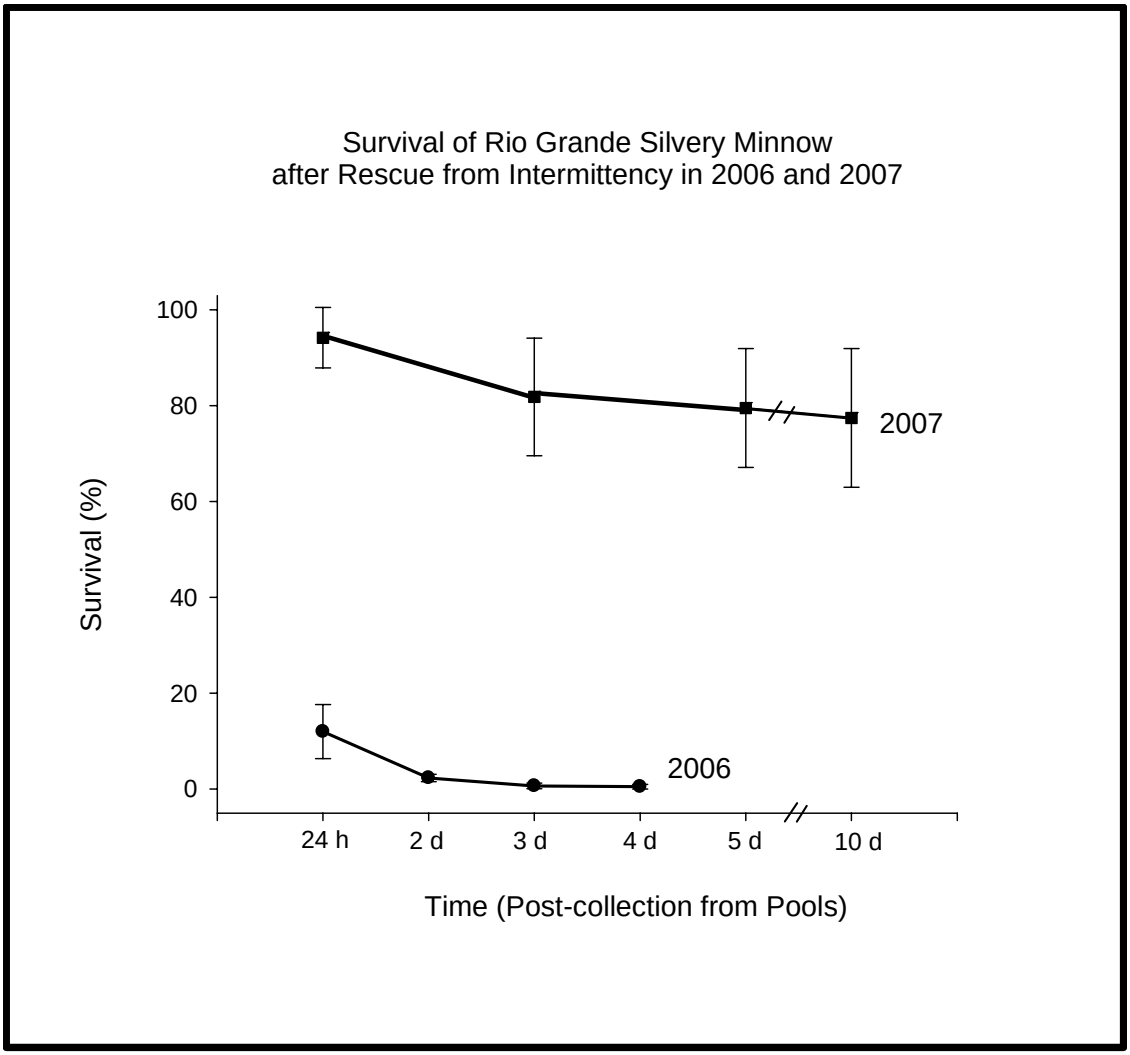
EFFECTS OF PROPAGATION, AUGMENTATION, AND SALVAGE ACTIVITIES ON RECOVERY AND SURVIVAL OF RIO GRANDE SILVERY MINNOW (*HYBOGNATHUS AMARUS*)

FINAL REPORT

to

U.S. BUREAU OF RECLAMATION AND THE MIDDLE RIO GRANDE ENDANGERED SPECIES COLLABORATIVE PROGRAM



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U.S. Bureau of Reclamation and the Middle Rio Grande
Endangered Species Collaborative Program

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In an attempt to re-establish populations of Rio Grande silvery minnow *Hybognathus amarus* within its former range, several initiatives were set forth designed 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 which increases vulnerability of the minnow to opportunistic pathogens and predation, possibly decreasing post-stocking survival. We assessed the physiological stress response to standard management practices by characterizing changes in plasma cortisol, glucose, and osmolality in the minnow. When subjected to the individual stressors of 30 s handling, 3 h confinement (density, 100 kg/m^3), or 3 h transport (density, 40 kg/m^3), and observed throughout recovery, moderate changes in plasma glucose were observed while changes in plasma osmolality were not detectably different. Plasma cortisol peaked at132 ng/mL after 3 h confinement and at158 ng/mL after 3 h transport. Within 6 h post-stress, plasma cortisol returned to unstressed levels. When subjected to consecutive stressors (30 s handling only, 30 s handling plus 3 h confinement, 30 s handling plus 3 h confinement plus 3 h transport), plasma glucose exhibited a cumulative increase that was not observed for plasma cortisol. This increase in plasma glucose was observed within 3 h post stress when 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 265 mOsm/kg compared to unstressed levels (279 mOsm/kg) when the minnow was subjected to three consecutive stressors indicating severe osmoregulatory dysfunction. Plasma cortisol, glucose, and osmolality returned to unstressed levels within 48 h indicating the species can regain its physiological homeostasis within a relatively short time as long as the stressors are reasonable in duration and intensity.

CHAPTER II. EFFECTS OF SALT ON CONFINEMENT AND TRANSPORT-INDUCED STRESS OF RIO GRANDE SILVERY MINNOW

Restoration and propagation efforts of the federally-endangered Rio Grande silvery minnow result in a myriad of stressors that include dipnetting, tagging, seining, handling, confinement, and transport that occur singly or consecutively with very little recovery time between stressful events. And while the fish might appear to recover from the effects of stressful practices, its chances for survival might be drastically reduced. Although salt appears to ameliorate the effects of stress in the species, too little might have no therapeutic effect while too much might have dire consequences. Not all fish species respond similarly to stressful stimuli, thus it is important that each species be carefully evaluated with respect to its tolerance within a range of effective salt concentrations. To assess the success of augmentation efforts, it was necessary to evaluate the effects that a range of salt concentrations during transport would have on the time to recovery once the species was released to the river. We demonstrated that salt levels at or below 0.9% during 3 h transport did not ameliorate the stress response in Rio Grande silvery minnow. The cumulative effects of severe 5 h confinement followed by 3 h transport in 0.0%, 0.5%, 0.9%, and 1.5% salt concentrations resulted in elevated plasma cortisol concentrations that were not detectably different from one another. A detectable increase in plasma glucose indicating secondary stress response and elevated plasma osmolality concentrations indicating osmotic dysfunction occurred in fish hauled in 1.5% salt. Minnow confined and transported in 1.5% salt experienced plasma glucose concentrations 47.2 mg/dL above levels of undisturbed fish of 20.1 mg/dL. Osmolality concentrations of 304.2 mOsm/kg were above baseline levels of undisturbed minnow as well (i.e., 279.1 mOsm/kg). Within 72 h post-stress, the physiological stress responses in the minnow throughout all treatments had returned to pre-stress or baseline concentrations. While we did not demonstrate the therapeutic value of salt in ameliorating the acute effects of transport stress in the Rio Grande silvery minnow, we recommend the continued use of salt (0.5 – 0.9%) throughout propagation and augmentation protocol to reduce the osmotic differential and thus increase chances for survival until a more thorough characterization can be done.

CHAPTER III. INFLUENCE OF CAPTURE, HANDLING, AND TRANSPORT ACTIVITIES ON RECOVERY AND SURVIVAL OF RIO GRANDE SILVERY MINNOW SUBJECTED TO RESCUE FROM RIVER INTERMITTENCY

In 2001, the U.S. Fish and Wildlife Service (Service) determined rescue of Rio Grande silvery minnow from intermittent and drying pools throughout the Isleta and San Acacia reaches in the middle Rio Grande a reasonable and prudent measure to minimize incidental take of the federally endangered fish. Protocol for rescue and transplant of the species was developed and implemented by the Service. This required locating isolated pools, collection, and transport by bucket, amphibious vehicle and distribution truck to optimal habitat of perennial flow. Within the protocol were detailed procedures to minimize stress due to temperature fluctuations, low dissolved oxygen levels, handling, and transport. Additional information, however, was needed to determine the effects of capture and transport, as well as survival of the minnow after release to reaches of the middle Rio Grande containing flowing water.

A systematic evaluation of the fish's physiological responses to rescue activities began in 2005, however, survival was not included for evaluation of long term effects of the Service's salvage and rescue program. Thus, our goal was to assess the physiological responses (i.e., recovery and survival) of wild Rio Grande silvery minnow subjected to collection and transport stressors associated with rescue activities. We describe the results of two years of field studies conducted the summers of 2006 and 2007 with Service personnel during the collection and translocation of wild minnow from intermittent pools to perennial areas within the middle Rio Grande.

In July of 2006, we characterized the magnitude and duration of the physiological stress response and survival of the minnow subjected to standard collection and transport activities during intermittency within the San Acacia reach. We used a suite of primary (plasma cortisol), secondary (plasma glucose and osmolality) and tertiary indices (parasite and incidence of disease) in wild minnow and compared these responses to those collected from a perennial reach (i.e., flowing water) and subjected to standard collection and transport activities. The cumulative effects of intermittency followed by capture and transport resulted in a greater magnitude of physiological responses with plasma cortisol and glucose concentrations as high as 423 ng/mL and 174 mg/dL, respectively. Within three days of rescue from the isolated pools, survival was less than 1%. The cumulative effects of intermittency as well as exposure within the isolated pools elevated the stress response and susceptibility to opportunistic pathogens. Disease diagnostics revealed heavy infestation of external parasites and bacterial infections. When the same protocol was repeated for the minnow within a flowing reach of the river, we observed lower plasma cortisol concentrations (272 ng/mL) but elevated plasma glucose concentrations (244 mg/dL). When survival of these fish were monitored for up to 18 days post-collection, survival was relatively high (63%) compared to fish collected from the intermittent reach. The results of the study indicated that modification of the rescue protocol was needed.

From July to August 2007, we repeated the work to assess changes to the rescue protocol. The following criteria were strictly adhered to if rescue was to occur within a pool: (1) water temperature must be less than 34^{o}C, (2) dissolved oxygen must be greater than 2.0 mg/L, (3) pH must be less than 9.0, (4) no moribund fish as indicated by lethargy, (5) no observable dead fish, and (6) no fish exhibiting hemorrhagic lesions. If any of the criteria were not met, salvage did not occur from that pool.

We had a unique opportunity to obtain physiological "snapshots" of the fish at the time of rescue from isolated pools. Collection and analysis of fish within a pool only occurred if all criteria for rescue were met. At the time of collection, isolation within a pool was unknown but likely varied between one and three

hours. Plasma cortisol concentrations in the minnow rescued from pools throughout the San Acacia reach in July 2007 varied from 90 to 455 ng/mL. Similarly, plasma cortisol concentrations in the minnow rescued from pools throughout the Isleta reach in August 2007 varied from 200 to 331 ng/mL. Using rescue criteria in the collection of the minnow from isolated pools in the Isleta reach, health diagnostics revealed very little external parasites and no internal disease. Fish rescued from isolated pools were placed into a series of cages to assess physiological response to the cumulative effects of stress on survival. The physiological responses were comparable to those obtained in the previous snapshots in the San Acacia reach in July and reflected moderate stress in fish within the isolated pools at the time of capture. Plasma cortisol and glucose were 200 ng/mL and 62 mg/dL, respectively. Plasma osmolality was 288 mOsmol/kg. Within 24 h of rescue and transport from the pools to the cages, survival was relatively high (94%). Plasma cortisol and glucose had increased to 354 ng/mL and 120 mg/dL, respectively. Plasma osmolality decreased to 230 mOsmol/kg indicating severe osmoregulatory dysfunction. Within 48 h of collection from the isolated pools, the fish had begun to recover as indicated by the return of the plasma variables to levels seen at the time of the collection from pools. Plasma cortisol and glucose had declined slightly to 333 ng/mL and 93 mg/dL, respectively. Plasma osmolality had increased to 251 mOsmol/kg reflecting an attempt by the fish to regain osmotic control. Within 72 h post-collection, survival had begun to decrease throughout the cages (82%). By 10 days post-collection, survival remained relatively stable with an overall average survival of 77.4% by the end of the cage study.

In conclusion, the effects of stressors associated with river intermittency and rescue activities resulted in a cumulative stress response in wild Rio Grande silvery minnow. Fish health diagnostics revealed fish in isolated pools incur a greater risk of exposure and susceptibility to pathogens (parasites and bacteria). We observed the stress response and subsequent disease effects were reduced resulting in greater survival of wild fish rescued from pools during optimal conditions described in modified protocol.

OVERVIEW AND JUSTIFICATION

Rio Grande silvery minnow (*Hybognathus amarus*) was once the most widespread and predominant fish species in the Rio Grande Basin (Bestgen and Platania 1991). Its original habitat ranged throughout the larger order streams of the Rio Grande Basin including the Rio Chama, the Pecos River and the middle and lower Rio Grande through to the Gulf of Mexico. Habitat alterations from water diversion for irrigation might be the most substantive factor in the decline of the species. 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 caused habitat modifications by reducing water flow resulting in large reaches of the river becoming dry. As a result of habitat modification and fragmentation, the species was found in less than 7% of its historic range from Cochiti Reservoir downstream to Elephant Butte Reservoir (Bestgen and Platania 1991) and was formally listed as a federally endangered species in 1994 by the U.S. Fish and Wildlife Service (USFWS 1994).

The Rio Grande Silvery Minnow Recovery Plan (USFWS 1999, 2007) was developed to re-establish, stabilize, and enhance populations within its former range. The Recovery Plan resulted in a series of initiatives designed to guide captive propagation, augmentation, and salvage of the species. Propagation of the species focuses on the establishment and management of broodstock in fish culture systems; augmentation contributes to the re-establishment of self-sustaining minnow populations in the middle Rio Grande by repatriating the species and evaluating restocking success; 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 part of the recovery initiatives, the Rio Grande silvery minnow is inevitably subjected to various physical stressors without recovery between the disturbances (e.g., netting, handling, confinement, transport, isolation in pools, poor water quality). The intensity and duration as well as pre-existing conditions are cumulative in their effects and ultimately result in increased stress and susceptibility to disease. Thus, a characterization of the physiological response to individual and cumulative effects of stressors associated with conservation efforts was undertaken to assist the Service with the recovery of the species.

CHAPTER I
PHYSIOLOGICAL STRESS RESPONSES OF RIO GRANDE SILVERY MINNOW: EFFECTS OF INDIVIDUAL AND MULTIPLE PHYSICAL STRESSORS OF HANDLING, CONFINEMENT, AND TRANSPORT

Introduction

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 resulting in large reaches of the river becoming dry. Within 40 years, the species was found in less than 7% of its historic range from Cochiti Reservoir downstream to Elephant Butte Reservoir (Bestgen and Platania 1991) and was formally listed as federally endangered in 1994 by the U.S. Fish and Wildlife Service (USFWS 1994).

The Rio Grande Silvery Minnow Recovery Plan (USFWS 1999, 2007) was developed to re-establish, stabilize, and enhance populations within its former range. The Recovery Plan resulted in a series of initiatives designed to guide captive propagation, augmentation, and salvage of the species from river intermittency. Species propagation focuses on the establishment and management of broodstock in fish culture systems. Augmentation contributes to self-sustaining minnow populations in the middle Rio Grande. 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, isolation in pools, poor water quality). 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 pre-existing conditions likely 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 individual stressors and consecutively without time for recovery between each stressor. While the intent of conservation and recovery efforts is to improve its chances for survival, the stress associated with management efforts is inevitable. It is our intent that the results of this research could be used to modify management practices to minimize stress effects on the Rio Grande silvery minnow and thereby improve its chances for survival.

The Physiological Stress Response

To better understand the fish's stress response to management activities, we describe below the generalized stress response and the series of physiological changes. A conceptualization of physiological responses to environmental stressors was best described as the generalized stress response by Hans Selye in 1950 with the General Adaptation Syndrome (GAS) and applies to all vertebrates (Selye 1950). Briefly stated, the GAS conceptualizes a series of physiological responses divided into three stages. The following is an adaptation of Selye's GAS and the generalized stress response in fishes (Wedemeyer 1970; Mazeaud et al. 1977; Schreck 1981).

(1) Alarm or Primary Response: The GAS begins with the perception of stressful stimuli by the hypothalamus which triggers the primary response in fish. This includes the release of catecholamines (epinephrine and norepinephrine) and glucocorticosteroids (cortisol) in teleosts. The Sympathetic Autonomic Nervous System stimulates the chromaffin tissue of the anterior kidney to release catecholamines. The primary role of plasma catecholamines is to facilitate oxygen supply to compensate for detrimental effects of stressors by modulating cardiovascular and respiratory functions (Reid et al. 1998). In response to stressful stimuli, the hypothalamic portion of the brain stimulates the pituitary to release adrenocorticotrophic hormone (ACTH) into circulation. Within minutes, ACTH triggers the release of cortisol from the interrenal tissue of the anterior kidney of teleosts. Although target sites for cortisol are varied, glycogen deposits in the liver of teleosts represent an important site of action that results in a secondary metabolic response to the stressor by mobilizing glucose from the energy stores. The primary target sites of mobilized cortisol are gills, intestine, and liver. Hydromineral balance and energy mobilization are two major functions of

cortisol (Wendelaar Bonga 1997). Thus, the purpose of cortisol release is to stimulate a series of compensatory processes necessary to achieve homeostasis or acclimation and thereby compensate for the initial negative effects of stressors.

(2) Resistance or Secondary Response: In the secondary response, metabolic pathways are activated and thereby provoke hematological changes in metabolites (glucose and lactate) and hydromineral (chloride, sodium, potassium) (Barton 2002). Hormones from the primary response initiate a suite of secondary changes including increased cardiac output, increased ventilation rate, stimulated branchial blood flow, increased branchial oxygen diffusion and increased glomerular filtration of the kidney, all of which simultaneously contribute to water and hydromineral balance in fish (Wedemeyer 1996), as well as the re-allocation of energy resources (Carmichael et al. 1984; Barton and Iwama 1991). The energy reserve of liver-glycogen is mobilized, thereby increasing circulating plasma glucose to compensate for the adverse effects of stressors. In particular, elevated plasma glucose provides energy substrate to the gills, brain, and muscles (Reid et al. 1998). The convergence of energy substrates to the main organs, however, decreases the energy supply to other tissues. It is this reallocation of energy supplies from normal life processes that negatively affect immunity, reproduction, and growth (Wendelaar Bonga 1997). Although the physiological responses to stressors are an effort to maintain homeostasis, if the stressors persist then energy repartition is followed by energy draining processes (Schreck 1990).

In addition to the metabolic changes described above, osmoregulatory function in fish can be disrupted. Given the large area of semi-permeable epithelial cells of the gill in direct contact with the hypo-osmotic environment, freshwater fish are constantly challenged by water influx and ion efflux. Hormones involved in osmoregulatory regulation are catecholamines such as adrenaline. This hormone is controlled through the Hypothalamic-Sympathetic-Chromaffin axis to stimulate sufficient uptake and delivery of oxygen to target tissues by increasing cardiovascular and respiratory functions (Reid 1998). By increasing blood flow and gill recruitment, available respiratory surface area at the gill enlarges (i.e., lamellar recruitment) to increase oxygen uptake. However, the increase in gill surface area results in an increase of osmotic influx of water and passive diffusional loss of ions from a freshwater fish to its environment. The result is a dilution of intra- and intercellular fluids of the freshwater fish. Within hours of hemodilution, swelling of erythrocytes and somatic cells occurs which leads to tissue swelling and reduction in ion levels or

ionoregulatory dysfunction. This dysfunction in ion levels is the causative failure (short term and long term) for a variety of physiological processes that include disruption of ion transport processes across permeable membranes (Bond 1996). It is these processes that ultimately result in delayed recovery or death in fish and are often overlooked by managers when evaluating the effects of stressful management practices.

The release of catecholamines at the onset of stress results in an increase in gill permeability and blood flow, resulting in increasing inward diffusion of water at the gills (Wendelaar Bonga 1997). Water influx at the gill results in the dilution of body fluids, disturbing hydromineral balance. Therefore, freshwater fish produce copious dilute urine and actively uptake ions at the gill to offset loss of ions and water gain. Often, the secondary physiological responses may become maladaptive resulting in an increase in branchial gill blood flow which leads to irrevocable osmoregulatory imbalance. If these responses persist, then a third tier of physiological changes become manifested and can be quantitatively measured as the tertiary response, which reflects the integration of physiological changes at the whole organismal level.

(3) Exhaustion or Tertiary Response: If the duration and/or severity of the stressor exceed the tolerance limits of the fish, compensatory physiological changes become maladaptive. Adverse physiological responses occur such as reduction in growth, altered behavior, and increased susceptibility to disease (Wedemeyer 1970). An important tertiary effect at the organismal level is stress-mediated disease (Wedemeyer 1970; Snieszko 1974; Wedemeyer et al. 1984). Disease in fish is not the result of a single event, but the result of multiple interactions between the fish, the pathogen, and the aquatic environment. Most often, stress-mediated diseases are the result of opportunistic pathogens that are ubiquitous or continuously present in the environment (Wedemeyer and Wood 1974). When unfavorable environmental conditions exist, opportunistic pathogens will flourish resulting in disease and death in fish with compromised immunity.

Corticosteroids are known for their anti-inflammatory and immunosuppressive effects in mammals and fish. Elevated blood cortisol levels compromises the fish's immune responses by inhibiting inflammatory reactions and phagocytosis (i.e., reduced lymphocytes and macrophages) and by retarding healing processes (Ellasaesser and Clem 1986; Pickering 1987). The end result is increased susceptibility to disease (MacArthur et al. 1984; Woo et al. 1987). Infectious fish diseases that indicate exceeded tolerance limits to stress include facultative bacterial pathogens such as

aeromonads (Angelidis et al. 1987) and pseudomonads (Wedemeyer and Wood 1974). Protozoan parasites (e.g., *Costia* sp.), mild fungal infections (e.g., *Saprolegnia* sp.), and monogenean trematodes (e.g., *Gyrodactylus*) may not be a problem unless stress exceeds homeostasis capability.

Methods

Experimental fish.- Adult Rio Grande silvery minnow (from 2004 wild stock but reared in captivity for 2 years; total mean length 8.56 cm and mean weight 5.70 g) were obtained from the A-Mountain Fish Culture and Research facility at New Mexico State University (NMSU). Before each experiment, the fish were transferred to a re-circulating system (total volume of 3,300 L or 870 gal) containing forty-eight 38-L (10 gal) glass aquaria. Well water was fed into a 950-L (250 gal) sump tank. A 1/8 h.p. pump moved the water through a bubble bead filter (Aquatic Eco-Systems, Inc., Apopka, Florida) and a Rainbow Lifegard UV97 ultraviolet-light sterilizer (Aquatic Eco-Systems, Inc., Apopka, Florida) attached behind the bead filter. From the sterilizer, water was pumped into a 378 L (100-gallon) head tank aerated with a 122-cm (4 ft) bio-weave type diffuser hose.

Fish were acclimated to the re-circulating 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 per day. 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 (^oC) were monitored daily using a dissolved oxygen meter (Yellow Springs, Ohio). This research received approval from NMSU Institutional Animal Care and Use Committee, the U.S. Fish and Wildlife Service (T&E Permit No. TE046517-0), and the New Mexico Department of Game and Fish (Permit No. 3033).

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 (unpublished data; S.J. Cho). There were no detectable changes in the circadian pattern of the minnow's plasma cortisol throughout 28 h when fish were sampled at 4-h intervals (i.e., 0800, 1200, 1600, 2000, 2400, 0400, 0800, and 1200 hours).

To avoid interrupting daily variation of plasma cortisol, 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 in all experiments, five fish were sampled only once from each aquarium. These five fish were pooled to form one replication in replicates of three aquaria ($n = 3$) for each post-stress time. In addition to the stress treatments, 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 anaesthetized in 200 mg/L solution of tricaine methanesulfonate (MS-222) to avoid additional sampling-related stressors. Fish were completely anaesthetized within 30 s. The anaesthetic 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 surgical blade (No. 22) 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 euthanized in a lethal dose of MS-222. Hematocrit tubes containing the pooled blood samples from five fish were centrifuged at 14,000 × *g* 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 prior to the experiment, 30 fish were placed in three aquaria (30 fish per aquarium) for the treatment group. Within each of the three aquaria, fish were captured and held in a dip net exposed to air for 30 seconds. Fish were immediately apportioned into six aquaria (i.e., five fish per aquarium) to assess their recovery. These five fish were sampled and pooled at 0.5, 1, 3, 6, 12, and 24 h post-handling. Two weeks prior to the experiment, the control group was represented by five fish released into 18 aquaria (i.e., 5 fish per aquarium 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 post-handling was 2 kg/m^3 for both treatment and control groups.

Experiment 2: 3 h confinement.-For each replicate, 35 fish were confined at a density of 100 kg/m^3 for 3 h. Confinement was conducted in the same aquaria to eliminate the stress effects of moving fish among aquaria. Within these aquaria, frames (3.5 × 14.5 × 24.5 cm; same inner

dimension of aquaria) with wire mesh (0.5 × 0.5 cm) were used to crowd and confine the fish. During confinement, dissolved oxygen concentration was maintained at saturation. After 3 h confinement, 5 fish were immediately sampled from each of the three replicates to reflect time 0 while the remaining fish were released into 18 recovery aquaria (5 fish per aquarium with a density of 2 kg/m^3). Five fish were sampled at 1, 3, 6, 12, 24, and 48 h post-confinement in replicates of three. Unstressed fish (five per aquarium) representing the control group was 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 0.5% of NaCl in transport water to mimic 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 transport, 5 fish were immediately sampled from each of the three replicate transport bags to reflect time 0 while the remaining fish were released into 18 recovery aquaria (5 fish per aquarium with a density of 2 kg/m^3). Five fish were sampled at 1, 3, 6, 12, 24, and 48 h post-confinement in replicates of three. Unstressed fish (five per aquarium) representing the control group remained in their respective aquaria throughout the acclimation and transport interval and 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 4: Assessment of the cumulative effects of multiple stressors.-Three groups of fish were subjected to one of three different combinations of physical stressors: (1) single stressor: 30 s handling; (2) 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 post-stress 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 per aquarium with a 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 post-stress. For the single stressor, the first blood sample was taken at 0.5 h post-stress in replicates of three. Due to limited time, control fish were sampled at slightly 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, anaesthetize, 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 non-radioactive cortisol (unlabelled cortisol) and radioactive cortisol (^{125}I-labeled cortisol) for the binding sites, which are cortisol-specific antibodies coated in a polypropylene tube. Non-radioactive cortisol from samples and radioactive cortisol labeled with radioactive iodine (^{125}I) was added to the antibody-coated tubes. Binding of non-radioactive 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. Intra-assay and inter-assay coefficients of variation (CV = 100 x standard deviation / mean) were measured to validate precision of the cortisol assay. Intra-assay CV was 12.7% (n = 15) for experiments 1, 2, and 3 and 13.5% (n = 6) for experiment 4. Inter-assay CV for all experiments was 9.6% (n = 4). Plasma glucose was measured colorimetrically (Stanbio Glucose LiquiColor, Procedure No. 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 by treatment interaction was non-detectable. A detectable time by treatment interaction would be expected to occur if initial effects of stress are apparent, yet

recovery occurs within the time frame studied. Tukey's post-hoc tests were used to identify where differences occurred. A two-way ANOVA was used in the multiple stress experiment 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 $P \leq 0.05$ 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) 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 ± 9.89 mg/dL) compared to the control group (36.0 ± 1.52 mg/dL) (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 by treatment interaction was identified ($P > 0.10$ for both). Within 1 h post-handling stress, plasma osmolality was lower in stressed fish than control fish and remained that way throughout the 24 h recovery (Figure 1).

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 vs. 48 h post-stress) as indicated by a Tukey's post-hoc test (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 confinement were nearly double those of control fish at 1 h recovery (Figure 2). 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).

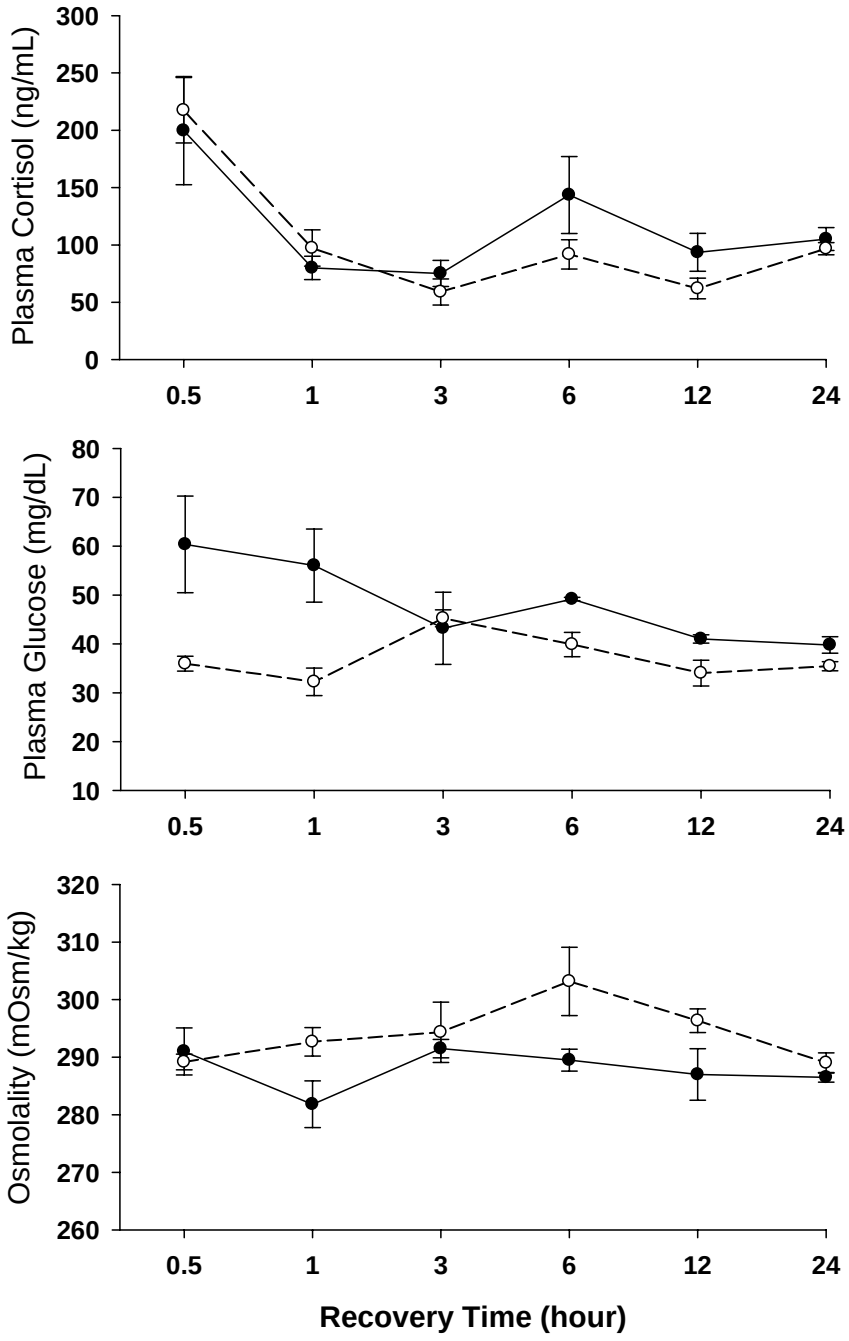

Figure 1.1. Concentrations of plasma cortisol, glucose, and osmolality of Rio Grande silvery minnow after 30 sec handling. Treatment (solid circles) and control (open circles) groups are represented by means and SE bars based on n = 3 replicates with five fish pooled per replicate.

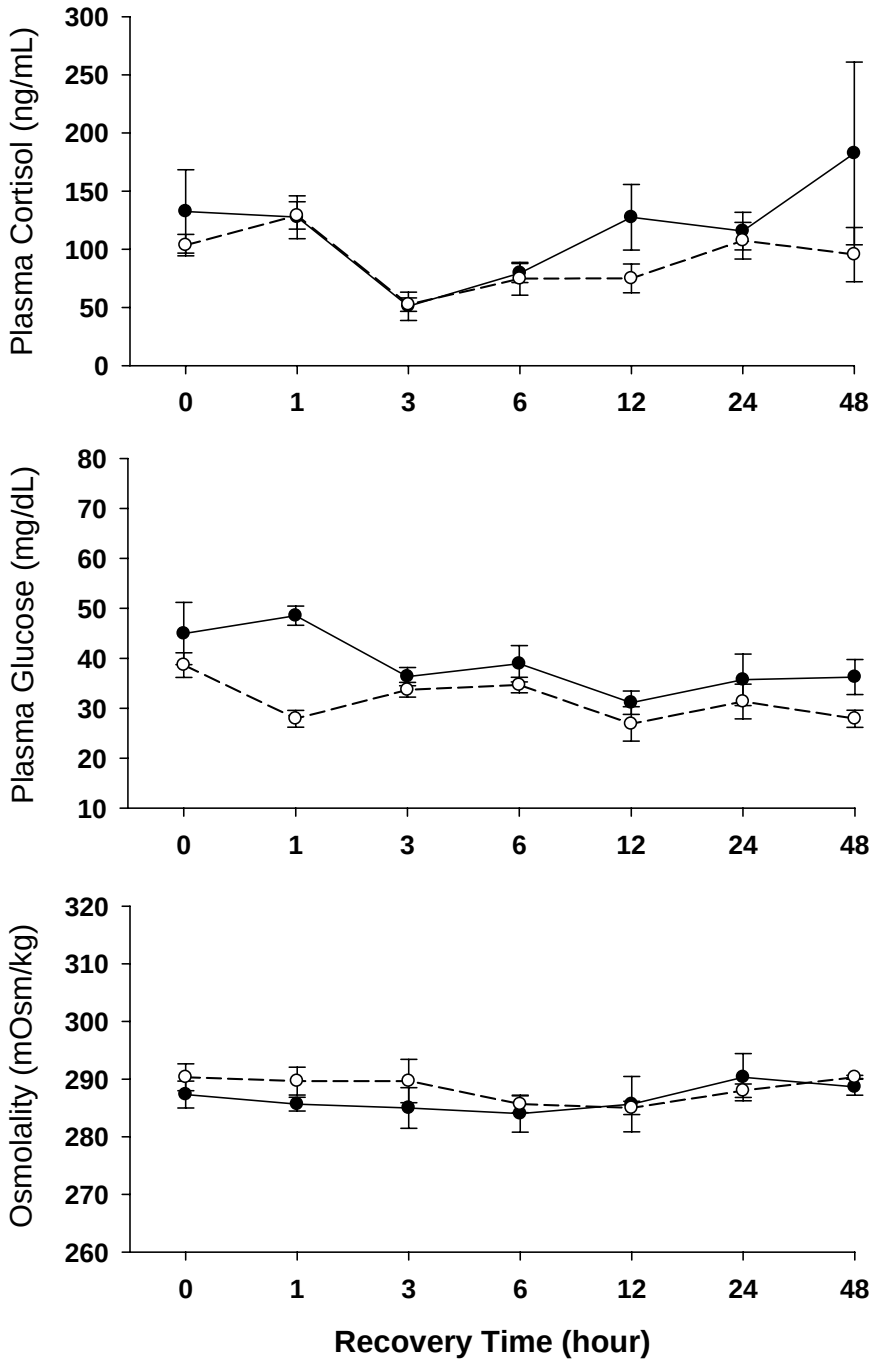

Figure 1.2. Concentrations of plasma cortisol, glucose, and osmolality in Rio Grande silvery minnow after 3 h confinement. Treatment (solid circles) and control (open circles) groups are represented by means and SE bars based on n = 3 replicates with fish pooled per replicate.

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) by a four-fold difference in the treatment group (158.2 ± 17.70 ng/mL) compared to the control group (46.1 ± 12.74 ng/mL) (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 by treatment interaction (P > 0.10) was observed. Plasma glucose concentration peaked at 53.5 ± 1.67 ng/mL within 3 h of recovery and was followed by a gradual decrease throughout the remainder of the 48 recovery period (Figure 3). Plasma osmolality did not differ between treatment and control groups or over time throughout the 48 h recovery (Figure 3).

Experiment 4: Assessment of the Cumulative Effects of 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 and 95% confidence limit; 31.9 ± 20.71 ng/mL, 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 by treatment interaction occurred at 3 h of recovery, whereby plasma glucose peaked at 62.6 ± 12.66 mg/dL in fish subjected to three stressors compared to 28.0 mg/dL (SE = 4.32) when subjected to two stressors and 22.0 mg/dL (SE = 2.16) in fish subjected to the single handling stress (Figure 4). The order of increasing concentrations with increasing severity of stressor for plasma glucose among treatment groups was maintained through 12 h recovery. Within 48 h, plasma glucose concentrations for all treatments returned to baseline conditions (20.1 ± 1.74 mg/dL, 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 by treatment interaction ($F_{7,37}$ = 2.8, P = 0.0180) for the triple stressor group at 3 h and 6 h as indicated by Tukey's post-hoc tests. Plasma osmolality had decreased from 282.2

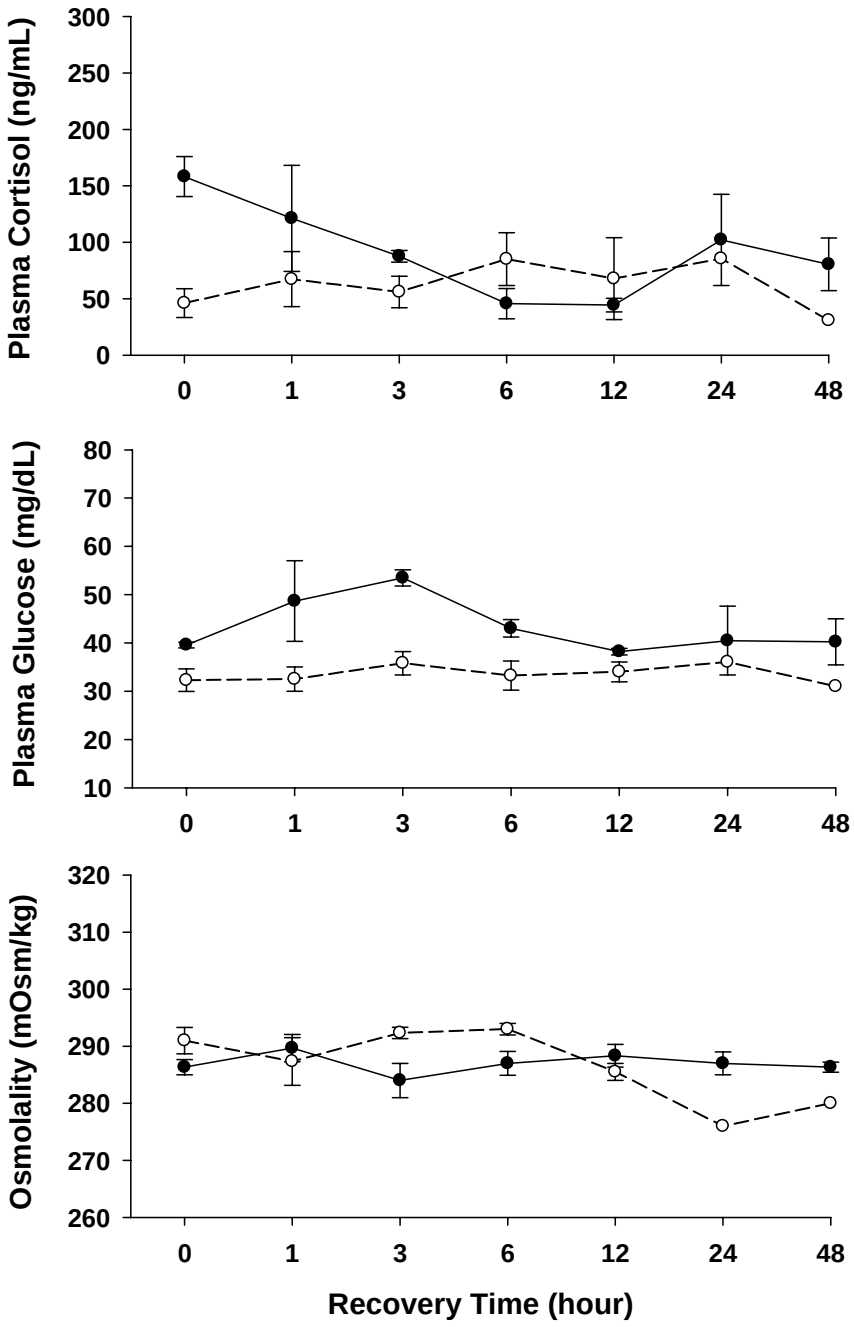

Figure 1.3. Concentrations of plasma cortisol, glucose, and osmolality in Rio Grande silvery minnow after 3 h transport. Treatment (solid circles) and control (open circles) groups are represented by means and SE bars based on n = 3 replicates with five fish pooled per replicate.

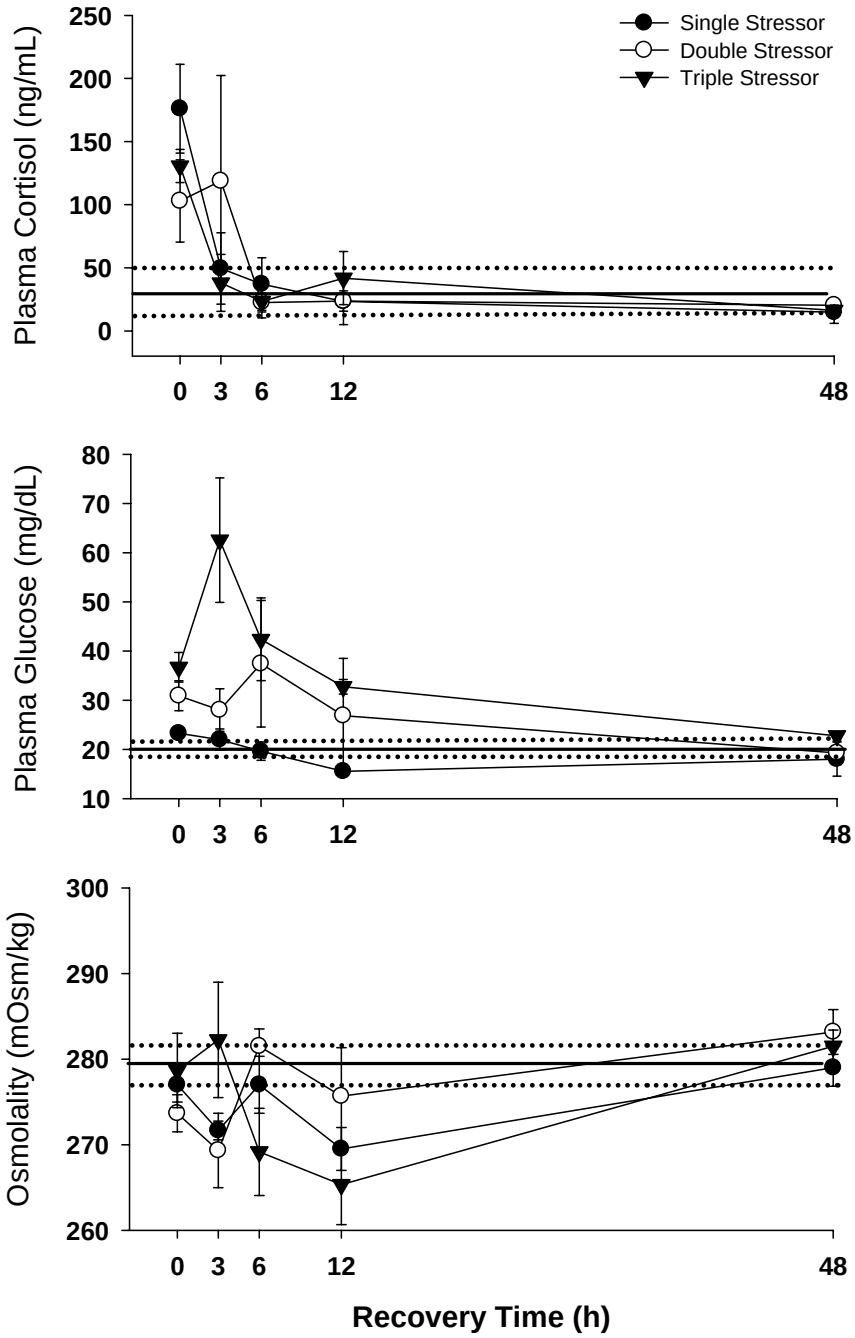

Figure 1. 4.4. Average concentrations and standard error of plasma cortisol, glucose, and osmolality in Rio Grande silvery minnow throughout 48 h recovery after subjected to single stressor (30 s handling), two stressors (30 s handling and 3 h confinement), and three stressors (30 s handling, 3 h confinement, 3 h transport). Time 0 represents fish sampled immediately upon release from stressors. Solid horizontal lines represent averaged control values collected throughout recovery and dotted lines represent 95% confidence intervals for the control based on n = 3 replicates with five fish pooled per replicate.

mOsm/kg (SE = 6.74) to 269.2 mOsm/kg (SE = 5.06) by 6 h recovery well below the overall average of the control fish (279.1 ± 2.46 mOsm/kg, n = 12) (Figure 4). 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. Investigators must anticipate the timing in activation of the hypothalamus-pituitary-interrenal (HPI) axis and the subsequent release of cortisol into circulation if stress effects in fish are to be adequately characterized. 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 the 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 minnow possibly perceived some unforeseen stressor which resulted in elevated plasma cortisol concentrations in the control fish. Our attempts to minimize disturbances prior to each experiment were met with lower plasma cortisol values in control fish. Despite our efforts, these uncontrolled-for 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 *Semotilus margarita* (50 – 230 ng/mL) (Rehnberg et al. 1987), juvenile

Chinook salmon *Oncorhynchus tshawytscha* (10 – 225 ng/mL) (Barton and Schreck 1987), and gilthead sea bream *Sparus aurata* (37 – 139 ng/mL) (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 3 h confinement (20 – 70 ng/mL) (Carmichael et al. 1984a) and coho salmon *O. kisutch* subjected to 4 h transport (10 – 160 ng/mL) (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 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 in the minnow in order to best mimic typical management practices throughout recovery efforts.

When subjected to 30 s handling, 3 h confinement, or 3 h 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 elongates* (Milston et al. 2006). The minnow recovered relatively quickly in response to each stressor as reflected by plasma glucose returning to control or pre-stress 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 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 1984b; Barton et al. 2005; Hur et al. 2007). However, sublethal stressors of 30 s handling, 3 h confinement, or 3 h transport were not severe enough to result in osmoregulatory disturbances in the 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 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 pre-stress levels within 48 h post-stress. 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 plasma glucose and

plasma osmolality but not by plasma cortisol. 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 compared with a longer time frame needed when recovering from the cumulative effects of the same stressors (i.e., 24 – 48 h). While stress associated with management practices of propagation and augmentation efforts for the minnow are simply unavoidable, we suggest reducing the severity and duration of the individual stressors and release the fish only when environmental conditions are deemed favorable to the minnow's 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 prior to the experimental treatments. These disturbances were not manifested in either plasma glucose or osmolality and thus these variables may provide a more accurate representation than that of plasma cortisol for the time course of the stress response and subsequent recovery throughout propagation and augmentation efforts.

CHAPTER II

EFFECTS OF SALT ON CONFINEMENT AND TRANSPORT-INDUCED STRESS OF RIO GRANDE SILVERY MINNOW

Introduction

Salt (NaCl) is undeniably one of the most important therapeutic agents in the prevention and treatment of fish diseases. Adding salt to the transport or holding tank removes excess mucus and debris associated with ectoparasite infestation and thereby facilitates oxygen diffusion across the gill surfaces increasing the effectiveness of therapeutic chemicals to eliminate pathogens. Salt is often used in the hauling or holding water to alleviate electrolyte disturbance between fish and the environment during stressful activities. In freshwater fish, stress will result in a dilution of the plasma through the net influx of water and loss of hydrominerals such as monovalent ions (Na^+ and Cl^-) at the gills (Wedemeyer 1996; McDonald and Milligan 1997). Increasing the salinity and thereby the osmotic pressure of the freshwater environment to that of the fish's blood facilitates compensatory regulation of salts (ionoregulation) and water (osmoregulation) to offset ion efflux and water influx at the gills, respectively. Salt can be used indefinitely with freshwater fish at concentrations similar or isotonic with respect to the fish's blood which can range from 0.8% to 1.2%. At higher concentrations (> 1.5%), salt can be used for short periods of time (1 – 3 h) as a treatment for external pathogens (Post 1987; Noga 2000).

Fish are often subjected to a variety of stress-related culture and management practices. Once the fish perceives a stressful stimulus, the stress response is initiated by the release of catecholamines (adrenalin and noradrenalin) (Mazeaud et al. 1977). These chemical messengers target the gills to increase blood flow through the secondary lamellae thereby increasing dissolved oxygen diffusion from the environment via lamellar recruitment. In freshwater fish, however, the tradeoff for this adaptation is the increase in potential loss of ions to the environment and osmotic flow of water into the fish. The severity of the stress is often reflected in the magnitude and duration of the osmo- and ionoregulatory disturbance and has been identified as one of the primary mechanisms associated with post-stocking mortality in fish (Barton et al. 2003). Decades ago, the relative importance of this physiological process was realized and the application of salt has become

a standard and cost effective tool to ameliorate the effects of stress in fish (e.g., Miles et al. 1974; Hattingh et al. 1975; Nikinmaa et al. 1983; Carmichael et al. 1984).

Propagation and restoration efforts result in a myriad of stressors to Rio Grande silvery minnow that include dipnetting, tagging, seining, handling, confinement, and transport that occur singly or consecutively with very little recovery time for the fish between stressful events. When Rio Grande silvery minnow are transported to other propagation facilities, ponds, or release to the wild, the fish are netted into transport tanks containing salt and then released into freshwater (tanks, pond, or river). And while the fish might appear to recover from the effects of stressful practices, chances for survival may be drastically reduced (Wood et al. 1983). Although salt appears to ameliorate the effects of stress in the species, too little may have no therapeutic effect while too much may have dire consequences. Not all fish species respond similarly to stressful stimuli, thus it is important that each species be carefully evaluated with respect to its tolerance within a range of effective salt concentrations.

To assess the success of management efforts for the Rio Grande silvery minnow, it was necessary to evaluate the effects that a range of salt concentrations during transport would have on the time to recovery once the species was released to the river. A salt concentration as low as 0.5% has been shown to ameliorate the effects of transport stressors in walleye (Barton and Zitzow 1995; Forsberg et al. 2001) and rainbow trout (Barton and Peter 1982) and is often the concentration selected when uncertainty is high regarding salt levels that are isotonic with respect to the fish's blood. Anecdotal observations by culturists of the Rio Grande silvery minnow have noted that salt levels as high as 1.5% for brief periods of time during prophylaxis treatment for disease has met with success in increasing survival (pers. comm. M. Ulibarri). Thus, the objective of this study was to evaluate a range of salt concentrations in ameliorating the physiological stress response in Rio Grande silvery minnow. Severe confinement and long term transport were chosen as the stress challenges because they typical of standard propagation and restoration practices throughout many recovery programs.

Methods

Experimental Fish and System.-Adult minnow (from 2004 wild stock reared in captivity; total mean length 8.68 cm and mean weight 5.90 g) were obtained from the A-Mountain Fish Culture and Research facility at New Mexico State University. Four weeks prior to the experiment, fish were transferred to a water recirculating system (total volume, 3300 L) containing 48 glass aquaria (38 L). Well water was fed into a 950-L (250-gallon) sump tank. A 1/8 h.p. pump moved the water through a bubble bead filter, then 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 bio-weave diffuser hose. Fish were maintained on a photoperiod of 12 h light and 12 h dark and fed three times each day (0900, 1300, 1700) at 1.5% body weight/day. Fish were offered a diet formulated for the species (Caldwell et al. 2009). Nitrite (mg/L) and ammonia (total N; mg/L) were monitored daily from the sump tank and aquaria using a HACH (DR/2010) spectrophotometer (Hach Chemical, Loveland, Colorado). Dissolved oxygen (mg/L) and temperature (oC) were monitored daily using a dissolved oxygen meter (Yellow Springs, Ohio. This research received approval from NMSU Institutional Animal Care and Use Committee and the U.S. Fish and Wildlife Service (T&E Permit No. TE046517-0) and the New Mexico Department of Game and Fish (Permit No. 3033).

Salt Concentrations.- Prior to the experiment, we empirically derived the NaCl concentration closest to the osmotic pressure of the minnow's plasma by preparing a range of salt concentrations (0.5%, 1.0%, 1.2%, 2.0%) using reagent-grade NaCl (Sigma Chemical Co.). From this range, we constructed a standard curve for osmolality or osmotic pressure using the vapor pressure osmometer (Wescor, Inc.). Osmolality reflects the number of un-dissociated solute molecules or ions per kg of solvent and can vary in freshwater fishes from 260 to 330 mOsmol/kg (Bond 1996). A 0.9% salt concentration exhibited an osmotic pressure of 281.0 mOsm/kg closest to the osmolality of the undisturbed minnow (mean ± SE; 279.1 mOsm/kg ± 1.12, see Chapter I). We selected the concentration of 0.9% to represent a treatment closest to osmotic potential of the fish's blood (i.e., isoosmotic with respect to the fish's blood). We selected 0.5% (156.2 mOmos/kg) because this salt level is hypotonic with respect to the fish's blood and managers have used this concentration for transport with the assumption that salt levels would be sufficiently high to ameliorate osmotic stress effects. We chose 1.5% (465.4 mOsmo/kg) to represent a salt concentration hyperosmotic with respect to the fish's blood and a concentration that managers have anecdotally shown to have

ameliorative effects of the stress response in captive propagation and augmentation practices for the Rio Grande silvery minnow.

Experimental Design.- Although prior research (see Chapter I) did not identify a diurnal rhythm of plasma cortisol in Rio Grande silvery minnow, the work demonstrated a heightened sensitivity to disturbance around their aquaria resulting in elevated plasma cortisol in control fish. Thus, to prevent an increase in plasma cortisol by repeated sampling from the same aquarium, five fish were sampled only once from each aquarium throughout the experiment. These five fish were pooled to form one replication in replicates of three aquaria (n = 3) for each treatment throughout recovery. Concurrent with the treatment groups, three replicates of five fish were pooled per replicate to characterize plasma cortisol, glucose and osmolality for the controls (unstressed fish). At each sample collection, fish were quickly captured by a dip net and anaesthetized in 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 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 euthanized 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^{o}C. The composited plasma was separated from the packed red cells and stored at –80°C until analyses were performed.

Two weeks prior to the experiment, 25 fish were placed in each of three aquaria within each of the four banks of the experimental system for a total of twelve aquaria or 300 fish. The fish were initially maintained at a low density of 5.0-7.5 kg/m^3. The onset of the experiment began with crowding of the 25 fish via a screen (5-cm wire mesh) into a reduced portion of each aquarium (3.5 x 14.5 x 24.5 cm) to achieve a density of 80 kg/m^3 for five hours. Confinement was conducted within the same aquaria to eliminate the stress effects of moving fish among aquaria. During confinement, dissolved oxygen concentration was maintained at air saturation. After 5 h confinement, fish from each of the twelve aquaria were netted and placed into twelve 2.2 L plastic bags containing either 0.0%, 0.5%, 0.9%, or 1.5% (replicates of 3) salt concentrations at a moderate confinement density of 40 kg/m^3 which was well below the optimum densities of 60 kg/m^3 for transporting fish (Carmichael and Tomasso 1988). These bags were placed into insulated coolers with lids and transported for three

hours. Immediately at the end of transport, blood was collected from a sample of 5 fish from each of the three replicate transport bags for all treatments and represented as time 0 h. The remaining 20 fish from each bag were immediately released into a series of aquaria (5 fish per aquarium) to assess physiological response to the cumulative stressors among the treatments at 3, 6, 12, and 72 h post-stress in replicates of three (see Chapter I for *Analyses of plasma constituents*).

Data analysis.- Two-way analysis of variance (ANOVA) was conducted in SAS (version 9.1) to compare response variables (cortisol, glucose, osmolality) among treatments (including the control group) and to compare each response variable over time. The limited time required to collect, anaesthetize and bleed the fish as well as the limited space precluded the concurrent analysis of a control group with each treatment; therefore, one control group (n = 3) was assessed throughout the experiment at time 0, 3, 6, 12, and 72 h. Unless otherwise indicated, the time by treatment interaction was non-detectable. A detectable time by treatment interaction would be expected to occur if initial effects of stress are apparent, yet recovery occurs within the time frame studied. Tukey's HSD with the SLICE=time statement was used to compare means among treatments at each recovery time to identify where differences occurred. Statistical detectability was determined at $P \leq 0.05$ 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

The cumulative effects of 5 h confinement followed by 3 h transport in 0.0%, 0.5%, 0.9%, and 1.5% salt concentrations resulted in no detectable differences of plasma cortisol among treatments ($F_{3, 38} = 0.75$ $P = 0.5316$). Although there was a notable time effect ($F_{4, 38} = 8.73$ $P < 0.0001$), there was no detectable time by treatment interaction ($F_{12, 38} = 0.96$ $P = 0.4988$) throughout the 72 h recovery (Figure 1). Within 6 h of recovery, plasma cortisol concentrations decreased to levels representative of undisturbed fish (31.9 ng/mL, 2 SE = 18.82, n=12, *see* Chapter I).

In contrast, detectable differences were observed for plasma glucose among the treatments ($F_{3, 38} = 6.75$ $P = 0.0009$) and by time ($F_{4, 38} = 7.04$ $P = 0.0002$) (Figure 2). Within 3 h of transfer

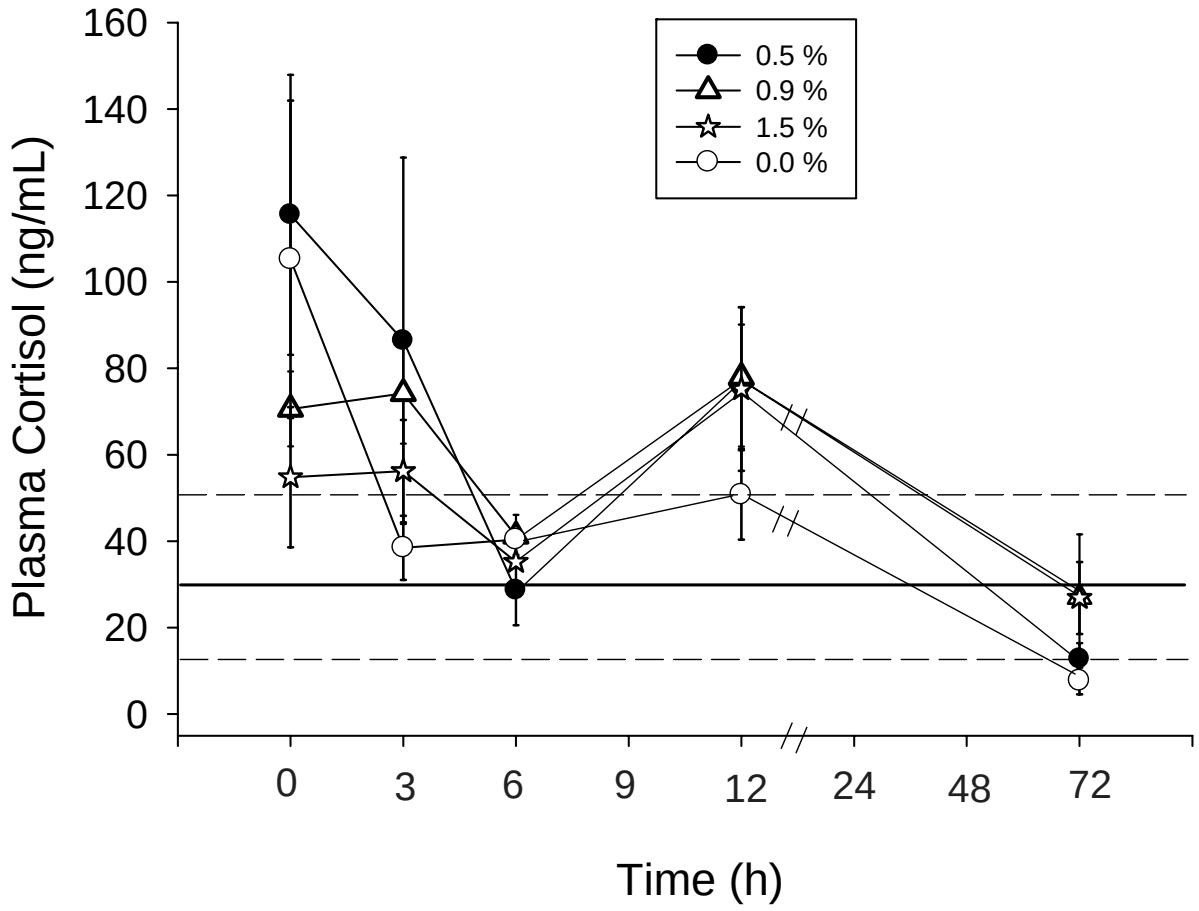

Figure 5.1. Plasma cortisol concentrations (ng/mL) of Rio Grande silvery minnow throughout 72 h recovery in freshwater after confinement for 5 h followed by transport for 3 h in 0.0% 0.5%, 0.9%, and 1.5% salt concentrations. Treatment groups are represented by means and SE bars on three replicates and five fish per replicate. Horizontal lines represent the mean and 95% confidence intervals for plasma cortisol concentration for non-disturbed fish (31.9 ng/mL ± 18.82, n=12).

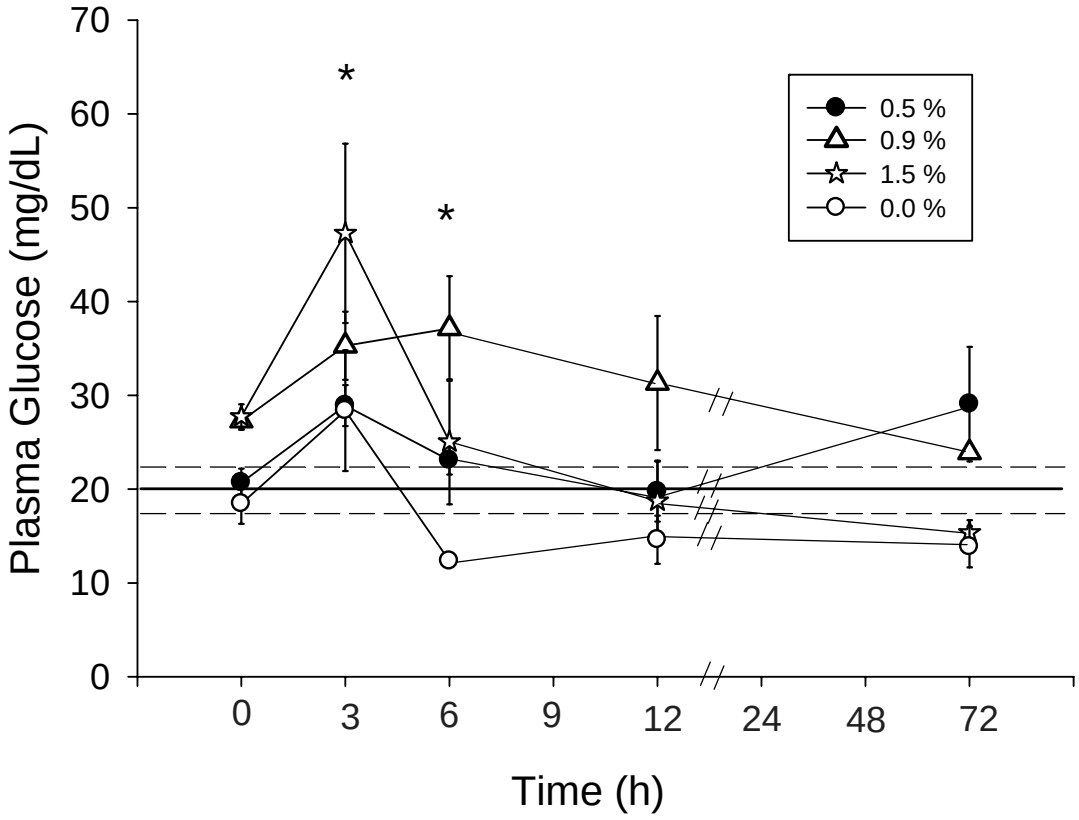

Figure 6.2. Plasma glucose concentrations (mg/dL) of Rio Grande silvery minnow throughout 72 h recovery in freshwater after confinement for 5 h followed by transport for 3 h in 0.0% 0.5%, 0.9%, and 1.5% salt concentrations. Treatment groups are represented by means and SE bars on three replicates and five fish per replicate. The horizontal line represents the mean and 95% confidence intervals for plasma glucose concentration for non-disturbed fish (20.1 mg/dL ± 1.57, n=25). Asterisks represent detectable differences ($P \leq 0.05$) among treatments.

from the hauling containers to aquaria, plasma glucose concentrations had increased in all treatments reflecting the stress of netting from the transport tank to the recovery aquaria. Fish that had been hauled in 1.5% exhibited detectably greater plasma glucose concentrations (47.2 mg/dL ± 9.53) compared to fish hauled in 0.9% (35.3 mg/dL ± 3.64), 0.5% (28.9 mg/dL ± 2.19), and 0.0% (28.4 mg/dL ± 6.44), (P = 0.0147). By 6 h in recovery, plasma glucose concentrations remained elevated in fish transported in 0.9% salt (37.1 mg/dL ± 5.82). By 72 h in recovery, plasma glucose concentrations had declined to levels near undisturbed fish (20.1 mg/dL ± 1.57, n=25; *see* Chapter I).

The cumulative effects of 5 h confinement followed by 3 h transport resulted in detectable differences among the salt treatments for plasma osmolality ($F_{3, 38} = 8.03$ $P = 0.0003$). Plasma osmolality at time 0 h was elevated in fish hauled in 1.5% (304.2 mOsm/kg ± 1.88) compared to fish hauled in 0.0% (293.2 mOsm/kg ± 1.74), 0.5% (293.3 mOsm/kg ± 1.45), and 0.9% salt (295.8 mOsm/kg ± 7.45) (Figure 3). Within 12 h recovery, plasma osmolality in all but the highest salt treatment (1.5%) had declined to osmolality levels representative of undisturbed fish minnow (279.1 mOsm/kg ± 2.24, n=25; *see* Chapter I) (P=0.0442). Mortality was not observed throughout confinement, transport and the entire post-stress recovery period of 72 h.

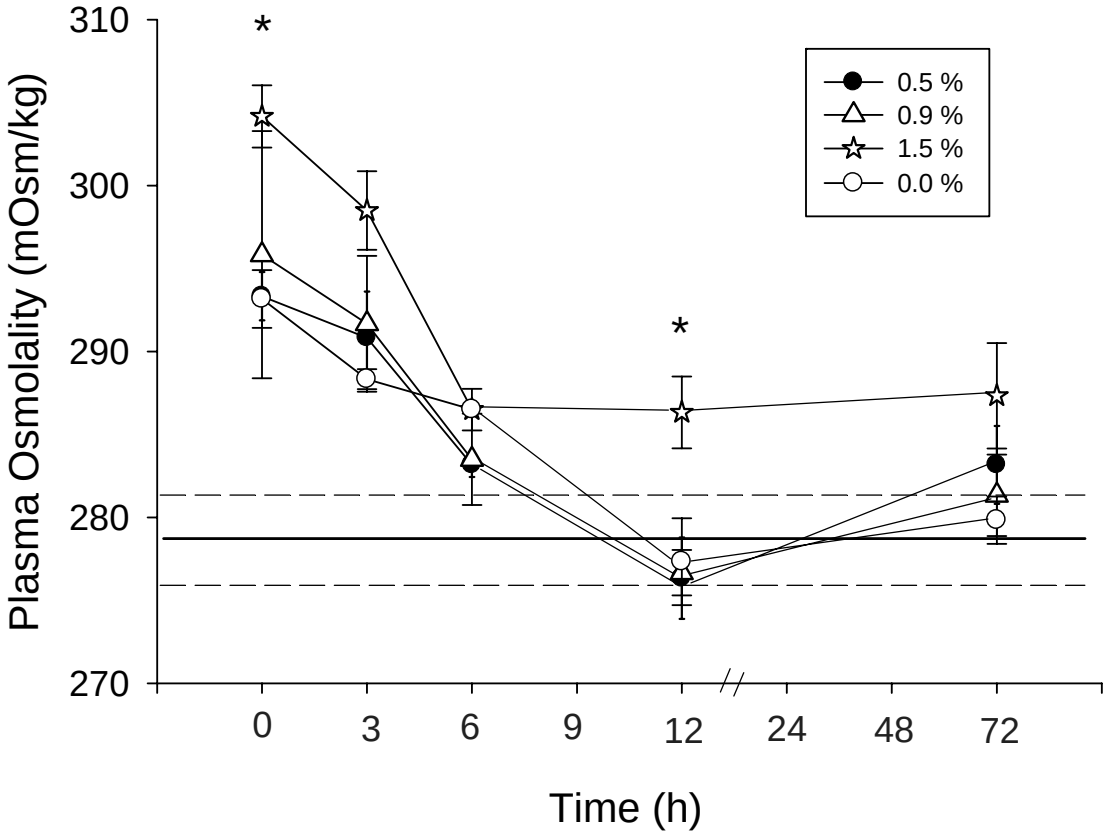

Figure 7.3. Plasma osmolality concentrations (mOsm/kg) of Rio Grande silvery minnow throughout 72 h recovery in freshwater after confinement for 5 h followed by transport for 3 h in 0.0% 0.5%, 0.9%, and 1.5% salt concentrations. Treatment groups are represented by means and SE bars on three replicates and five fish per replicate. The horizontal line represents the mean and 95% confidence intervals for plasma osmolality concentration for non-disturbed fish (279.1 mOsm/kg, ± 2.24, n=25). Asterisks represent detectable differences ($P \leq 0.05$) among treatments.

Discussion

Previous research revealed stress in this species was cumulative with increasing time to recovery as additional stressors were placed on the Rio Grande silvery minnow (*see* Chapter I). The purpose of this study, however, was not to characterize the duration and magnitude of a series of disturbances of the minnow. Our intent was to evoke a stress response severe in magnitude that would allow us to assess the effects a range of salt concentrations (i.e., hypo-osmotic, iso-osmotic, hyper-osmotic) would have in attenuating the stress response and time to recovery. Concentrations of plasma cortisol in Rio Grande silvery minnow varied throughout the treatments resulting in no detectable effects of salt in ameliorating the primary stress response. The minnow appears to be an 'excitable species' in which the stress response, once elicited, exhibits cortisol concentrations that varied in magnitude and duration (*see* Chapter I). Despite the variability among treatments, plasma cortisol concentrations returned to pre-stress conditions within 3 h of recovery. These results were similar to those reported in Chapter I in minnow subjected to the cumulative effects of three stressors (30 s dip net, 3 h confinement, 3 h transport) and plasma cortisol concentrations returned to baseline within 6 h of recovery. Although plasma cortisol has been used as an indicator of the primary stress response in fishes for decades (see reviews of Barton and Iwama 1991; Portz et al. 2006), little attention has been paid to plasma cortisol's complexity regarding its multiple effects on a wide spectrum of target tissues that include gills, liver, gonads, and kidney (Mommsen et al. 1999). A sample of cortisol in the fish's blood represents a snapshot of a sum of dynamic processes (i.e., release, transport, uptake and clearance) and might not always be illuminating regarding the effects of disturbance in fish such as was seen here.

Plasma glucose provides a more utilitarian approach when evaluating stress effects in fish by providing snapshots of mobilized energy for maintenance and performance of routine and stress-related activities. Plasma glucose concentrations were higher than expected when the minnow was transported in salt levels presumably closest to those levels isotonic with respect to its plasma (i.e., 0.9%). These values ranged from 23.9 mg/dL to 37.1 mg/dL compared to baseline levels of 20.1 mg/dL in non-stressed Rio Grande silvery minnow. In the highest salt treatment (1.5%), plasma glucose concentrations increased to as high as 47.2 mg/dL by 3 h in recovery while plasma glucose concentrations were lower than expected when the minnow was transported with no salt (0.0%) and low salt (0.5%). The cumulative effects of stress in this study were not sufficient to deplete

glycogen stores to result in lower glucose concentrations. Although the minnow were not fed throughout the 72 h recovery period, the fish in this study had been maintained since hatch on an experimental diet specifically formulated for the species (Caldwell et al. 2009).

At the onset of recovery, the minnow exhibited osmolality levels in all treatments that were elevated compared to levels of non-disturbed fish observed in Chapter I (279.1 mOsm/kg). Elevated levels of osmolality have also observed for other species subjected to severe stress. Earlier work by Wood et al. (1983) revealed an influx of plasma Na^+ and Cl^- ions across the gills of rainbow trout (*Oncorhynchus mykiss*) that was subsequently followed by a progressive reduction indicative of an efflux or loss of the ions to the environment. Stress-induced release of catecholamine stimulates lamellar recruitment to increase blood flow through the gills thereby increasing diffusion of oxygen (Randall 1982; Postlethwaite and McDonald 1985). And while oxygen diffusion will be greater, the increase in gill branchial permeability exacerbates the loss of ions to the surrounding environment. Thus, the return of plasma osmolality in fish transported in 0.0%, 0.5% and 0.9% salt to pre-stress levels in the minnow within 6 h of the recovery period reflects the fish were able to adjust the permeability of its gill to prevent additional loss of precious ions without compromising oxygen diffusion. This is in contrast to plasma osmolality in fish transported in 1.5% salt which remained elevated throughout the 72 recovery. While these elevated levels of osmolality do not represent detrimental or osmotic dysfunction, elevated levels of osmolality may incur expenses at the cellular level that are costly to the fish. Stress-related costs associated with metabolic requirements and recovery from disturbance place greater energy demands on a fish and should be carefully considered when evaluating physiological responses to stressors (Schreck 1981; 1982). The costs for maintaining osmolality levels will be at the expense of available energy stores (Redding and Schreck 1983), however, this was not evident in plasma glucose concentrations throughout the treatments. Osmotic costs can be as high as 3 to 5% of total daily caloric intake (Wedemeyer 1996).

Although unavoidable, physical stressors associated with standard culture and management practices will not only delay recovery from stress but result in disruption of osmotic balance that will be manifested long after the stressors have been removed (Carmichael et al. 1984a.). Salt has long been used to reduce stress-related osmotic dysfunction and the addition of salts to the recovery water is also beneficial to recovery of fish. Osmotic stress has led to altered behavior, increased risk to predation, and reduced survival (Weirich et al. 1992; Forsberg et al. 2001; Barton et al. 2003).

While we did not demonstrate an amelioration of stress-related effects of transport in a range of salt concentrations, fish culturists and managers have long known that if physiological challenges exceed homeostatic threshold in fishes, then one should expect changes in the short term (hours and days) manifested in altered behavior, reduction in feeding and swimming ability or performance.

Summary and Management Recommendations

Successful restoration efforts of the federally-endangered Rio Grande silvery minnow require a standardized approach to propagation, augmentation and rescue activities with an effort to minimize stressors to increase the chances of recovery upon release to the wild. Despite continued efforts to assess and improve culture and management practices, stressors resulting from these efforts are simply unavoidable. The application of salt concentrations of 0.5% to ameliorate the acute and long term effects of stress has long been considered an important tool in the propagation and augmentation of the imperiled minnow. We demonstrated that salt levels at or below 0.9% during 3 h transport did not ameliorate the stress response in Rio Grande silvery minnow. After 3 h of transport in 1.5% salt, the minnow exhibited osmotic dysfunction that was manifested in elevated osmolality and mobilized energy that was manifested in elevated plasma glucose concentrations. While we demonstrated that no differences were observed in stress physiology of an imperiled minnow subjected to salt levels at or below salt threshold levels of the plasma, we urge managers to continue the use of salt therapy between 0.5% and 0.9% to reduce osmotic costs.

CHAPTER III

INFLUENCE OF CAPTURE, HANDLING, AND TRANSPORT ACTIVITIES ON RECOVERY AND SURVIVAL OF THE RIO GRANDE SILVERY MINNOW

Introduction

In 2001, the U.S. Fish and Wildlife Service (Service) determined rescue of Rio Grande silvery minnow from intermittent and drying pools throughout the Isleta and San Acacia reaches in the middle Rio Grande a reasonable and prudent measure to minimize incidental take of the endangered fish. From 2001 to 2007, approximately 550,000 RGSM have been rescued from isolated and receding pools and transported to perennial reaches of the middle Rio Grande (Table 1) (Pers. com.; Remshardt, USFWS).

Table 3.1. Estimated number of Rio Grande silvery minnow rescued each year by the U.S. Fish and Wildlife Service from isolated pools and transported upstream to perennial reaches of the Middle Rio Grande.

Year	Total Number Rescued
2001	240
2002	3,662
2003	713
2004	12,864
2005	450,000
2006	69,079
2007	13,504

The purpose of this research was to provide U.S. Fish and Wildlife Service personnel with a characterization of physiological responses (i.e., recovery and survival) of the Rio Grande silvery minnow subjected to collection and transport stressors associated with annual rescue activities. We hypothesized the cumulative effects of stressors associated with isolation in pools (e.g., elevated temperature and reduced dissolved oxygen concentration), collection, and transport of the Rio Grande silvery minnow to perennial reaches for release would result in greater magnitude or severity of stress and ultimately reduced survival through increased incidence of disease. Preliminary work identified elevated physiological stress responses in addition to severe disease effects in fish collected from isolated pools in the San Acacia and Isleta reaches of the middle Rio Grande

(Appendix A; Memo to USFWS Caldwell 2005). Thus, an objective of this research was to characterize time to recovery (magnitude and duration of the physiological stress response) and survival of the minnow subjected to rescue activities using a suite of primary (plasma cortisol), secondary (plasma glucose and osmolality) and tertiary indices (disease incidence). We report the results of a series of field studies conducted during federal rescue activities in 2006 and 2007 within the San Acacia and Isleta reaches of the middle Rio Grande.

Methods

2006 Intermittency Study

Fish Collections.- With the issuance of the Biological Opinion (29 June 2001), a Coordinator for 2006 rescue activities of the minnow was appointed by the Service. The Coordinator (M. Hatch) evaluated river conditions throughout the summer to determine the best location for rescue efforts. Rescue efforts were conducted in accordance with standard operating procedures written by the Coordinator (see Appendix B).

The authors of this report (C. Caldwell and S.J. Cho) accompanied the Coordinator on 19 July 2006 to rescue the minnow within the San Acacia reach (RM 135-139). Rescue operations began at approximately 1015 and salvage crews seined isolated pools within a 2.4 – 3.4 km stretch of the river. Periodically, temperature was monitored within individual pools (range 25 - 28^{o}C). At each pool, the minnow were removed from the seine and placed either in a bucket containing river water or transferred directly into an aerated hauling tank containing approximately 0.5% salt and dissolved oxygen concentrations (15 - 17 mg/L). The hauling tanks containing the fish were attached to all-terrain vehicles which were also used to transport rescue personnel. Rescue operations ceased at approximately 1220 and all salvaged fish were transported via dip nets without acclimation to a hauling tank containing fresh water (0.5% salt; 23^{o}C; dissolved oxygen 12 – 15 mg/L). The transport vehicle departed the collection site at 1250 for delivery to the release site containing flowing water.

Study Site and Cage Study to Assess Intermittency Effects.- At approximately 1440, the transport vehicle arrived at the study site containing six cages in the lower portion of the Albuquerque reach ("Shirk Site"). The cages were deployed in a single row along the east bank of

the river, in the lower portion of the Albuquerque reach, south of the AMAFCA channel. The cages (3 ft x 3 ft x 3 ft = 27 ft^3 or 0.76 m^3) were constructed with 1.5 in (3.91 cm) PVC pipe and covered with a plastic mesh (1/4 in; 8 mm) to facilitate water flow but prevent escape of fish. Each cage was covered with mesh to prevent depredation and provide access to fish for sample collections. Guidelines for optimum fish densities of 15 – 20 kg/m^3 are recommended to minimize metabolic stress by providing sufficient water flow, dissolved oxygen concentrations near saturation, un-ionized ammonia below 0.01 mg/L (Banks et al. 1979). Each cage received 200 fish, resulting in densities that ranged from 0.69 kg/m^3 to 0.98 kg/m^3, well below maximum densities that would result in crowding and deterioration of water quality.

Due to time constraints, fish were counted immediately from the transport tank (~25^{o}C) to cages within the river (29.5^{o}C). A subsample totaling 30 fish that appeared healthy (no apparent disease or lethargy) was collected from the hauling tank and shipped overnight to the USFWS Region 2 Fish Health Unit at the Dexter National Fish Hatchery and Technology Center in Dexter, New Mexico (hereafter referred to as the Dexter Fish Health Unit) for diagnostics of external parasites, bacteria and viruses.

While fish were counted into the cages, a sub-sample of 33 fish was removed from the hauling tank and immediately euthanized (100 mg/L Tricaine Methanesulfonate or Finquel™). Blood was collected from the severed caudal peduncle into a heparinized capillary tube to assess physiological status of the fish at the time they were placed into the cages (time 0). Length (mm) and weight to the nearest 0.01 g were recorded at the time blood was collected for evaluation of Fulton's condition index (KtL) for comparison among wild minnow throughout the study. Due to the small size of fish (<3 g), blood from 10-15 fish was pooled into a series of capillary tubes to provide sufficient volume for physiological parameters. The capillary tubes containing the composited blood were centrifuged for 4 min using a hematocrit centrifuge operated by a gas-powered generator at the site. The plasma was removed from the capillary tubes and placed on ice and frozen for chemistry analysis at a later date. Throughout the recovery period, dead fish were collected and recorded each day to characterize total cumulative mortality.

Three cages were chosen randomly for characterizing cumulative mortality (from north to south: cage 1, 3, 4). The remaining three cages (2, 5, 6) were used to characterize physiological responses and time to recovery from stress associated with isolation in intermittent pools, capture,

transport and handling. Fish within these cages were netted, anaesthetized and blood collected within approximately 20 h in the cage. Dead and dying fish were removed from all the cages, enumerated and examined for any unusual external anomalies.

2006 Perennial Cage Study

Shortly after assessing intermittency effects, heavy river flow in the Albuquerque reach destroyed the six cages. One cage (4 ft x 4 ft x 4 ft = 64 ft^3 or 1.81 m^3) was obtained from Service personnel to characterize the effects of capture, transport and survival of the minnow obtained from a perennial or flowing reach of the middle Rio Grande. On 21 August 2006, Caldwell accompanied USFWS personnel (J. Remshardt and L. Torres) to collect Rio Grande silvery minnow from upstream of Corrales Siphon, Rio Rancho WWTP 2 (River mile 200.0). Between 1030 and 1300 (river temperature 24^oC), a total of 160 minnow was collected. An additional 11 minnow were collected at U.S. 550 Bridge (River mile 203.8) between 1430 and 1630 (river temperature 26.8^oC). Fish from both sites were combined and transported in plastic bags containing well oxygenated river water (14 mg/L; 21.5^oC) to the Shirk site. Fish were not sampled for physiological analysis at the time of arrival to the Shirk site due to time constraints and limited number of fish. The estimated density of the fish within the cage was 0.76 kg/m^3 (based on an average of 2.0 g per fish and the cage 2/3 submerged) at the start of the recovery period.

The following morning at 1000 or 18 h post-collection, 20 fish were removed from the cage and euthanized. Blood was collected from the severed caudal peduncle into a heparinized capillary tube to assess physiological status of the fish. Length (mm) and weight to the nearest 0.01 g were recorded at the time blood was collected for comparison of wild minnow collected throughout the study. Due to the small size of fish (<3 g), blood was pooled from 10 – 15 fish into a series of capillary tubes to provide sufficient volume for physiological parameters. The capillary tubes containing the composited blood were centrifuged for 4 min using a hematocrit centrifuge operated by a gas-powered generator at the site. The plasma was removed from the capillary tubes and placed on ice and frozen for frozen to determine physiological indices at a later date.

Fish within the cage were checked every 2 to 3 days during the first 10 days of the study, and then every 3 to 4 days thereafter (18 days total) until the study was terminated on 8 September 2006.

Dead or moribund fish were removed at each time. Fish were not assessed for external parasites or pathogens due to a shortage in personnel at the Dexter Fish Health Unit at the time of the study.

Water Quality.- Due to time constraints, water quality was not characterized while collecting fish in both intermittent and perennial reaches. Water quality, however, was characterized above, below and within cages throughout both studies. Conductivity (µmhos; VWR Scientific, West Chester, PA), DO (mg/L) and temperature (^{o}C; YSI 58, Yellow Springs Instrument Company, Yellow Springs, OH), pH (LaMotte pHPlus meter and probe calibrated prior to use, LaMotte Company, Chestertown, MD) were measured near the center and at mid-depth within each cage, and in the river approximately 1 m above and below the row of cages. In addition, a sub-surface grab sample was collected in cages 1, 3, 4, 5 and in the river above and below the cages for analysis of ammonia. Water samples were collected in 250 mL and 1 L low density polyethylene (LDPE) bottles and placed in a cooler filled with wet ice. In the laboratory, the water samples were preserved with 0.4% H_2SO_4 and kept at 4^{o}C until analysis for total ammonia as nitrogen (TA-N) and un-ionized ammonia (APHA 1995; Orion 1990). The 1 L sample bottle was submitted to the NMSU Soils, Water and Testing Laboratory at NMSU for analysis of alkalinity and hardness (as mg/L total $CaCO_3$; APHA 1995).

2007 Intermittency Study

Fish Collections and Study Areas.- River conditions for 2007 were evaluated beginning June 2007 by the Coordinator (J. Remshardt) to determine the optimal time and location for rescue efforts in accordance with revised standard operating procedures (Appendix C). Briefly stated, the primary criterion for rescue operations to be conducted was an ambient air temperature < 34^{o}C. If air temperature was <34^{o}C, and a pool was identified for rescue of the minnow a set of secondary criteria for the pool was immediately assessed to determine whether rescue efforts should be initiated. The secondary criteria for rescue included: (1) water temperature within the pools must be < 34^{o}C, (2) dissolved oxygen must be >2.0 mg/L, (3) pH < 9.0, (4) no observable dead fish, (5) no moribund fish as indicated by lethargy, and (6) no fish exhibiting hemorrhagic lesions. If any of these secondary criteria were not met, the pool was not rescued.

Assessment of Physiological Indices and Health in Fish Rescued from Pools.- With the beginning of intermittency July 2007 throughout the San Acacia reach (River Mile 135 - 155), Caldwell and Cho accompanied the Coordinator to assist with the rescue of the minnow from isolated pools. Between 16 July and 20 July, a total of 88 adult fish (greater than 70 mm) was seined from 24 isolated pools in the San Acacia reach and held for physiological assessment. Collection began at approximately 0700 and lasted until approximately 1200 or when air temperatures began to exceed 34^{o}C. At each pool, water quality and fish health were assessed immediately prior to seining. Once water quality parameters were deemed acceptable, the pool was seined and if minnow were captured, they were counted and placed into buckets containing fresh river water obtained from a flowing section of the river. The total time between when fish were captured in the seine, removed, anaesthetized, and bled was between 5 and 10 min. We were unable to assess the physiological response of the fish within a pool due to the small size and number of Rio Grande silvery minnow within each pool. Thus, fish were composted only from adjacent pools (distance less than 3 to 5 m) with similar water quality parameters. This resulted in a sample size of 16 blood composites (n = 16) representative of Rio Grande silvery minnow rescued from a total of 24 pools from which physiological variables were analyzed. Lengths and weights were recorded for each fish and fish were euthanized after blood collection.

On 17 July, a total of 14 fish was rescued from pools meeting rescue criteria and shipped overnight to the Dexter Fish Health Unit for disease diagnostics. Prior to shipment, none of the fish exhibited lethargy or disease. Fish were shipped in plastic bags containing water saturated with dissolved oxygen (approximately 10 mg/L).

From 13 to 17 August 2007, 92 fish were rescued from eight pools throughout the Isleta reach for physiological assessment. Similar to the protocol described in July, fish were bled and blood composited when rescued from adjacent pools with similar water quality parameters. This resulted in a sample size of five composites (n = 5) from which physiological variables were analyzed. Lengths and weights were recorded for each fish and fish were euthanized after blood collection.

Cage Study.- On 13 August, 370 fish were rescued from three pools and distributed among six cages deployed in a single row mid-river in the Isleta reach within Sevilleta National Wildlife Refuge. Prior to transporting fish from the rescue location to the cage site, a sub-sample of 47 fish

were euthanized and bled to assess physiological status at the time of rescue but prior to transport. Each cage (2 ft x 2 ft x 2 ft = 8 ft^3 or 0.23 m^3) was constructed with 1.5 in (3.91 cm) PVC pipe and covered with a nylon bag mesh (1/8 in; 6 mm; Memphis Net and Twine Co., Memphis, TN) to facilitate water flow but prevent escape of fish. Ten fish were submitted to the Dexter Fish Health Unit for disease diagnostics and the remaining 313 fish were distributed among the 6 cages resulting in a density of 0.3 kg/m^3 per cage (52-53 fish per cage). Fish submitted for assessment were netted from the holding tank containing minnow rescued from a series of pools that did not exhibit lethargy or external signs of disease prior to shipment. Fish were shipped overnight to the Dexter Fish Health Unit in plastic bags containing water saturated with dissolved oxygen (approximately 10-12 mg/L).

Throughout the recovery period, dead fish were removed from each cage and recorded each day to characterize cumulative mortality. The small size and number of fish within each cage precluded replicate analysis of the physiological variables on every sample date. Thus, at 24, five fish were removed from each cage and composited to obtain sufficient blood for one sample for physiological analysis (n = 1). At 48 h, 15 fish were removed from each cage and composited to obtain sufficient blood for four composites (n = 4). Dead and dying fish were removed from all the cages, enumerated and examined for any unusual external anomalies on days 1 (24 h), 3, 5, and 10 d post-collection. Water quality was assessed within each cage on these days.

Water Quality.- Within all pools throughout both reaches, dissolved oxygen, temperature and pH were characterized. At the cage site, the water quality parameters were measured inside each cage near the center at mid-depth and in the river approximately 1 m above and below the row of cages. In addition, a sub-surface grab sample was collected in cages 1, 3, 4, 5 and in the river above and below the cages for analysis of alkalinity and hardness. The water samples were collected in 1 L Nalgene® bottles, preserved with 0.4% H_2SO_4 and kept at 4^oC until submitted to the NMSU Soils, Water and Testing Laboratory for analysis.

Physiological Stress Indices.- Plasma cortisol, glucose and osmolality were characterized similar to that described in Chapter I. Briefly stated, plasma cortisol was obtained using radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). The assay relies on competition between non-radioactive and radioactive cortisol (^{125}I-labeled cortisol) for the binding sites (cortisol-specific antibodies coated in polypropylene tubes). The cortisol assay was modified to accommodate small sample volume (10 µL). Plasma glucose was measured colorimetrically

(Stanbio Laboratory, Boerne, TX) using glucose oxidase, peroxidase, phenol, and 4-aminoantipyrine to form a red-violet quinone complex. The intensity of color is proportional to glucose concentration. The glucose assay was also modified to accommodate a 10 µL sample volume. Plasma osmolality was measured by a vapor pressure osmometry (VAPRO 5520, Wescor, Inc., Logan UT) using 10 µL sample volume.

Data Analysis.- A two-tailed Students t-test for unequal samples sizes was conducted in EXCEL (version for Windows 2007) to compare the response variables (plasma cortisol, glucose, osmolality) of between minnow rescued from pools in the Isleta and San Acacia reaches. Differences were considered detectably different at an α level of 0.05.

Results

2006 Cage Study

Physiological Effects of River Intermittency.- Within 4 h of collection from the river and just prior to placing in cages, plasma cortisol concentration in the minnow was 336.7 ng/mL (Table 2). This concentration was well above mean baseline or resting levels of 31.9 ng/mL (mean and 95% confidence limit ± 20.71, n = 12) observed for the species in controlled laboratory conditions (see Chapter I). Plasma glucose and osmolality were 185.5 mg/dL and 234 mOsm/kg, respectively (Table 2). The glucose concentration was well above baseline or resting levels of 20.1 mg/dL (± 1.74, n = 12) while the osmolality level was well below baseline or resting levels of 279.1 mOsm/kg (± 2.46, n = 12) for species in controlled laboratory conditions (see Chapter I).

At 24 h post-collection or rescue from the isolated pools, all six cages were inspected by partially removing each cage from the water. Dead and moribund fish were removed and counted. Due to the high mortality in cages 2, 5, and 6, we chose to characterize the blood chemistry from the remaining fish within these cages. Fish within cages 1, 3, and 4 were monitored for survival throughout the remainder of the cage study. Plasma from fish remaining in cage 2 (total of 30 fish), cage 5 (total of 37 fish), and cage 6 (total of 15 fish) was collected, composited, and analyzed for the physiological stress indices. Within 24 h post-collection, plasma cortisol concentrations had increased to 423.1 ng/mL (standard error ± 41.08, n = 3). Plasma glucose declined slightly to 174.5 mg/dL (± 4.82, n = 3) and osmolality increased slightly to 248 mOsm/kg (± 6.84, n = 3) (Table 2).

Cumulative Effects of Isolation, Rescue and Transport on Survival.- Total mortality by day 1 (24 h post-collection) among the three remaining cages ranged from 81.5% to 91.5% or an average of 88% (Table 3). By day 2, cumulative mortality was 97.7% and by day 4, all but three of the 600 fish placed into the three cages had died resulting in 99.5% cumulative mortality.

Physiological Effects of Perennial Flow.- In an attempt to characterize the degree that isolation within a pool has on the cumulative effects of rescue and release, we chose to separate out the isolation event by assessing the difference in fish collected from perennial or flowing reach of the river with the fish collected previously from the intermittent reach. Fish within the perennial reach were captured and transported to the cage site to assess survival and recovery. At approximately 18 h post-collection and transport, plasma cortisol concentration in the wild minnow was 272.4 ng/mL (represented by one composite sample of 20 fish). To place this in perspective, this value was lower than that obtained from the previous value for minnows rescued from intermittent pools (i.e., 24 h, 423.1 ng/mL). Although this plasma cortisol concentration was 8.5 times greater than the baseline or resting level of plasma cortisol for laboratory-reared species (31.9 ng/mL; see Chapter I) indicating that stress was severe, it was 36% lower than previously reported for cortisol concentrations in minnow captured from isolated pools and transported.

At 18 h post-collection, plasma glucose concentration was 244.4 mg/dL which was 40% greater than that observed in the minnow rescued from the intermittent site (24 h, 174.5 mg/dL). Plasma osmolality was 220 mOsm/kg which was 11% lower than plasma osmolality in fish rescued from isolated pools and transported to the perennial site for release to the cages. Mean length and weight of the minnow collected from the perennial reach and intermittent reach were 70.6 mm (range 58 - 80 mm) and 2.34 g (range 1.11 - 3.42 g), respectively.

Cumulative Effects of Collection and Transport on Survival from Perennial Flow.- By 18 h post-collection, 21% of the fish had died and the remaining fish were exhibiting fin rot and lethargy. By the third day, 32% of the fish had died. By the sixth day, no dead or dying fish were observed. Fish were netted and removed from the cage for close inspection and all appeared healthy and vigorous (i.e., no scale loss or signs of disease). Fish within the cage were monitored on days 8, 10 and 15 until the study was terminated 18 d post-collection. Between days 8 and 15, an additional five fish had died and were removed resulting in a cumulative mortality of 37% by the end of the 18 d cage study.

Table 3.2. Mean plasma cortisol (ng/mL), plasma glucose (mg/dL) and plasma osmolality (mOsm/kg) from Rio Grande silvery minnow collected from the San Acacia reach on 19 July 2006. Fish were transported to the perennial reach and placed into cages for characterization of plasma constituents at 4 h after collection (4 h Post-collection) and at 24 h after collection (24 h Post-collection) from the San Acacia reach. The 4 h sample collection is representative of a composite of 33 fish (n = 1). The 24 h post-collection samples represent a composite of fish for each cage resulting in a sample size of 3 (n = 3). Standard error followed by range of values is reported in parenthesis for the 24 h post-collection samples.

	Cortisol (ng/mL)	Glucose (mg/dL)	Osmolality (mOsm/kg)
4 h Post-collection	336.7	185.5	234
24 h Post-collection	423.1 (41.08, 349.3 – 491.3)	174.5 (4.82, 169.6 – 184.1)	247.7 (6.84, 234 – 255)

Table 3.3. Average cumulative mortality (%) of Rio Grande silvery minnow collected from the San Acacia reach on 19 July 2006. Fish were transported to the perennial reach and placed into cages for monitoring recovery and survival at days 1 (24 h post-collection), 2, 3 and 4. Average cumulative mortality (%) is reported for the three cages followed by standard error in parenthesis.

	Mean Cumulative Mortality (%)
Day 1 (24 h)	88.0 (3.25)
Day 2	97.7 (0.45)
Day 3	99.3 (0.33)
Day 4	99.5 (0.29)

Fish Disease Diagnostics.- The results of the fish health analysis for skin and gills revealed "moderate" to "heavy" infection of opportunistic pathogens in the wild minnow that included ciliated protozoans (*Costia*, *Trichodina*, *Trichophyra*) (see Appendix D.2). Gas bubbles were identified in the gills presumably from supersaturation (>12 mg/L) from compressed oxygen gas attached to the hauling tanks. As the hauling water heated throughout the day, oxygen solubility would have decreased resulting in discernable bubbles within the gill lamellae.

In addition to external parasites, a virus was observed and confirmed by visualization through electron microscopy at the University of Montana after the isolates were screened and prepared by staff at the USGS, Western Fisheries Research Center. At the time of this report, the virus has not been identified. A virologist at New Mexico State University was contacted to assist in its identification. Funds, however, would be needed to identify the virus using molecular tools.

Water Quality.- Water quality (dissolved oxygen, temperature, pH, conductivity, un-ionized ammonia) were measured on day 1 (20 July) of the study in the intermittent reach (Table 4). Although temperature was somewhat elevated in the river at the time of the study (average 30.5^{o}C), this value was within the range of tolerance for the species (Pers. com.; K. Buhl, USGS). Despite elevated river temperatures, dissolved oxygen concentrations were within an acceptable range (average 6.31 mg/L) in all six cages. Water quality did not differ above or below the cages throughout the study.

Water quality (dissolved oxygen, temperature, pH, conductivity, ammonia) was measured within the cage throughout the perennial study from day 1 (22 August) through day 18 (8 September) (Table 5). Although water quality was somewhat variable from sample to sample, the values were not indicative of deteriorating or poor conditions within the river.

Table 3.4. Water quality collected on 20 July 2006 at the Shirk site containing six cages in which Rio Grande silvery minnow were placed for physiological assessment and survival from isolated pools in the intermittent reach. Water quality was measured above (Upstream) and below (Downstream) of the cages.

Site	Dissolved oxygen (mg/L)	pH	Conductivity (µmho/cm)	Temperature[1] (°C)	Ammonia[2] (mg/L as N)
Upstream	6.33	7.67	492	30.3	0.006
Downstream	6.28	7.67	494	30.5	0.006
Cage 1	6.27	7.62	506	30.7	0.006
Cage 2	6.34	7.68	493	30.3	-
Cage 3	6.27	7.64	503	30.6	0.006
Cage 4	6.30	7.65	501	30.6	0.006
Cage 5	6.34	7.67	493	30.4	0.006
Cage 6	6.32	7.66	494	30.4	-
Average Value	6.31	7.65	498	30.5	0.006

[1] Temperature recorded mid-day
[2] Ammonia presented as un-ionized ammonia
- parameter not collected

Table 3.5. Water quality collected from the middle Rio Grande at the Shirk site containing one cage with Rio Grande silvery minnow from perennial reach near Albuquerque. Water quality was assessed within the cage from 22 August to 8 September 2006.

Date	Dissolved Oxygen (mg/L)	pH	Conductivity (µmho/cm)	Temperature[1] (°C)	Alkalinity (mg/L)	Hardness (mg/L)
22 August	6.37	7.55	436	22.6	111	126
24 August	6.28	7.51	499	26.7	117	130
26 August	-	7.58	485	27.2	122	123
28 August	6.82	7.60	465	23.4	123	124
31 August	6.47	7.50	502	26.5	126	124
5 September	6.77	7.65	486	26.9	-	-
8 September	7.25	7.75	429	20.9	-	-

[1] Temperature recorded mid-day
- parameter not collected

2007 Intermittency Study

Physiological Responses of the Minnow in Isolated Pools.- There was no detectable difference for plasma cortisol concentrations in minnow rescued from pools between the two reaches (P=0.7741). At the time of rescue, mean plasma cortisol concentrations was 209.2 ng/mL (± 28.72 ng/mL; range 32.4 – 321.4 ng/mL; *n* = 16) in minnow within pools throughout the San Acacia reach and 289.8 ng/mL (± 30.15; 200.2 - 330.9 ng/mL; *n* = 4) in minnow within pools throughout the Isleta reach (Figure 1). Mean plasma glucose concentrations were similar for the minnow collected throughout all pools in the San Acacia reach (42.2 mg/dL ± 3.72; 28.9 - 83.7 mg/dL; *n* = 16) and the Isleta reach (58.5 mg/dL ± 9.19; 34.5 - 79.1 mg/dL; *n* = 4) (P=0.7569) (Figure 1). Mean plasma osmolality values were also similar for the fish collected throughout all pools in the San Acacia reach (278.8 mOsm/kg ± 3.18; 245.0 – 300.0 mOsm/kg; *n* = 16) and the Isleta reach (281.4 mOsm/kg ± 8.75; 257.5 – 299.0 mOsm/kg; *n* = 5) (P=0.9033) (Figure 1).

Of note, Rio Grande silvery minnow from the Isleta reach were half the size of fish from the San Acacia reach. The average weight of the minnow collected throughout the San Acacia reach was 3.6 g (± 0.36; range 1.2-6.5 g; *n* = 85) and mean total length (TL) was 78.7 mm (± 1.04; range 58-96 mm; *n* = 85). In contrast, the average weight of the minnow collected throughout the Isleta reach one month later was 1.1 g (± 0.30; range 0.32–5.2 g; *n* = 110) and mean TL was 49.9 mm (± 3.42; range 37–91 mm; *n* = 110).

Physiological Effects of Intermittency, Capture, and Transport.- Presumably, values for physiological stress indices at the time of collection (0 h post-collection) represent the effects of environmental stressors associated with isolation in pools within the intermittent reach. These values were reflected by elevated concentrations of plasma cortisol (200.2 ng/mL) and plasma glucose (62.3 mg/dL) (Table 6). At the time of capture, however, the physiological effects of isolation were not severe and did not result in osmo- or ionoregulatory dysfunction. Plasma osmolality was near levels representative of undisturbed fish at the time of collection from the pool (0 h) (288 mOsm/kg) (see Table 6). Within 24 h of capture and transport to the cages, however, substantive physiological changes occurred as reflected by a 77% increase in plasma cortisol (354.2 ng/mL), a 92% increase in plasma glucose (119.8 mg/dL), and a 20% decrease in plasma osmolality (230 mOsm/kg) (Table 6). Within 48 h of collection from the pools, fish within the cages began to

recover as reflected by a 6% decrease in plasma cortisol (332.8 ng/mL ± 41.03, *n* = 4), a 19% decrease in plasma glucose (92.8 mg/dL ± 13.39, *n* = 4) and a 9% increase in plasma osmolality (251 mOsm/kg ± 1.25, *n* = 4) (Table 6). An elevation in river levels from a storm resulted in the loss of the fishes from the cages at day 10 post-collection. Thus, we were unable to fully characterize time to recovery. Although stocking densities within the cages (0.3 kg/m^3) were well below recommended densities necessary to maintain optimal water quality when transporting fish (i.e., 15 – 20 kg/m^3), we cannot rule out that the fish experienced some degree of stress while confined within the cages. Fish within the cages ranged from 48 to 70 mm in length (mean 62.8 mm) and from 1.10 to 2.54 g in weight (mean 1.77 g).

Cumulative Effects of Isolation, Rescue and Transport on Survival.- Within 24 h of rescue and transport from the pools to the cages (post-collection), survival was relatively high with total mortality from 3.3 to 16.7% (Table 7). By day 3 post-collection, survival within the cages had decreased with cumulative mortality from 3.3 to 33%. Four of the six cages did not experience additional fish loss between days 5 and 10 post-collection (Table 7). The cage study was terminated at day 10 due to the rise in river flow from a storm that flooded the cages and the fish were lost.

Water Quality.- Water quality parameters (dissolved oxygen, temperature, pH) were measured at day 1 throughout day 10 of the cage study. Although temperature was slightly elevated (31.0 ^oC) in the river at the time of the study (Table 8), this value was well within the range of tolerance for the species (Pers. com.; K. Buhl, USGS). Despite the elevated temperature, dissolved oxygen concentrations and pH were deemed acceptable throughout the entire study within all six cages (Table 8). Although not reported, water quality parameters did not differ above or below the cages (personal communication, Cho).

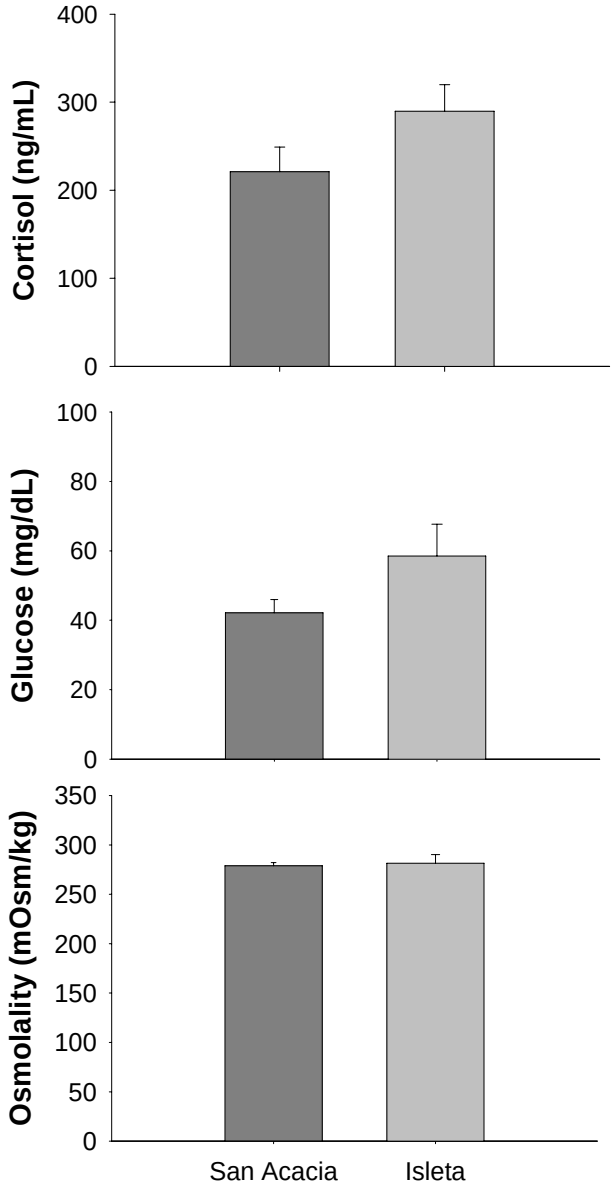

Figure 8.1. Average plasma cortisol (ng/ml), glucose (mg/dL), and osmolality (mOsm/kg) in Rio Grande silvery minnow captured in isolated pools within the San Acacia reach July 2007 (n = 16 composites) and within the Isleta reach August 2007 (n = 4 composites) of the middle Rio Grande. Standard error bars are represented.

Table 3.6. Plasma cortisol (ng/mL), plasma glucose (mg/dL) and plasma osmolality (mOsm/kg) of Rio Grande silvery minnow collected from the Isleta reach on 12 August 2007 (time 0) and placed into six cages to observe recovery from the cumulative stress effects of isolation, capture, and transport.

Time (h) Post-collection	Cortisol (ng/mL)	Glucose (mg/dL)	Osmolality (mOsm/kg)
0[a]	200.2	62.3	288
24[b]	354.2	113.4	230
48[b]	332.8 (41.03, $n = 4$)	92.2 (13.39, $n = 4$)	251 (1.25, $n = 4$)

[a] The time 0 h sample denotes a composite of 47 fish ($n = 1$) that were bled immediately upon capture at the pool site and represents physiological status of RGSM prior to transport the cage site.

[b] The 24 h and 48 h Post-collection samples represent an average of the physiological status of fish within six cages at 24 h and 48 h into the recovery period. The 24 h post-collection represents one composite of 30 fish, $n = 1$. The 48 h post-collection represents 4 composites of 30 fish ($n = 4$) among the six cages.

Table 3.7. Average percent cumulative mortality of Rio Grande silvery minnow after collection from the Isleta reach (12 August 2007) and placed into six cages for 10 d to observe recovery from the cumulative effects of isolation, capture, and transport. Standard error and sample size are presented in parentheses followed by the range in cumulative mortality among the cages within the collection period.

Time Post-collection	Cumulative Mortality (%)
Day 1 (24 h)	5.8 (2.58, 6) 0 – 16.7
Day 3	18.2 (5.01, 6) 3.3 – 33.3
Day 5	20.5 (5.06, 6) 3.3 – 35.9
Day 10	22.6 (5.90, 6) 3.3 – 41

Table 3.8. Average dissolved oxygen concentrations (mg/L), temperature (°C) and pH within cages containing Rio Grande silvery minnow collected from the Isleta reach (12 August 2007). Standard error and sample size are in parentheses.

Time Post-collection	Dissolved Oxygen (mg/L)	Temperature (°C)	pH
Day 1 (24 h)[a]	-	20.8	8.8
Day 3	8.0 (0.21, 6)	28.7 (0.15, 6)	8.5 (0.09, 6)
Day 5	7.7 (0.15, 6)	29.2 (0.02, 6)	8.5 (0.01, 6)
Day 10	7.6 (0.31, 6)	31.0 (0.0.07, 6)	8.5 (0.01, 6)

[a] Water quality was assessed in only Cage 1 due to time constraints.
- Dissolved oxygen was not collected due to instrument malfunction

Fish Disease Diagnostics.- The sub-sample of ten wild Rio Grande silvery minnow collected from isolated pools within the San Acacia reach revealed 8 of the 10 fish contained *Costia*, *Trichodina*, and *Gyrodactylus* (monogenean trematode) on the gills. Parasites were not observed on the skin of the fish and no bacteria or viruses were isolated. Of note, 2 of the 10 fish collected from the San Acacia reach were identified as "clean" indicating no parasites were observed on either the gills or skin. The sub-sample of 10 wild minnow collected from the Isleta reach revealed no parasitic infection on skin or gills with one monogenean trematode observed on the gills of 1 of the 10 fish. Presence of monogenean parasites in 1 out of 10 fish was considered incidental by the fish health specialist and represents little to no pathogenesis in the population (Pers. Comm. P. Hines).

Physiological Responses, Survival, and Disease Prevalence of Rescued Rio Grande Silvery Minnow.- When comparing physiological variables of rescued wild minnow across collection years, rescue and recovery efforts resulted in greater physiological stress responses in 2006 compared to 2007 (Table 9). Within 24 h of collection and transport from isolated pools, plasma cortisol was 16% higher in minnow during the 2006 collections compared to the minnow collected in 2007. Similarly, plasma glucose was 35% higher in minnow collected in 2006 compared to 2007. Plasma osmolality in minnow rescued in both years was considerably lower in both years when compared to baseline or reference values of hatchery-reared minnow at rest. Although sample size was small in both years and environmental variables differed greatly between years, adherence to the modified rescue protocol in 2007 contributed to the reduced stress responses and greater survival.

Notably, we observed negligible survival of wild minnow after collection from isolated pools and transported to cages for recovery in the perennial reach in 2006 compared to 82% survival when the study was repeated in 2007 (Figure 4). The majority of the wild fish collected in 2006 were heavily infected with parasites and exhibited symptoms of disease positively identified by the USFWS Fish Health Unit. In contrast, the majority of the wild fish collected from intermittent reaches in 2007 were relatively free of parasites and bacterial disease. Although these differences may have been influenced by a variety of environmental factors (duration and timing of intermittency) that would have varied between years, our results suggest the modified protocol contributed to the reduction in the cumulative effects of capture and transport.

Table 3.9. Plasma cortisol (ng/mL), plasma glucose (mg/dL) and plasma osmolality (mOsm/kg) of Rio Grande silvery minnow collected from the San Acacia reach in 2006 and the Isleta reach in 2007 and placed into cages to observe recovery within 24 h from the cumulative stress effects of isolation, capture, and transport. Blood from fish within a pool or cage were composited due to the small size of the fish. Standard error is reported in parentheses for 2006. "–" indicates no standard error can be reported due to small sample size.

	Cortisol[a] (ng/mL)		Glucose[b] (mg/dL)		Osmolality[c] (mOsm/kg)	
Year	2006	2007	2006	2007	2006	2007
24 h	423.1 (41.08)	354.2 -	174.5 (4.82)	113.4 -	247.7 (6.84)	230.0 -

[a] Baseline or reference value for plasma cortisol was 31.9 ng/mL (± SE 9.41) in resting RGSM reared in laboratory conditions.
[b] Baseline or reference value for plasma glucose was 20.1 mg/dL (± 0.79) in resting RGSM reared in laboratory conditions.
[c] Baseline or reference value for plasma osmolality was 279.1 mOsm/kg (± 1.12) in resting RGSM reared in laboratory conditions.

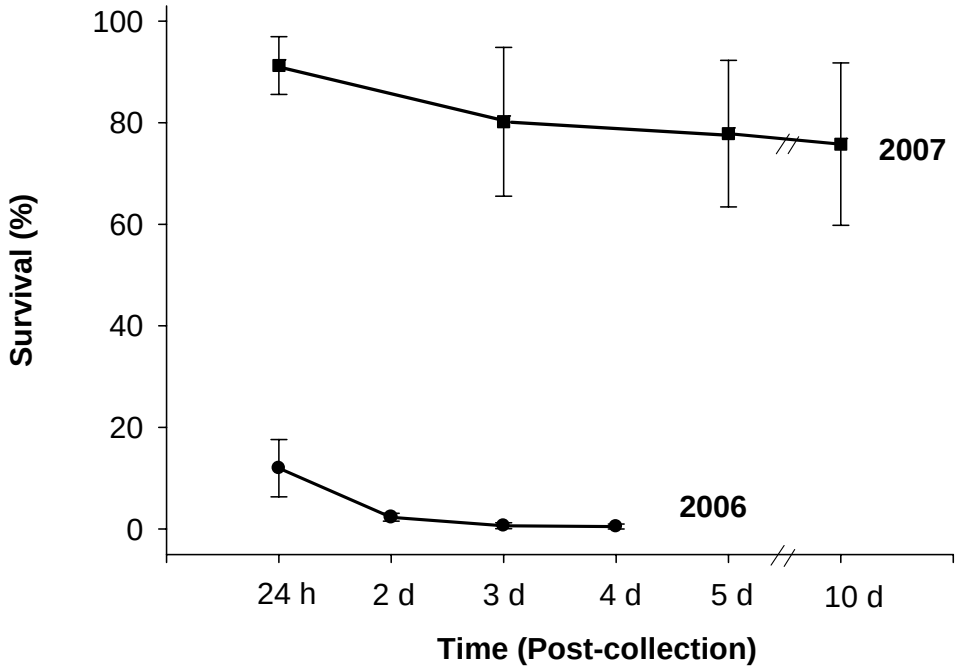

Figure 9.2. The cumulative effects of rescue from intermittent pools and transport to perennial sites on survival (%) of Rio Grande silvery minnow in the San Acacia reach (2006) and the Isleta reach (2007). Values are averages with standard error bars.

Discussion

The Rio Grande Silvery Minnow Recovery Plan (USFWS 1999) was developed to re-establish, stabilize, and enhance populations within its current range. While the Recovery Plan established a series of initiatives designed to guide recovery efforts for the species, an evaluation of the overall success of the recovery efforts was needed. Despite precautionary measures throughout all aspects of recovery, it is inevitable that the fish will be subjected to various physical challenges (i.e., stressors) that could affect conservation efforts. In particular, salvage or the emergency translocation of the minnow from isolated and drying pools to flowing reaches in an effort to reduce fish loss has been conducted since 2001. A rescue protocol was developed and implemented by the Service in 2003 which required locating isolated pools, collection of fish, and transport by bucket, amphibious vehicle, or distribution truck to optimal habitat of perennial flow (Smith and Cryan 2003; Hatch 2004; Appendix B). As we learned about the biology and ecology of the species, these rescue procedures evolved with time (Remshardt 2006; Appendix C).

Despite continued improvement of salvage protocol, the long term effects of isolation within intermittent pools compounded by collection and transport stress was of concern to Service personnel. If conditions within isolated pools were detrimental to the fish (i.e., elevated temperatures, reduced dissolved oxygen levels), then it would be necessary to modify capture and transport protocol accordingly to increase the fish's chances for survival. Thus, characterizing the stress response of Rio Grande silvery minnow throughout rescue and transport during river intermittency was deemed necessary to determine if conservation efforts were successful.

Stress in fish results in a series of physiological changes that can be characterized to assess if modifications of management actions to reduce stressors are necessary (*see* Chapter I: The Physiological Stress Response). In response to acute stress, plasma cortisol concentrations will increase within minutes (5 - 15 minutes depending on species) reaching peak concentrations within 1 h (Strange et al. 1977; Barton et al. 1980). Snapshots of plasma cortisol, however, can be obtained if fish are anaesthetized immediately upon capture and bled (Strange and Schreck 1978). This precludes the effects of the capture and handling if blood can be obtained within the first few minutes of disturbance to the fish. In addition to the severity and duration of the stress, the timing of cortisol release and concentration will vary among species (biological variation) and within a species (nutritional status, prior stress, age, sex). When capturing 'snapshots' of plasma cortisol, glucose

and osmolality, one must carefully consider all these variables to adequately assess stress effects in wild fish.

In 2006, we observed elevated levels of plasma cortisol (336.7 ng/mL), glucose (185.5 mg/dL) and reduced osmolality (234 mOsmo/kg) in wild Rio Grande minnow within 4 h of collection from the intermittent pools. Despite 24 h of recovery, plasma cortisol levels had continued to increase (423.1 ng/L) with a range of values from 349.3 to 491.3 ng/mL indicating that the cumulative effects of isolation, capture, and transport using the 2004 protocol was severe. Although the effects of cage confinement on stress responses of the fish cannot be ruled out, it is very likely that the fish were undergoing severe stress prior to and at the time of the rescue. Disease diagnostics of fish collected from the isolated pools revealed heavily parasitized fish. Thus, it was not surprising when nearly 100% of the fish captured within the isolated pools died within 4 days of our observational period. The role that environmental stress has in pre-disposing fish to disease has been well documented (Wedemeyer 1970; Sniesko 1974, Pickering 1993).

When the salvage protocol was modified in 2006 and implemented for the first time in 2007, plasma cortisol concentrations in wild Rio Grande silvery minnow from isolated pools were elevated (209.2 - 289.8 ng/mL) compared to levels in non-stressed laboratory-reared minnow (31.9 ng/mL, *see* Chapter I). Plasma glucose concentrations in isolated minnow (42.2 - 58.5 mg/dL) were also elevated above non-stressed fish (20.1 mg/dL, *see* Chapter I). Together, these two variables reflect a fish undergoing stress within the isolated pools unrelated to rescue despite optimal water quality and no prior disturbance. However, plasma osmolality of 278.8 – 281.4 mOsm/kg reflect that these fish were not experiencing osmotic results of stress. These levels were well within those of non-stressed laboratory-reared Rio Grande silvery minnow (279.1 mOsmol/kg; *see* Chapter I). We recognize that the prevailing physical and chemical conditions within the pools as well as time in isolation varied greatly among pools which would result in varied responses by the minnow to current conditions within their pool. Despite this variation, the physiological assessment reflects moderate stress from isolation in the wild minnow at the time of capture that was not yet manifested in osmotic dysfunction. The implication of osmotic dysfunction was discussed in Chapter I and is related to the severity and duration of the stressor which dictates whether a fish can recover from a stressor.

In 2007, the effects of capture from isolated pools and transport to perennial reach were comparable to those observed in 2006. At 24 and 48 h into recovery, plasma cortisol, glucose, and

osmolality levels reflected wild Rio Grande silvery minnow were experiencing stress similar in severity to fish rescued under less protective rescue protocol in 2006. Disease diagnostics of fish rescued in 2007, however, revealed lower prevalence of parasitism and disease of fish rescued from isolated pools when adhering to the more stringent rescue protocol. This was further supported in greater survival (67.0 - 96.7%) of the wild fish in recovery.

To place this in perspective, we can compare cortisol concentration in these wild minnow subjected to cumulative effects of stress during rescue to the cumulative effects of similar stressors in laboratory-reared minnow reported in Chapter I. Briefly stated, when laboratory-reared fish were subjected to multiple stressors without recovery, plasma cortisol concentrations peaked at 350 ng/mL, plasma glucose concentrations peaked at 63 mg/dL, and plasma osmolality declined to the lowest value of 266 mOsm/kg. Within 12 h post-stress, all stress responses returned to baseline or resting levels seen in laboratory-reared fish. The relative short time to recovery in laboratory-reared fish is in contrast to the elevated stress response seen well into 48 h in the wild counterpart. This was due to the prior stress the fish were experiencing while in the pools at the time of collection. We recognize the limitations when comparing physiological responses between wild and hatchery-reared fish. The comparisons, however, allow us to begin to assess the effects that environmental pressures may have when very little is known about the species fish (Caldwell and Strange 1987).

Wild and captive-reared fish can differ in the magnitude and duration of their physiological responses to stressors (Barton and Iwama 1991). Caldwell and Strange (1987) demonstrated a higher magnitude in the stress response of wild rainbow trout compared to hatchery-reared fish of the same species. Dietary carbohydrate can affect carbohydrate metabolism in fish subjected to stress (Hemre et al. 1991). Presumably, this would result in greater energy reserves in hatchery-reared fish to compensate for the negative effects of stressors than that of their wild counterparts. In addition, regular exposure of the hatchery-reared fish to physical stressors such as sorting, netting, confinement, transport, and cleaning activities would result in the fish becoming better adapted to repeated short-term physical stressors. The magnitude and duration of the stress response in fish might also depend on other factors such as health (Barton and Schreck 1987), reproductive status and developmental stage (Barton et al. 1985), and external factors such as rearing temperature, salinity, water quality and background color of recovery tanks (Barton and Iwama 1991). Thus,

careful consideration should be taken into account when interpreting the management implications when comparing wild species with their hatchery-reared counterparts.

Disease in fish is not the result of a single event, but of multiple interactions among fish, pathogen, and the aquatic environment. The presence of most fish pathogens will not result in disease unless unfavorable conditions (stressors) compromise the host's defense system (Sniesko 1974). Infectious diseases such as those observed in wild Rio Grande silvery minnow collected from intermittent reaches in 2006 indicated that stress tolerance limits were exceeded while either in the pools or shortly after rescue and transport to the perennial reach for release. The occurrence of pathogenic parasitism by *Costia* on wild minnow rescued from isolated pools was high. *Costia* (also referred to as *Ichthyobodo*) is a flagellated protozoan that adheres to the host via flagella. Relatively few of the flagellated protozoans result in pathogenesis of their fish host except for *Costia* (Noga 2000). Fish culturists consider *Costia* to be one of the most dangerous of the external protozoans if culture conditions shift in favor of the parasite. *Costia* is ubiquitous in the aquatic environment and often found on the skin and gills of their fish hosts in very small numbers if the host is healthy. However, the relationship shifts quickly to pathogenic if the fish host becomes stressed due to changes in environmental conditions that are beneficial to the parasite but harmful to the host (e.g., low dissolved oxygen, elevated temperature, elevated ammonia, elevated nutrients).

Trichodina is a ciliated protozoan that works similarly to *Costia* by infesting the gills and skin of their hosts resulting in epithelial tissue damage (Post 1987). This parasite was also found in large numbers on the wild minnow collected from isolated pools in 2006. While *Trichodina* infestation will not usually result in pathogenic effects to its host, damage to the epithelial tissues results in secondary bacterial infections followed by large-scale mortality in the infected population. In fish populations experiencing poor nutrition and overcrowding, moderate infestation results in loss of appetite with low levels of mortality (1% of the population; Noga 2000).

Trichophyra was also observed in wild Rio Grande silvery minnow rescued from isolated pools in 2006. *Trichophyra* is a ciliated protozoan but referred to as an 'ectocommensal' protozoan because it does not penetrate the host epithelium but feeds off sloughed epithelial cells and external bacteria. As a bacteriovore, *Trichophyra* will flourish in waters, similar to isolated pools of high nutrient or organic levels observed during intermittency throughout the Middle Rio Grande. Elevated nutrient levels will often tip the balance in favor of the protozoan resulting in excessive

parasite numbers on the gills. The impacts include excessive mucous production and prevention of oxygen diffusion from the environment through the lamellae.

Monogenean trematodes (flukes) are common parasites found on the gills and skins of fish hosts and are usually indicators of deteriorating water quality (overcrowding, high ammonia, organic pollution) (Noga 2000). These parasites feed on the epithelial layers of gills and skin resulting in irritation and excessive mucus production. In addition, Monogeneans attach to their hosts using posterior hooks that damage tissue often creating entry points for bacterial invasion and secondary infections. They are usually found in small numbers on host fish and unless their host becomes stressed from other conditions, their presence does not indicate pathogenesis or reveal adverse health effects.

The reproductive potential of the protozoans increase exponentially and result in death of the fish host if there is a change in environmental conditions that are beneficial to the parasite (e.g., low dissolved oxygen, elevated temperature, elevated ammonia, elevated nutrients). An increase in food supply to the protozoan (usually in the form of bacteria) is indicative of stress to the fish host and is followed by exponential population growth of the protozoan within a few hours. Temperatures between 10^{o}C and 25^{o}C favor rapid protozoan reproduction with several generations produced in a few hours (Post 1983). In addition to external parasites, two types of bacteria were isolated in the wild minnow rescued from the isolated pools in 2006. Bacterial gill disease was identified and confirmed by the presence of a flexibacter while bacterial septicemia resulted from the presence of an aeromondad. Both types of bacteria are representative of opportunistic pathogens that are ubiquitous in the aquatic environment but harmless to fish unless their immune systems become compromised.

The results of the disease diagnostics revealed that the majority of the wild minnow collected throughout pools in both reaches the summer of 2007 were relatively free of parasites and bacterial pathogens. The modified rescue protocol was designed to ensure fish were rescued from isolated pools of optimum water quality and thereby increase their chances for recovery and survival upon release. Although we were uncertain of the exact length of time the fish were in the pools, we surmised optimum temperature and dissolved oxygen levels as well as low parasitism and moderate physiological stress indicate fish were rescued within an adequate time frame that would increase chances of survival upon release to the perennial reach.

There is very little information in the literature describing the pathogenic effects of isolation in pools of stream or riverine fishes. Post (1987) described elevated mortality in wild fish populations caused by a protozoan (i.e., Trichodina) resulting from low flow conditions in which fish were isolated in shallow pools (similar to what occurs in the middle Rio Grande). The author inferred elevated parasitism was the result of proliferation of the protozoan populations. Robinson et al. (1998) observed parasite infestation (Trichodina, Ichythyophthirius, white grubs, tapeworm) with reduced health indices in wild razorback suckers (*Xyrauchen texanus*) and Colorado pikeminnow (*Ptychocheilus lucius*) throughout the Verde River, AZ. The authors were not able to relate disease prevalence with either water quality or time of year. To our knowledge, this study reflects the first of its kind in which stress and fish health were assessed for wild fish in isolation during intermittency.

In 2007, we observed two size classes of wild Rio Grande silvery minnow which presumably reflects fish collected from the Isleta reach may have been at least one year old while fish collected from the San Acacia reach may have been at least two years old. This is noteworthy in that Alò and Turner (2005) demonstrated reduced genetic diversity of wild Rio Grande silvery minnow was related to habitat fragmentation by diversion dams throughout the fish's current range. The diversion structures act as physical barriers to egg and larvae dispersal as well as subsequent recruitment and dispersion throughout the species range. Our observation of two distinct size (i.e., age) classes within the river reaches lends additional support to the consequences of the effects of impediments throughout the species current range. Stress from repeated river intermittency can increase the deleterious effects of reduced genetic diversity (Franham 2005). The loss of genetic diversity can increase the susceptibility and thus the consequences of stress-related disease in a population of individuals.

Management Implications

Conservation and recovery efforts of the imperiled Rio Grande silvery minnow often result in a range of physical and environmental stressors placed upon the fish. During the irrigation season when large sections of the middle Rio Grande begin to dry, the fish becomes isolated in pools. This results in crowding, reduced food availability, temperature fluctuations, low dissolved oxygen, high pH, and an increased concentration of opportunistic pathogens. Add to these, the effects of capture with seines, dip nets and transport to perennial reaches, the concern becomes whether or not rescue of the minnow is a viable management practice in meeting recovery goals of the species. Therefore, managers and agencies executing the Rio Grande Silvery Minnow Recovery Plan must consider the detrimental effects that individual as well as multiple stressors have on the fish's chances for survival upon release during repatriation activities. Appropriate protocol for propagation and rescue activities should undergo annual review and revision to ameliorate management-related stressors as they relate to current environmental conditions (i.e., drought, extreme temperatures). Prior to rescue, fish isolated in pools may experience temperature fluctuations and sub-optimal water quality that are not seen in water quality assessment at the time of the rescue. While rescue procedures have been modified through the years, physical stressors are simply unavoidable when rescuing and relocating fish to more suitable habitat. The underlying concern, associated with acute short-term stress, is that the stimulus ceases long before the response to the stress is complete and the time course of when the fish fully recovers will be strongly influenced by the severity and duration of the stress. Thus, it is necessary to identify not only the magnitude and duration of the stress response, but to identify whether the fish recovers from the combined effects of the physical stressors and to determine the timeline for recovery. While there is no doubt that the cumulative effects of stressors associated with pre- and post-stocking activities may result in reduced survival long after the release of fish (Barton et al. 1986), the long term effects are often manifested weeks post-stress and are often not observed by managers or monitoring programs.

In summary, the direct and indirect effects of river intermittency on habitat, physiological responses and health of Rio Grande silvery minnow are complex. Figure 5 is a diagrammatic attempt to summarize the relationships between response groups (variables directly and indirectly affected). While a portion of these response variables have been characterized in captive populations of the minnow under controlled conditions, the majority of the potential effects have yet

to be elucidated. Until these pathways are explored, it is unlikely that we will reach the recovery goal of a self-sustaining population of Rio Grande silvery minnow.

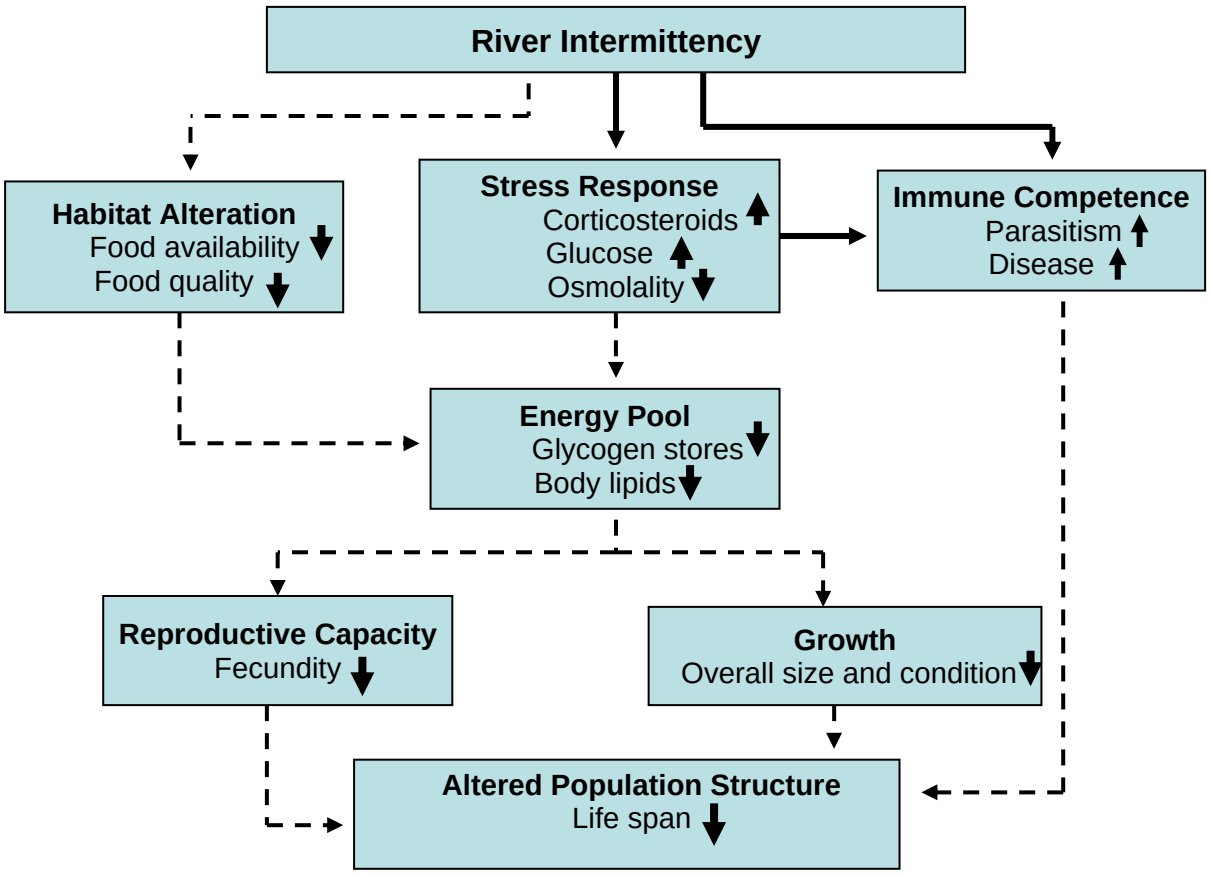

Figure 5. Direct and indirect effects of river intermittency on habitat alteration, physiology and health (immune competence) of Rio Grande silvery minnow populations within the Middle Rio Grande. Solid lines represent known relationships between response groups and dashed lines represent presumed relationships between response groups (adapted from Adams et al. 1992).

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APPENDICES

APPENDIX A

Memo to the U.S. Fish and Wildlife Service on Observations of Diagnostics and Blood Chemistry of Rio Grande Silvery Minnow Collected During 2005 Rescue Operations

New Mexico Cooperative Fish and Wildlife Research Unit
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December 5, 2005

Memorandum

TO: Brian Hanson, Field Supervisor, U.S. Fish and Wildlife Service, Ecological Services Field Office, Albuquerque, New Mexico

FROM: Colleen Caldwell, Acting Leader, New Mexico Cooperative Fish and Wildlife Research Unit, New Mexico State University

SUBJECT: Diagnostics and blood chemistry of Rio Grande silvery minnow collected during rescue operations

On 14 September 2005, Colleen Caldwell, SungJin Cho (Graduate Student, NMSU), and Jason Remshardt (Fisheries Resources Office) assisted Michael Hatch (Ecological Services Field Office) with rescue of Rio Grande silvery minnow from the Peralta reach (RM 153) of the Rio Grande. Work began at around 0830 with seining of intermittent pools throughout several miles of the Peralta reach. Field notes reflect moderate conditions (air = 24-26 ^{o}C; water = 20-26 ^{o}C, slight breeze, sunny). Rio Grande silvery minnow were collected in small numbers that varied from 1 to as many as 20 fish from one pool. Fish from each pool were placed into a 1.5 L bag containing water from the pool, salt and Stress Coat™. Dissolved oxygen from a compressed oxygen tank was added to the water and the bag carefully tied. The bagged fish were placed into a cooler attached to the rear of an all-terrain vehicle.

At approximately 1400, Caldwell, Cho and Remshardt returned to the vehicles to begin the characterization of physiological effects of "reference" RGSM not subjected to isolation in pools and rescue efforts. The objectives at this time were to collect RGSM from areas of the river that were flowing and not subjected to isolation. Rio Grande silvery minnow were collected in a series of seine hauls (two to three attempts were necessary to collect sufficient numbers of fish). These fish were immediately returned to the vehicle where the fish were anaesthetized and blood collected by severing the caudal peduncle. Blood was collected by touching the tip of a heparinized hematocrit tube to the severed caudle area. The tubes were centrifuged for 4 minutes using a hematocrit centrifuge powered with a generator. After centrifugation, tubes containing separated plasma and packed red blood cells were placed on ice. Within a six hours of the blood collection, we separated the plasma from the packed cells and froze the plasma for later analysis of cortisol (see below). Average total time between seine collection and anaesthetization of the fish was between 15 and 20 minutes.

Rescue and salvage of RGSM continued until approximately 1600 when the field crew returned to the vehicles, loaded equipment and drove to the release site whereupon fish were released at approximately 1730. The majority of the fish appeared to swim vigorously away from the release point. At the release site, Caldwell, Cho and Remshardt obtained blood from a small sample of RGSM just prior to release. The objectives at this time were to characterize the cumulative physiological stress effects of isolation, rescue, handling, and transport of fish. In addition, 10 (apparently healthy) RGSM were selected from a series of the earliest collections (0830–noon), bagged and labeled Peralta #1. A second set of 10 (apparently healthy) RGSM were selected from a series of the later collections (noon-1400), bagged and labeled Peralta #2. The objective of separating the fish into two bags was to identify if diagnostics of pathogens and survival differed

between the earlier and later collection periods. These fish were shipped via FED EX overnight (next morning delivery) to the U.S. Fish and Wildlife Service Pinetop Fish Health Center for diagnostics of external parasites and bacteriology. Of note, two of 10 fish from Peralta #1 survived the shipping to Pinetop Fish Health Center. Four of the 10 fish from Peralta #2 survived the shipping.

The report by Hines (PFHC; see attached) describes the presence of three external parasites (Costia, Cryptobia, Trichodina). Costia (also referred to as Ichthyobodo) and Cryptobia are flagellated protozoans that adhere to their host via flagella. Relatively few of the flagellated protozoans result in pathogenesis of their fish host *except* for Ichthyobodo while Cryptobia is weakly pathogenic (Noga 2000). Fish culturists consider Ichthyobodo to be one of the most dangerous of the external protozoans if culture conditions shift in favor of the parasite. Trichodina is a ciliated protozoan that adheres to the host similar to a suction cup with bristles resulting in extensive damage to skin and gills. Icthyobodo and Trichodina typically occur on both skin and gills of their host in very small numbers while Cryptobia will be found in small numbers in the gut and gills. If present, and the host is healthy, both host and the protozoans described above exist in a symbiotic relationship deriving some benefit from one other feeding on sloughed epithelial cells (Post 1983). The relationship shifts quickly to pathogenic if the fish host becomes stressed. The reproductive potential of the protozoans increase exponentially if there is a change in their environment to conditions that are beneficial to the parasite but harmful to the host (e.g., low dissolved oxygen, elevated temperature, elevated ammonia, elevated nutrients). An increase in food supply to the protozoan (usually in the form of cell sloughing) is typically indicative of stress to the fish host and followed by enormous population growth of the protozoan within a few hours. Temperatures between 10 and 25^{o}C favor rapid reproduction with several generations produced in hours. Of interest regarding rescue of RGSM from isolated pools, Post (1983) reported elevated mortality of wild fish populations due to Trichodina in which low flow conditions isolated fish in shallow pools resulting in increased protozoan populations.

In addition to external parasites, Hines (PFHC) isolated and identified four species of bacteria. These represent opportunistic species that are ubiquitous in the aquatic environment and are not harmful until the fish become compromised. Disease in fish is not the result of a single event, but the result of multiple interactions between the fish, the pathogen, and the aquatic environment. Infectious fish diseases that indicate tolerance limits to stress have been exceeded include facultative bacterial pathogens such as Aeromonads and Pseudomonads observed in RGSM by Hines. Thus, pre-existing protozoan parasites (Ichthyobodo) infections may not be a problem until the fish is pre-disposed to stress. The presence of most fish pathogens will not result in disease unless unfavorable conditions compromise the host's defense system.

Plasma cortisol was assayed by Caldwell and Cho to determine the magnitude of the cumulative effects of stress (i.e., isolation in pools, handling, transport) and whether the cumulative effects could be identified and separated. Environmental stressors are cumulative; thus, it is important to identify which of the stressors associated with rescue result in the greatest stress effects. It is also important to acknowledge the stress in fish isolated in pools and to separate the effects of isolation from those of collection and transport. We chose plasma cortisol because it is the 'primary stress hormone' in teleosts. Glycogen deposits in the liver of teleost fish represent an important site of action for cortisol that results in a secondary metabolic response by mobilizing glucose or energy stores. Thus, the purpose of cortisol release is to stimulate a series of compensatory processes necessary to achieve homeostasis or acclimation by the fish. Release of these energy stores assists with regulation and maintenance of the stress effects. If the duration and/or severity of the stressor exceeds the tolerance limits of the fish, compensatory physiological changes become maladaptive. Adverse physiological responses occur such as reduction in growth, altered behavior, and increased susceptibility to disease (Wedemeyer 1970).

The assay for plasma cortisol required compositing five RGSM for each value reported below. Total plasma cortisol concentrations in the 'stressed' fish (collected and transported throughout the day) ranged from 236.3 to 259.5 ng/mL (average of 3 composites = 240.0 ng/mL; standard deviation = 17.92). Total plasma cortisol concentrations in 'reference' fish (bled within 20 minutes of capture) ranged from 178.9 to 222.4 ng/mL (average of 4 composites = 192.5 ng/mL; sd = 20.60). Total plasma cortisol concentrations in 'stressed' RGSM were relatively high. Other cyprinids exhibited plasma cortisol concentrations from 120 to as high as 583 ng/mL when subjected to a range of handling, transport, and confinement conditions (see Barton and Iwama 1991). We anticipated 20 minutes as sufficient in which to capture and bleed a fish to characterize basal or prestress cortisol levels. Although prestress or basal plasma cortisol levels vary among species of fish (see Barton and Iwama 1991 for review), the elevated average concentration observed here (192.4 ng/mL) was higher than expected for resting levels and reflects a shorter duration in time to initiate or mobilize cortisol in the blood stream. The elevated plasma cortisol levels may also reflect the stress response of a wild fish which is greater in magnitude to hatchery-reared fishes (see Caldwell and Strange 1987). For example, baseline or prestress levels of plasma cortisol in RGSM reared in culture conditions at NMSU ranged from 1.0 to 30.0 ng/mL. When subjected to 30 min of handling stress, plasma cortisol in RGSM ranged from 38.3 to 195.0 ng/mL. Despite these differences, corticosteroids are known for their anti-imflammatory and immunosuppressive effects in mammals and fish. Elevated blood cortisol levels compromise the fish's immune responses by inhibiting imflammatory reactions and phagocytosis (i.e., reduced lymphocytes and macrophages) and retarding healing processes resulting in increased susceptibility to disease. Additional work characterizing the stress response in RGSM is currently underway at the USGS/NMSU laboratory of Caldwell and Cho.

Summary and Recommendations

The cumulative effects of stress in fish result in disruption of physiological equilibria leading to pathogenic outbreak of parasites, diseases and ultimately reduced survival. When one considers the combined effects of elevated plasma cortisol, elevated gill parasites, and opportunistic bacterial infections observed in RGSM subjected to rescue efforts, then management strategies in future rescue operations of RGSM from isolated pools within the Rio Grande should be reconsidered and revised accordingly. The results described here indicate that efforts and resources used to rescue and relocate RGSM may be fruitless if (1) pools containing RGSM are revisited several times when water quality has degraded over time and (2) RGSM are transported for long periods of time prior to their release. Rio Grande silvery minnow in pools may have slightly increased chances of survival if they are not intermittently disturbed by repeated seining efforts (resulting in resuspension of sediments and nutrients) as well as handling and transport (resulting in cumulative effects of stress) with the chance that a singular rain event or increased flow provides connectivity to the river. Recommendations are that the USFWS consider implementing rescue activities *only* during optimal times of the year (low temperatures and elevated dissolved oxygen) and when fish are subject to isolation in pools for only a brief time. If rescue occurs, consider the following to reduce cumulative stress and thus increase the chances for RGSM survival: transport fish in clean river water having elevated salt concentrations of 1.0%, reduce confinement densities, and reduce hauling time during rescue. One last note of concern pertains to the relocation of diseased fish into areas containing healthy RGSM. Relocation efforts should include an inventory of RGSM populations and their health status throughout areas prior to introducing salvaged fish. The addition of any number of diseased fish into a healthy population may have severe consequences and thus negate not only rescue efforts but the RGSM propagation and augmentation efforts as well.

Additional research is planned by Caldwell and Cho for the spring of 2006 in which blood chemistry, susceptibility to diseases and survival will be more thoroughly characterized in RGSM subjected to a range of handling, transport, and confinement stressors.

Cc Michael Hatch, Fisheries Biologist, U.S. Fish and Wildlife Service Ecological Services Field Office

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APPENDIX B

U.S. Fish and Wildlife Service, 2004 Protocol for Rescue and Salvage Operations of Rio Grande Silvery Minnow

U.S. Fish and Wildlife Service, 2004 Protocol for Rescue and Salvage Operations of Rio Grande Silvery Minnow

A) Handling Protocol for distribution truck

Handling and transportation of Rio Grande silvery minnow after reaching the distribution truck will follow the proposed protocol set forth by Service protocol, as follows:

- Water temperature (determined with thermometers) should be about 5°F lower in the hauling truck than in the river.
- Drivers must be informed of and follow a specific route.
- Hauling water will contain 0.5 percent NaCl (18.9 grams/gallon) and 0.26ml/L stress coat (1 ml/gallon).
- Oxygen levels will be >6.0 mg/L as determined with an oxygen meter.
- Nets must be functional. Aeration equipment must be in place and must be used. A fish-holding container will be at least 5 gallons in size and fish densities will not exceed 10 grams of fish per liter of water. Small Delta mesh (1/16") nets will be present to transfer the fish from one container to another, although it is preferred to have water-to-water transfer. Oxygenation/aeration equipment will be in place and working.
- Prior to loading and after the fish are concentrated, they should be quickly placed in the transport tank. When using nets to place fish in the transfer buckets, nets should not be overloaded or the fish on the bottom could be crushed. Using a “wet transfer” with buckets or other containers that contain water is preferable. When emptying the nets and buckets, care will be taken to avoid adding algae and mud to the transport tank. Before loading, dissolved oxygen levels should be at saturation.
- Immediately after loading, all equipment on the transport vehicle should be re-checked and then the vehicle should depart. Oxygen concentrations and temperatures should be monitored at a minimum of every 30 minutes.
- During unloading, water will be tempered, and thermometers should be used to match water temperatures. Hauling water temperature should be equal to receiving water temperature.
- Silvery minnow mortality will be preserved. Final disposition of specimens will vary with developing plans and operational needs.

B) Hauling Protocol when transporting silvery minnows in bag

This protocol is similar to that for the distribution truck. In some instances, it may be more feasible to transport silvery minnows in aerated plastic bags rather than a distribution truck. The designated crew leader will make this determination with concurrence from the Coordinator.

The Protocol is as follows:

- Only plastic bags designed for fish transport will be used.
- Plastic bags will contain no less than half their capacity in water.
- Ice chests of sufficient size to hold 1 to 3 bags will be used to protect bags from ambient conditions.
- Temperature (determined with thermometers) should be about 5°C lower in the bags than in the river.
- Water in the bags will contain approximately 0.5 percent salt (18.9 grams/gallon) and 0.26ml/L stress coat (1 ml/gallon). This will be measured by scales and graduated cylinders.
- Oxygen levels will be >6.0 mg/L as determined with an oxygen meter.
- Fish densities should not exceed 10 grams of fish per liter of water.
- Small Delta mesh (1/16") nets will be present to transfer the fish from one container to another. Using a “wet transfer” with buckets to bags containing water is preferable.
- When emptying the nets and buckets, care will be taken to avoid adding algae and mud to the transport bag.

- Dead specimens will be preserved and disposed of properly.

C) Transporting of silvery minnows in the field and distribution to flowing water

This will occur when it is feasible to distribute fish to flowing water near a dry reach. This method will be used when the crew leader and Coordinator determine that transporting fish to distribution trucks will cause undue stress to the silvery minnows. This will reduce silvery minnow mortality in some cases.

The protocol is as follows:

- Each ATV and/or amphibious craft (ARGO) will be outfitted with an ice chest with a water capacity of greater than 10 gallons.
- Temperature (determined with thermometers) should be equal or lower in the ice chests than in the river.
- Water in the ice chest will contain approximately 0.5 %salt (18.9 grams/gallon) and 0.26ml/l stress coat (1 ml/gallon). Scales and graduated cylinders will be employed to measure this.
- Fresh water will be added to the ice chest every 15 minutes if available. If water is not available, then existing water will be agitated within the ice chest using a bucket. This will ensure some level of dissolved oxygen within the ice chest during transport.
- Fish densities should not exceed 10 grams of fish per liter of water. When estimated densities exceed this concentration, fish should be transported to the distribution truck, then placed in plastic bags at the truck, or released into flowing portions of the river.
- Silvery minnows will be transferred from one container to another in a water medium using buckets. When this "wet transfer" method is impractical, fish will be captured and transferred using small delta mesh (1/16") nets.
- When emptying the nets and buckets, care will be taken to avoid adding debris to the ice chest.
- Before fish are released into flowing river, water temperatures will be within 1°C of each other.

General Distribution and Release

The following protocol will be used for distribution and release of silvery minnows. This will insure that the silvery minnows are not subjected to any undue stress during transport and release.

- Drivers must be informed of and follow a specific route.
- Immediately after loading, all equipment on the transport vehicle should be re-checked and then the vehicle should depart. Oxygen concentrations and temperatures should be monitored at a minimum of every 30 minutes.
- During unloading, a tempering water pump should be present and functional, and thermometers should be used to match water temperatures. Hauling water should be equal to receiving water. Bags should be placed in the receiving waters until temperatures within the bags are equal to or within 1°C of receiving water.

APPENDIX C

U.S. Fish and Wildlife Service, 2006 Protocol for Rescue and Salvage Operations of Rio Grande Silvery Minnow (REVISED)

U.S. Fish and Wildlife Service, 2006 Protocol for Rescue and Salvage Operations of Rio Grande Silvery Minnow (REVISED)

This document serves as a protocol for conducting silvery minnow salvage activities beginning in 2007. The primary goal for salvage of Rio Grande silvery minnow (silvery minnow) from intermittent reaches of the Rio Grande is to reduce the probability that the mortality associated with water operations will exceed the limit for incidental take. Every effort will be made to increase the efficiency of salvage efforts towards this primary goal while improving the effects of releasing salvaged fish on the remaining silvery minnow population. To this effort we are proposing a set of criteria which would determine when and if salvage and subsequent releases would occur. We will test the effectiveness of these criteria in 2007, evaluate the data, and make the necessary changes to the criteria for future salvage efforts.

Interim results from recent research indicates that cumulative effects of intermittency, capture, and transport of salvaged fish result in greater physiological stress responses and lower survival compared to fish collected from perennial areas (Caldwell et al. 2006). Therefore, the priority for salvage activities will concentrate on newly (on an annual basis) intermittent stretches of river where salvage would minimize incidental take and survival of salvaged fish would be highest. The cumulative effects of intermittency may increase the silvery minnow's susceptibility to disease as well as increase the chances of exposure to opportunistic pathogens (Caldwell et al. 2006). In the instances where salvage is deemed necessary and feasible, every effort will be made to ensure that any fish to be moved have the highest probability of survival. There is also a potential for unwanted spread of disease and pathogens through the intentional release of potentially health-compromised silvery minnow. Included in this protocol as Appendix C is a Hazard Analysis Critical Control Point (HACCP) plan to remove or reduce the risks of introducing hazardous non-target species of plants, animals, and pathogens into new locations associated with salvage activities.

Determination of Priority for Salvage and Incidental Take due to Channel Drying

Salvage activities enable officials to minimize take of silvery minnow due to water management activities and secondarily enumerate take. In the recent past, intermittent conditions have existed in as many as 68.0 miles of the river between Isleta Diversion Dam and Elephant Butte Reservoir. Although incidental take can occur due to factors other than river drying (e.g., incidental take limit of 100,000 eggs due to entrainment into irrigation canals), salvage is not an effective management option to lessen these effects. Therefore, salvage activities relate only to that portion of incidental take due to channel drying.

Silvery minnow mortality that occurs as a result of federal water operations in the Middle Rio Grande is evaluated under measures established in the March 17, 2003 Biological Opinion (BO) (U. S. Fish and Wildlife Service 2003) and as amended on August 15, 2005 (U. S. Fish and Wildlife Service 2005). The August 15, 2005 amendment specifies that incidental take limits due to channel drying apply to silvery minnows > 30 mm standard length (SL) or approximately 35 mm total length. The number of silvery minnow < 30 mm SL is difficult to quantify and therefore no take limit has been applied to this size class. All smaller sized post-embryonic silvery minnow are presumed to be taken as a result of federal water operations when the river dries downstream of Isleta Diversion (U. S. Fish and Wildlife Service 2003). Silvery minnow less than 30 mm SL are fragile and desiccate quickly and post-salvage survival of these small fish after handling and transport is doubtful. As amended, the incidental take for adults and young-of-year > 30 mm SL is now estimated annually using a formula that incorporates data from fall recruitment, spring runoff, and augmentation. Estimates of incidental take are derived by multiplying the observed mortality by 50, based on the assumption that the probability of observing a single mortality is 0.02. This estimate originates from past salvage activities and field observations.

Efforts to salvage silvery minnows from intermittent reaches of river may reduce silvery minnow mortality of individuals > 30 mm SL that can occur with channel drying. It will also reduce the probability that the mortality associated with water operations will exceed the limit for incidental take. Silvery minnow salvage operations will generally progress in synchrony with river recession, but will always be prioritized in river reaches where probability of take of silvery minnow due to federal water operations is highest.

The definition of incidental take for slivery minnows > 30 mm SL depends on the environmental and operational conditions that lead to "take." Mortality of silvery minnows > 30 mm SL that occurs in portions of the river rewetted due to acts of nature (e.g., runoff from thunderstorms) or the diurnal expansion-contraction cycle that attends river recession is not considered incidental take under the March 17, 2003 Biological Opinion (U. S. Fish and Wildlife Service 2003). Generally, flows in the river retract quickly following a storm event and may result in silvery minnow mortality. However, such mortality cannot be said to be an effect of water operations and therefore is not considered incidental take. Similarly, the diurnal expansion-contraction cycle that attends river recession may result in mortality of silvery minnow > 30 mm SL but also will not be regarded as incidental take. Silvery minnow mortality that occurs outside of the active river channel is generally not considered to be incidental take under the March 17, 2003 BO (U. S. Fish and Wildlife Service 2003); the exception to this generalization involves areas outside of the active channel that dry as a consequence of federal water pumping operations. For example, when supplemental pumping from the low flow conveyance channel has ended, silvery minnows may become stranded in the artificially constructed canals that connect to the river.

All silvery minnow that are "salvaged" become the responsibility of the U.S. Fish and Wildlife Service and therefore fall under the Southwest Regional Director's permit. If these individuals perish before they are subsequently released they will not be considered incidental take. Silvery minnow that exhibit advanced clinical signs of poor health (e.g., lethargy and/or hemorrhagic lesions) during the salvage process will not be salvaged and not count towards incidental take.

Salvage Criteria

Once a location is identified as a potential for salvage, a set of primary and secondary biological criteria will be applied to determine whether salvage should occur. These criteria were defined using field experience (air temperature and secondary fish health) and after reviewing tolerances of silvery minnow to environmental variables (secondary water quality, K. Buhl, unpublished data). Documentation of conditions, incidental take (if appropriate), and preservation of individuals follows. The primary criteria (ambient air temperature > 34 C) will first be applied and if not met, salvage activities will not continue. The secondary (water quality and fish health observation) criteria will be applied to individual isolated pools as differences in water quality and fish health vary, but if any one of these secondary criteria is not met for a particular isolated pool, then salvage will not occur from that pool.

Primary	1. Ambient air temperature > 34 C
Secondary (Water Quality)	1. Water temperature > 34 C 2. Dissolved Oxygen < 2.0 mg/liter 3. pH > 9.0
Secondary (Fish Health)	1. Dead fish (any species) in pool 2. Lethargy and/or hemorrhagic lesions noticed from fish (any species) in pool

Enumeration of Incidental Take and Salvage

When the number of taken silvery minnow in an isolated pool precludes absolute counts, an estimate of take will be necessary. The first step will be to estimate the total surface area of the isolated pool by measuring the average width and length of the pool. Then, a single seine haul through the pool will be made and measured (width of seine and length seined noted). The ratio of area seined to the area of the pool will allow an estimated number of take for that pool to be calculated. All fish collected in the seine will be preserved for later lab enumeration. Each sample of candidate incidental take specimens will first be processed to remove as much debris as practical, species of fish other than silvery minnows will be removed, and specimens of silvery minnows that are undersized (< 30 mm SL) will be removed from the sample and counted separately. This information may be used to estimate how many silvery minnows less than 30 mm SL were taken. The total sample will be weighed to the nearest 0.20 of a gram (estimated weight of a single silvery minnow 30 mm SL) after excess fluids have drained. Immediately after the total sample is weighed, the sample will be sub-sampled at least three times (without replacement) and sub-samples will be weighed to the nearest 0.20 of a gram. Silvery minnows in each sub-sample will be counted, taking care to insure that only silvery minnows that are > 30 mm SL are included in the count. These data will yield at least three proportion estimates of the number of silvery minnows to sub-sample weight. A mean and sample standard deviation will be calculated for the proportion estimates, and a 95 percent confidence interval will be calculated for the mean (i.e., $\bar{y} \pm t_a s/\sqrt{n}$, where t_a is obtained from a *t*-distribution table, with $a = 0.025$ and $df = n - 1$). All preserved specimens will be subsequently accessioned to the Museum of Southwestern Biology, University of New Mexico.

Counts or estimates of the number of salvaged silvery minnow will be made at the time of capture. When high numbers of silvery minnow are collected and absolute counts would cause undue stress on the fish, visual count estimates will be made by the crew leader. To document the variation associated with these field estimates, visual estimates will be made of preserved (see above paragraph) specimens prior to processing. These lab estimates of preserved specimens will then be compared to the actual count of silvery minnow in that sample. This data will allow us to construct 95% confidence intervals around our estimate of salvaged silvery minnow.

Fish Capture and Transport Procedures

Once a potential salvage area is identified, adequate transport water must be acquired. Water from a nearby continuous section of the Rio Grande is preferred, but a nearby section of a riverside drain may be used as well. Under no circumstance will water from isolated pools be used as transport water. The water from isolated pools is of poorer quality than that of flowing sections of water. Fish capture will be by seine. Seines with a variety of dimensions may be used, depending upon the size of fish to be salvaged and the habitat to be salvaged. Generally, approximate dimensions of seines will be 3-5 m long x 1-2 m deep. Seine mesh will be 3.2-6.4 mm. Seines will have a double weighted lead line and will be clean of fish, debris, and sediment prior to use in a new salvage habitat. Captured silvery minnow will be placed into five gallon buckets filled with hauling water and transferred to tanks or plastic bags for transport. Tanks will be employed to transport silvery minnow in instances when their use is logistically possible.

Transport tanks equipped with water-tight lids will be filled with water to near capacity to reduce sloshing and vibration within the tank during fish transport. Bags will be filled with river water to approximately 2/3 capacity (approximately 3 liters; 0.8 gallons); the remaining volume of the bag will be inflated with pure oxygen and its opening sealed to prevent the loss of the bag's contents. Rock salt will be added to water in hauling vessels to achieve a 1.0 percent solution, and Stress Coat will be added at the rate of 0.26 ml/l (1 ml/gallon). Preferred fish density (10 grams per liter) and water conditioning formulations specific to the various hauling vessels are presented in Appendix A.

Pure oxygen will be supplied to transport tanks through diffuser stones. The flow of oxygen will be adjusted to maintain dissolved oxygen levels between 90% - 100% saturation. A preferred temperature of 25° C will be set for transport of silvery minnow. When ambient water temperature is at or below this level, the strategy will be to stabilize the water temperature. Keeping the bags covered/inside ice chests (without ice) should be adequate to stabilize temperature. When ambient water temperatures are between 25° C and 30° C, the strategy will be to reduce the water temperature to 25° C by adding small amounts of ice around the bags. When ambient water temperatures are between 30° C and 34° C (maximum temperature for salvage), the strategy will be to reduce the water temperature by 5° C by adding ice around the bags. In each case, the change in temperature should be slow (1° C / 5 minutes) and monitored frequently. Bags with fish will be inflated with oxygen and conditioned with salt and Stress Coat and placed in ice chests cushioned with bubble wrap.

Fish Community Characterization

For each pool salvaged, all fish species (presence/absence), life stage (young of year, adult), and relative abundance within the habitat (common/rare) will be recorded on field forms (Appendix B.).

Release Procedures

The potential reaches for salvage in the middle Rio Grande are defined by their upstream irrigation structure, (i.e., Angostura, Isleta, and San Acacia). Salvaged silvery minnow will be transported to perennial portions of the reach in which they originated. Priority for release will be focused on sites that have a high expectation of remaining wet throughout the irrigation season. The criteria that are being proposed for identifying salvageable pools (air temperature, water quality, fish health) will also be applied during release. That is, when the field crew reaches the potential release site, all the same parameters will be measured/observed. If any of these criteria are not favorable, then release will not occur until all criteria are met. All the remaining occupied reaches within the middle Rio Grande are important to the continued existence to the species, including the Isleta and San Acacia reaches, where intermittency is most likely to occur. Retaining salvaged individuals within their respective reaches is important to provide the potential for re-establishment when persistent re-wetting occurs and lessen the need for augmentation. In addition, the potential for unintentional movement of non-target, invasive species and disease and pathogens is lessened.

Prior to release of fish into the river, water in the transport tanks will be tempered by slowly adding river water to the transport tanks or placing unopened bags into the river until it (transport water) is within 1° C of the ambient water temperature. Counts or estimates of silvery minnow transport loss (those that perished during handling and transport prior to release) will be made. Salvage and stocking locations will also be recorded using Global Positioning System (GPS) coordinates (Appendix B.).

Rio Grande Silvery Minnow Origin

The occurrence of hatchery-produced (i.e., marked) Rio Grande silvery minnow will be noted during salvage operations. Hatchery-produced fish have been stocked at various locations within the species' contemporary range in the Rio Grande. A portion of each batch of hatchery-produced fish is distinguished by a unique visible implant elastomer mark. When marked Rio Grande silvery minnow are observed during salvage operations, unique attributes of the mark will be noted (i.e., color and body location), along with the GPS location that the marked fish was encountered.

Non-Salvage Monitoring Activities

Monitoring activities during intermittency are necessary, regardless of the determination of take and/or if salvage occurs. During the times when salvage is not feasible and/or incidental take does not apply (i.e., secondary drying events), every effort will be made to accurately document the conditions present at these

sites including species composition, age, water quality, GPS locations. These activities will include the documentation and preservation of mortalities. Preserved specimens will be returned to the lab and identification and enumeration of each species will be recorded. Other data, including standard and total lengths and external appearance will be recorded, as appropriate. Preserved specimens will be processed similar to methods described for enumeration of incidental take and will be offered to the Museum of Southwestern Biology, University of New Mexico. All of this information could provide invaluable information for future management.

Continuation of Research on Effects of Salvage

Results from research, including those conducted in 2006 have had significant effects on developing this protocol. In 2007, we will work with Dr. Colleen Caldwell from the New Mexico Cooperative Fish and Wildlife Research Unit in continuing this research as required in the Terms and Conditions Item 1.3 in the BO (USFWS, 2003). Four (4) tasks are identified for determining the direct effects of salvage on the silvery minnow. This design would be repeated a minimum of 4 times per year. These are:

1) To assess direct effects of salvage (do the adults survive the short term effects of salvage), salvaged adult silvery minnows (at least 15 individuals) would be held in cages in the river for a minimum of two weeks.

2,3) To assess the effects of the cage on the silvery minnow an equal number of adult silvery minnows taken from a propagation facility, and an equal number of adult silvery minnows caught from the wild in a location where the river was always connected would also be held in separate cages at the same time, in the same locality, and for the same length of time as the salvaged fish.

4) In addition, an equal number of salvaged silvery minnows would be taken to a propagation facility and held in captivity under typical holding conditions to assess survivorship in a benign environment.

While all of these tasks have been conducted at various times over the last several years, the required number of replications (4) has been difficult to obtain but will be attempted in 2007. In addition, we will also work with Dr. Caldwell and others in conducting experiments on the indirect effects of salvage, optimal salinity concentrations for hauling, and other research as needed.

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Water Conditioning Formulations for Transport Vessels

Large Transport Tank:

Each half holds 211.2 L (55.80 gal) of water.
To render this volume a 1.00 percent salt solution requires 2,112 grams (2.1 kg) of NaCl, which volumetrically equals about 1.50 cups.
The prescribed amount of stress coat is 0.26 ml/L (1.00 ml/gallon), with 56.00 ml (or approximately 0.25 cups) of stress coat added to each half of the large tank.
Optimal fish density for 211.2 L @ 10 grams/L = 2,112 g. 4,000 young-of-year (35 mm TL = 0.5 g), or 700 adult (65 mm TL = 3.0 g)

Small Transport Tank:

The tank holds 138.2 L (36.50 gal) of water.
To render this volume a 1.00 percent salt solution requires 1,382 grams (1.4 kg) of NaCl, which volumetrically equals about 0.66 cups.
The prescribed amount of stress coat is 0.26 ms/L (1 ml/gallon)and 36.00 ml (or approximately 0.12 cups) of stress coat will be added to the small tank.
Optimal fish density for 138.2 L @ 10 grams/L = 1,382 g. 2750 young-of-year (35 mm TL = 0.5 g), or 450 adult (65 mm TL = 3.0 g)

Bags:

Bags will be filled with river water to approximately 0.66 of bag capacity (approximately 3.00 liters; 0.80 gallons).
To render this volume a 1.00 percent salt solution requires 30.00 grams of NaCl, which volumetrically equals about 2.66 teaspoons.
The prescribed amount of stress coat is 0.26 ml/L (1.00 ml/gallon) and 1.00 ml of stress coat will be added to each bag.
Optimal fish density for 3 L @ 10 grams/L = 30 g. 60 young-of-year (35 mm TL = 0.5 g), or 10 adult (65 mm TL = 3.0 g)

APPENDIX D

Disease Diagnostics of Wild Rio Grande Silvery Minnow: Reports from the U.S. Fish and Wildlife Service Fish Health Unit, Dexter National Fish Hatchery and Technology Center

D.1. October 2005 Disease Diagnostics of Wild Rio Grande Silvery Minnow

D.2. July 2006 Disease Diagnostics of Wild Rio Grande Silvery Minnow

D.3. July 2007 Disease Diagnostics of Wild Rio Grande Silvery Minnow

D.4. August 2007 Disease Diagnostics of Wild Rio Grande Silvery Minnow

D.1. October 2005 Disease Diagnostics of Wild Rio Grande Silvery Minnow

U.S. Department of the Interior
Fish and Wildlife Service
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October 13, 2005

To: Dr. Colleen Caldwell, New Mexico Cooperative Fish and Wildlife Research Unit

From: Phil Hines, Pinetop FHC

Subject: Diagnostic examination of Rio Grande silvery minnow

CHN 05-45

On 9-15-05 we received two groups of wild Rio Grande silvery minnow for diagnostic examination. These fish were shipped live to our lab. The Peralta #1 sample contained 8 fish, only 2 were alive when we received them. The second set of samples Peralta #2 contained 9 fish, 5 were alive. Theses fish were examined for external parasites and bacteria from kidney tissues on BHI media.

Peralta #1 - only the 2 fish that were alive were examined – No parasites were observed on the skin scrape samples. The gill tissue samples had trichodina, costia and cryptobia present. Trichodina were 5 to 0 per sample, costia were present in excess of 500 per sample, cryptobia were up to 50 per sample.

Peralta #2 – we examined 4 of the live fish. Skin scrapes were all negative, gill tissue samples had trichodina, costia and cryptobia in similar numbers to the Peralta #1 samples.

Bacteria were detected and identified from both groups of samples as Aeromonas hydrophila, Pseudomonas fluorescence, Plesiomonas shigelloides and Shewanella putrifaciens. All species of bacteria were identified using API biochemical test strips. All bacteria identified are opportunistic species of bacteria present in most aquatic environments. Typically the parasites stress the fish first and then the bacteria infect the fish as secondary pathogen.

These fish were considered to be in poor health and any additional stressors such collection and relocation could lead to high rates of mortality during relocation efforts or the fish could experience delayed mortality up to week or more after the fish are relocated.

D.2. July 2006 Disease Diagnostics of Wild Rio Grande Silvery Minnow

DEPARTMENT OF THE INTERIOR
U.S. Fish and Wildlife Service

FISH HEALTH INSPECTION REPORT

Wild Fish H..lth Survey

This report is **NOT** evidence of future disease status. To determine current status, contact inspecting biologist below.

Name of Fish Source	Address or location of Fish Source	Name of Owner/ or Manager	Inspection Dates	Classification
***Rio Grande	Valencia County, New Mexico N 34.8172° W 106.7125°	State of New Mexico	This 7-20-06 Prior	N/A

Species[1]	FISH EXAMINED Lot Number	Age[2]	Number in Lot	Obtained as Eggs(E) or Fish(F) FROM:	Pathogens Inspected for and Results[3] BF	BR	BK	SW	VE	VH	VP	VC	*A	*B
**RGM		Unk.		(F) Rio Grande, NM	20	20					20		20	20
					-	-					-		-	-

Type of Fish Examined
☐ Hatchery X Feral
☐ Salmonid X Non-salmonid

Type of Water Supply
☐ Spring ☐ Well
X Stream ☐ Impoundment
☐ Enclosed ☐ Free of Fish

Inspecting Biologist Signature

Concurred (signature & title)
Fish Health Unit Leader

Inspecting Biologist Address
Region 2 Fish Health Unit @ Dexter
P.O. Box 219
Dexter, NM 88230

Remarks: CHN 06-32 RGM = Rio Grande silvery minnow
*A = *Onchorynchus masou* virus. *B = Largemouth Bass Virus ** Negative for *Edwarsiella ictaluri* and *Edwarsiella tarda*.
***Parasite exam on 10 fish revealed the following: costia, trichodina, trichophrya, gas bubbles in the gills, bacterial gill disease and bacterial septicemia in the mouth area. Additionally, we isolated an unknown virus from one pool of fish. Presence of a virus was confirmed by visualization through electron microscopy by staff at the University of Montana. Samples were prepared by staff at the USGS Western Fisheries Research Center for electron microscopy.

1 - Use standard FES abbreviations (see back of this page)
2 - For hatchery fish give age in months: for feral fish use symbols e=eggs or fry; f = fingerlings; y = yearlings; b = older fish.
3 - See list of pathogen abbreviations on back of page: finding report as number examined / results where "-"=negative and + = positive; Other pathogens listed in remarks.

Form 3-226(8/82)

D.3. July 2007 Disease Diagnostics of Wild Rio Grande Silvery Minnow

DEPARTMENT OF THE INTERIOR
U.S. Fish and Wildlife Service

Wild Fish Survey

FISH HEALTH INSPECTION REPORT

This report is **NOT** evidence of future disease status. To determine current status, contact inspecting biologist below.

Name of Fish Source	Address or location of Fish Source	Name of Owner/ or Manager	Inspection Dates	Classification
*** Rio Grande	Socorro County, New Mexico N 33.9204° W 106.8510°	State of New Mexico	This 7-16-07 Prior	N/A

Species[1]	FISH EXAMINED Lot Number	Age[2]	Number in Lot	Obtained as Eggs(E) or Fish(F) FROM:	Pathogens Inspected for and Results[3] BF	BR	BK	SW	VE	VH	VP	VC	* A	* B
**RGS		unk.			10	10			10	10	10		10	10
					-	-			-	-	-		-	-

Type of Fish Examined
☐ Hatchery X Feral
☐ Salmonid X Non-salmonid

Type of Water Supply
☐ Spring ☐ Well
X Stream ☐ Impoundment
☐ Enclosed ☐ Free of Fish

Inspecting Biologist Signature: Jason Woodling

Concurred (signature & title): John C. Hoesen, Fish Health Unit Leader

Inspecting Biologist Address
Region 2 Fish Health Unit @
Dexter
P.O. Box 219
Dexter, NM 88230

Remarks: CHN 07-47

** Negative for *Edwardsiella ictaluri* and *Edwardsiella tarda*. Parasite exam on the skin and gill show infestations of trichodina, costia and gyrodactylus. See the attached parasite datasheet for more details on parasite examination of the skin and gills.

* A = Largemouth bass virus
* B = *Oncorhynchus masou* virus.
RGS = Rio Grande silvery minnow

*** Revised 9-13-7

1 - Use standard FES abbreviations (see back of this page)
2 - For hatchery fish give age in months: for feral fish use symbols e=eggs or fry; f = fingerlings; y = yearlings; b = older fish.
3 - See list of pathogen abbreviations on back of page: finding report as number examined where "-"=negative and + = positive; Other pathogens listed in remarks. results

Form 3-226(8/82)

Rio Grande Silvery Minnow Data Sheet

CHN	NMSU	Name of Location	Site #	Collection Date
07-47	from Colleen Caldwell	Rio Grande		July 17, 2007

Sample No.	Tag (color) if present	Location of Tag if present	Gills	Skin
1			53 Trichodina	Clean
2			19 Trichodina, 1 Costia	Clean
3			18 Trichodina	Clean
4			200+ Trichodina	Clean
5			41 Trichodina, 8 Costia	1 Trichodina
6			Clean	Clean
7			Clean	Clean
8			1 gyrodactylus, 3 costia, 1 trichodina	Clean
9			3 Trichodina	Clean
10			2 costia	no sample
11				
12				
13				
14				
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29				
30				

Collector(s): Hines, Woodland

Notes:

D.4. August 2007 Disease Diagnostics of Wild Rio Grande Silvery Minnow

DEPARTMENT OF THE INTERIOR
U.S. Fish and Wildlife Service

Wild Fish Survey

FISH HEALTH INSPECTION REPORT

This report is **NOT** evidence of future disease status. To determine current status, contact inspecting biologist below.

Name of Fish Source	Address or location of Fish Source	Name of Owner/ or Manager	Inspection Dates	Classification
Rio Grande	Valencia County, New Mexico N 34.6939° W 106.7450°	State of New Mexico	This 8-15-07 Prior	N/A

Species[1]	FISH EXAMINED Lot Number	Age[2]	Number in Lot	Obtained as Eggs(E) or Fish(F) FROM:	Pathogens Inspected for and Results[3] BF	BR	BK	SW	VE	VH	VP	VC	*A	*B
**RGS		unk.			10	10			10	10	10		10	10
					-	-			-	-	-		-	-

Type of Fish Examined
☐ Hatchery X Feral
☐ Salmonid X Non-salmonid

Type of Water Supply
☐ Spring ☐ Well
X Stream ☐ Impoundment
☐ Enclosed ☐ Free of Fish

Inspecting Biologist Signature

Concurred (signature & title)
Fish Health Unit Leader

Inspecting Biologist Address
Region 2 Fish Health Unit @ Dexter
P.O. Box 219
Dexter, NM 88230

Remarks: **CHN 07-50**

** Negative for *Edwardsiella ictaluri* and *Edwardsiella tarda*. Parasite exam on the skin and gill of 10 fish showed infestations of 3 costia and 1 monogentic trematode on one fish only. See the attached parasite datasheet for more details on parasite examination of the skin and gills.

* A = Largemouth bass virus
* B = *Oncorhynchus masou* virus.
RGS = Rio Grande silvery minnow

1 - Use standard FES abbreviations (see back of this page)
2 - For hatchery fish give age in months: for feral fish use symbols e=eggs or fry; f = fingerlings; y = yea ; b = older fish.
3 - See list of pathogen a viations on back of page: finding report as number examined where "-"=ne ve and + = positive; Other pathogens listed in remarks.

Rio Grande Silvery Minnow Data Sheet

CHN		Name of Location	Site #	Collection Date
07-50	from Colleen Caldwell	Rio Grande		August 15, 2007

Sample No.	Tag (color) if present	Location of Tag if present	Gills	Skin
1			Clean	Clean
2			Clean	Clean
3			3 costia	1 monogenetic trematode
4			Clean	Clean
5			Clean	Clean
6			Clean	Clean
7			Clean	Clean
8			Clean	Clean
9			Clean	Clean
10			Clean	Clean
11				
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29				
30				

Collector(s): Woodland

Notes: