United States Department of the Interior

Fish and Wildlife Service

Dexter National Fish Hatchery & Technology Center

P.O. Box 219, 7116 Hatchery Road

Dexter, New Mexico 88230

575-734-5910, 575-734-6130 fax

Evaluation of the Rio Grande Silvery Minnow Health in Relation to Changes in Water Quality, Pathogens and Other Environmental Stressors

(Fish Pathogen Assessment Section)

Study Number: RFP 06-SF-40-2452

May 12, 2009

Submitted by: Jason Woodland, USFWS Dexter Fish Health Unit
Dexter National Fish Hatchery and Technology Center
Dexter, New Mexico 88230

Rio Grande Silvery Minnow Fish Heath Study – Fish Pathogen Assessment

2.2.1 Fish Pathogen Assessment - Abstract

Assessment of pathogens affecting the Rio Grande silvery minnow *Hybognathus amarus* is crucial in understanding impacts to recovery efforts of this federally listed endangered fish. Viral, bacterial and parasite pathogen examinations were conducted on Rio Grande silvery minnow in this study to provide a comprehensive pathogen assessment. The prevalence of isolated pathogens was determined from up to 60 fish for bacteriology and virology and up to 30 fish for parasitology from each sampling. Collections occurred quarterly at six sites along the middle section of the Rio Grande between July 2006 and July 2008. The top four most prevalent parasites observed were from the family Trichodinidae and the genera *Apiosoma, Cryptobia*, and *Ichthyobodo*. No significant bacteria or viruses targeted nationally by the U.S. Fish and Wildlife Service were isolated. There were also no significant overall differences in the mean parasite prevalence between the sites. Correlations between types of infection indicate a majority of 'parasite only' infections (65%) compared to 'bacteria only' (19%) or 'parasites and bacteria' present (16%). The parasites observed in this study range from being normal ectocommensals to possessing potential for acute pathogenesis and are generally indicators of poor water quality. Longer term study and standardization of parasite examination protocols will provide additional information on occurrence trends and potential impacts of the parasites observed. Results of this study provide a baseline of information on the types of pathogens currently observed with Rio Grande silvery minnow in the middle reach of the Rio Grande.

7.1 Introduction/Background

Assessment of pathogen presence in the federally listed endangered Rio Grande silvery minnow *Hybognathus amarus* is important for understanding the species' current health status for conservation efforts. This comprehensive study concentrates on pathogen presence for the RGSM extant to the middle section of the Rio Grande. Traditional targeted pathogen categories used for identifying pathogen presence include viruses, bacteria, and external parasites. Depending on the host species, environmental conditions, and pathogens present, pathogen impacts on fish health varies. Many microbes are ubiquitous in nature and may not impact fish health due to natural immunity. However, if environmental conditions or other stressors compromise the fish's immunity, these ubiquitous microbes can cause disease and mortality. Currently there are no known pathogens that specifically target the Rio Grande silvery minnow.

Observing disease or mortality events in the Rio Grande is difficult due to the hydrology, water operations, and turbidity of the water, obscuring monitoring efforts for these small fish. Consequently, to better understand if pathogens are impacting Rio Grande silvery minnow survival and recruitment it is best to take a proactive approach by conducting pathogen examinations on live fish. Dead fish do not generally have much utility for conducting pathogen examinations for several reasons. First, external parasites exit the host quickly after fish death. Second, viruses are obligate intracellular pathogens and may be degraded unless tissue samples are preserved properly for testing since various cellular lytic cascades are initiated postmortem. Third, bacteria multiply exponentially in dead fish that are no longer capable of mounting protective immune responses resulting in skewed or biased colony counts using bacteria diagnostic methods.

The primary purpose of the pathogen and parasite surveys described herein is to determine the distribution pattern of specific pathogens and parasites on or in Rio Grande silvery minnows collected in the middle reach (New Mexico portion) of the Rio Grande. This research is one component of an interagency objective to establish a baseline health status of the Rio

Grande silvery minnow and the Rio Grande. Additionally, relevant bacterial and viral pathogen survey results will add to the base of knowledge for the US Fish and Wildlife Service's National Wild Fish Health Survey (NWFHS) and will be uploaded to the survey's database website for public access.

7.2 Parasitology - Methods

Rio Grande silvery minnow, collected primarily by the US Fish and Wildlife Service's New Mexico Ecological Services and New Mexico Fish and Wildlife Conservation Office, were examined for external parasites from six locations along the middle section of the Rio Grande. Collection sites were identified based on their proximity to nearby cities: Site 1, Bernalillo; Site 2, Alameda; Site 3, Los Padillas; Site 4, Los Lunas; Site 5, La Joya; and Site 6, San Antonio. The targeted sample size for external parasite examinations was 30 fish per site per time point.

Parasite examinations were conducted on fish sampled quarterly during each of two field sampling years. The sampling dates were as follows: July 2006, October 2006, January 2007, April 2007, October 2007, January 2008, April 2008, and July 2008. Generally, three sites were sampled each week over the course of two consecutive weeks during each collection month mentioned above. Prior to parasite examination, live Rio Grande silvery minnow were euthanized by overdose with tricaine methanesulfonate (Finquel; Argent Chemical Laboratories, Inc., Redmond, WA). Examination of external parasites was conducted using wet mounts from skin scrapes and gill clippings from each fish. Skin scrapes were performed by collecting mucus on a cover glass along one side of the fish and placed on microscope slides with a drop of distilled water and covered with cover glasses. Gill clips were collected with necropsy scissors and placed on slides similar to skin scrapes. Wet mounts of the skin scrapes and gill clippings were examined at 40x, 100x, 200x, and 400x magnifications using a compound light microscope.

Parasites were identified and enumerated for each of the samples. The number of parasites recorded varied, in part due to the lack of a standardized sampling protocol. Before

April 2008 several parasite counts were estimated by collectors. A plus sign was added to indicate additional parasites present than counted and to also estimate a general number of parasites present. After April 2008 each type of parasite was counted up to one hundred. If there were more than 100 parasites present for each parasite type, it was noted with a plus sign (Appendix 13.7a – Parasite Records).

Parasites examined with the compound light microscope were identified to the species level when possible. If parasites could not be identified to species level the taxonomic family, class and/or genus was recorded. Any visual implant elastomer (VIE) tags present were also recorded based on their color and location. VIE marked fish are captive reared produced fish stocked into the river.

7.3 Bacteriology – Methods

Samples for bacteriology were aseptically collected from the kidney tissue of each fish with sterile 1-µL inoculation loops (Thermo Fisher Scientific, Rochester, NY). Up to 60 fish per site were sampled for bacteria (Table 1). Tissue samples were streaked onto brain heart infusion agar (BHIA) slants (Becton Dickinson, Sparks, MD) and incubated at 20°C in an ambient air incubator. Individual bacterial colonies that grew in culture within four days were further processed for identification.

Identification of bacteria was conducted following procedures used in sampling and screening of targeted bacterial pathogens described by the Standard Procedures for Aquatic Animal Health Inspections (USFWS and AFS-FHS 2005). The bacteria information recorded follows several assumptions in determining if isolates of bacteria were different (Appendix 7.1). Biochemical testing performed in addition to the initial tests of cytochrome oxidase, motility and gram stain or KOH tests were conducted using an API 20E™ (bio Mérieux® SA, Marcy I'Etolle, France) identification system. Bacterial identifications based upon lab results and lab codes (assigned to each culture for tracking back to the individual fish) were recorded (Appendix 13.7b

– Bacteria Records). Isolates of bacteria identified as targeted pathogens by API, as listed by U.S. Fish and Wildlife Service's Aquatic Animal Health Policy (Part 713 FWS 1 Exhibit 2), were compared to API codes of known targeted pathogens and further tested by direct fluorescence antibody test (DFAT) following protocols listed in Standard Procedures for Aquatic Animal Health Inspections (USFWS and AFS-FHS 2005).

7.4 Virology - Methods

Whole viscera samples including the kidney were sampled for viral testing. Up to 60 fish per site were sampled for viruses (Table 1). Samples of viscera were pooled into groups of five fish pools and diluted 1:10 in Hank's Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, CA). The HBSS contained the following supplements (all obtained from Invitrogen): penicillin (600 IU per mL), streptomycin (1600 µg per mL), amphotericin B (40 µg per mL) and sodium bicarbonate (375 µg per mL) (HBSS anti-inc medium). Samples were maintained at 4ºC no longer than 72 hours prior to processing *in vitro* at the Dexter Fish Health Unit.

Two methods were used to process the samples *in vitro*. Samples were diluted to a final concentration of 1:100 in HBSS anti-inc medium and either filtered through a sterile 0.45 µm membrane (Pall Corporation, Ann Arbor, MI) or centrifuged (Eppendorf Centrifuge 5810R, Brinkman Instruments Inc., Westbury, NY) to pellet sample debris at 2500 x *g* for 15 minutes. This filtration method allowed for quicker processing of the samples on the same day by physically removing non-viral particles from the sample. Centrifugation of the samples was performed after a two hour incubation at 15ºC or overnight incubation at 4ºC and this method allowed for additional antibiotic disinfection of the sample prior to inoculation of tissue cultures.

In general, sample processing and testing was conducted following procedures described by the Standard Procedures for Aquatic Animal Health Inspections (USFWS and AFS-FHS 2005). However, since the samples from this study originated from the wild, our test had some slight modifications in virology procedures. These procedures followed the National Wild Fish

Health Survey laboratory manual which does not require conducting blind passes for cell culture and the observation period for virus isolation is 21 days. The other exception was the time period between collection of the samples and processing samples *in vitro* for virology. This time period varied with the day the samples were collected from all sites for the week and return of the samples for *in vitro* processing at the end of the week.

Fat head minnow (FHM) and chinook salmon embryo (CHSE-214) cells were used for virus isolations. Cultures in 24 well multi-well cell plates (Becton Dickinson, Franklin Lakes, NJ) were inoculated in duplicate, excluding negative (no inoculum) controls, and incubated at 25ºC for FHM cultures and 15ºC for CHSE-214 cultures. After inoculation, cells were rocked at the appropriate incubation temperature (25ºC for FHM, 15ºC for CHSE-214) for 45 minutes to facilitate viral particle attachment and then overlaid with 1 mL of Eagle's Minimum Essential Medium (EMEM; Invitrogen) supplemented with 5% fetal bovine serum (Thermo Fisher Scientific, Logan, UT), penicillin 100 IU/mL, streptomycin 100 µg/mL, amphotericin B solution 2.5 µg/mL, 15 mM HEPES and sodium bicarbonate 2.2 mg /mL (pH 7.2) for each well. Samples were monitored and observations recorded at least biweekly for viral cytopathic effect (CPE) during the incubation period of the test (Appendix 13.7c – Virology Records).

Any samples with observed CPE were tested by polymerase chain reaction (PCR) assay for largemouth bass virus (LMBV) and reverse-transcriptase PCR (RT-PCR) for infectious pancreatic necrosis virus (IPNV). LMBV and IPNV were the only suspected viruses for this environment that were also considered listed pathogens as determined by U.S. Fish and Wildlife Service's Aquatic Animal Health Policy (Part 713 FWS 1 Exhibit 2). DNA extraction for LMBV was conducted using the BuccalAmp DNA extraction kit (Epicentre Biotechnologies, Madison, WI) per established laboratory procedures along with the manufacturer's directions with the kit. PCR of LMBV was conducted as described in the National Wild Fish Health Survey Laboratory Procedures Manual. Extractions of RNA for IPNV were conducted using a QIAamp® MinuElute® Virus Spin Kit (QIAGEN Inc.- USA, Valencia, CA). The RT-PCR for IPNV was conducted as described by the Standard Procedures for Aquatic Animal Health

Inspections (USFWS and AFS-FHS 2005). PCR reactions were run on 1.5% agarose gels in tris-acetate-EDTA buffer and stained with ethidium bromide and compared with a 1 Kb DNA ladder (Invitrogen) to estimate amplicon size.

7.5 Data treatment and statistical analysis

Parasite prevalence was determined from the mean and median observations from all sampling periods and sites. The most dominant parasite prevalence between each site for the entire study was then compared. The mean plus and minus the standard error of the mean (± s.e.m.) was used to compare differences in the overall prevalence of infection for selected parasites between sites. The statistical analysis of differences observed between sites for each type of parasite, based on the mean ± s.e.m., was considered statistically significant by *t*-test when the *P* value was < 0.05.

Correlations were determined between three levels of observations, including identifying both bacteria and parasites on the same fish, identifying parasites only on a fish or bacteria only on fish. The percentage of each type of correlation mentioned above from each location and sampling period was then determined. The results of this correlative analysis are then compared to provide an overall summary.

7.6 Results

Four external parasites were dominant in the external parasite examinations (Figures 1 and 2). In order of greatest to least prevalence, these were identified as Trichodonidae,

Apiosoma, *Cryptobia* and *Ichthyobodo*. Additional parasites observed in the study and identified in order of greatest to least prevalence included: Monogenea (primarily *Gyrodactylus* spp.), *Epistylis*, *Ambiphrya*, *Lernaea* (anchor worm), *Icthyophthirius, Clinostomum, Trichophrya*, and *Chilodonella*.

No significant differences were observed between sample sites for the top four most prevalent parasites ($P > 0.05$). Significant differences tested were based on the observable differences between the mean ± s.e.m. (Table 2).

Comparison between bacterial and parasite isolations observed did not reveal any discernable patterns from the data collected for the individual sites at different collection time points (Table 3). Fish from Site #6 San Antonio did show consistent parasite infestations but there was no data collected from this site during October 2006 and April 2007 so no statistical significance to this observation could be determined (Table 3). When considering the majority of types of infections with bacteria or pathogenic parasite isolations per sampling period, 65% of the sampling periods resulted in a majority of 'parasite only' isolations from fish while the remaining sampling periods resulted in a majority with both 'parasites and bacteria' 16% of the time and 19% of the time with 'bacteria only'. Table 4 describes the percentage observed with parasites and bacteria for each site and collection. The overall mean prevalence of pathogenic parasites infestations compared to bacterial isolations was 70% and 37%, respectively, for the entire study when averaging each site's data. The San Antonio site had the greatest prevalence of parasite infestations at 78% followed by Los Lunas at 72%, Los Padillas and La Joya at 69%, Alameda at 67% and Bernalillo at 66%. The La Joya site had the greatest prevalence of bacterial isolations (44%), followed by Bernalillo at 41%, Los Padillas and Los Lunas at 40%, Alameda at 34% and San Antonio at 22%.

No bacteria or viruses isolated from this study were considered targeted fish pathogens per the U.S. Fish and Wildlife Service's Aquatic Animal Health Policy (Part 713 FW 1 - Exhibit 2) (Appendix 13.7b - Bacteriology and 13.7c - Virology). Of the bacteria identified by the API test, 33 different genera were represented. One isolate was presumptively identified by API as *Aeromonas salmonicida*, a targeted bacterial pathogen, but later determined as negative by DFAT. Several gram positive bacteria, not characterized by API, were isolated from the Rio Grande silvery minnows. One unidentified virus was isolated from one pool out of twelve collected in October 2007 at the Los Lunas sampling site. This sample was confirmed negative for LMBV and IPNV when tested by PCR. A sample of this virus was identified as a putative aquareovirus based on its CPE (J. Winton, U.S. Geological Survey - Western Fisheries Research Center, Seattle, WA, USA, personal communication). Having been characterized as a non-targeted pathogen, no additional characterization of the virus was attempted.

7.7 Discussion

Potential pathogen trends were examined in this study to provide a baseline of knowledge in determining possible health concerns for the Rio Grande silvery minnow. This study examined the specific areas of parasitology, virology, and bacteriology in determining pathogenic trends. Isolation of individual parasites, viruses and/or bacteria does not always indicate a health concern as all organisms, including fish, harbor normal microflora and commensal parasites. Therefore, the significance of microbes isolated in this study are discussed in context of detailing potential impacts for causing disease or mortality and by providing possible explanations for their isolation based on their prevalence in specific sites.

Most external parasites observed in this study are ubiquitous and are expected to be found in low numbers in free ranging fish populations (Beverly-Burton 1994, Ewing 2002, Taylor and

Goodwin 2002). In culture conditions these parasites can rapidly multiply and transmit to other fish with crowded conditions, causing disease. In hatchery conditions, water flow and exchange may also be reduced which can allow potential fish pathogens to persist and infect fish. In the wild, fish have greater capability to flee undesirable environments that may lead to pathogen infestation. As the health of a fish is compromised by increases in parasite burden the potential for pathogenesis is also increased. The degree of parasite infestation should then be the primary area of interest when determining whether parasites are having a negative impact on an individual fish's health (Taylor and Goodwin 2002).

However, because our data collection lacked a standardized approach for counting numbers of parasites, selection of gills for examination and the area of observation, the most accurate use of the data was to determine parasite prevalence. Although parasites observed were counted, sample collection counts of parasites may not accurately reflect the degree of infestation uniformly.

The prevalent trichodinids, *Apiosoma*, *Cryptobia,* and *Ichthyobodo* isolated from this study can all be found on the skin and gills of fish (Hoffman 1999, Mitchum 1995, Noga 2000). *Cryptobia* completes its life cycle on the gills and is generally only found on the skin in heavier infestations (Hoffman 1999, Mitchum 1995). Of the four most prevalent parasites, *Apiosoma* and *Cryptobia* are primarily ectocommensal organisms while the trichodinids and *Ichthyobodo* are considered ectoparasitic (Hoffman 1999, Mitchum 1995, Noga 2000).

The sessile ectocommensals isolated in this study, including *Epistylis* and *Ambiphrya*, primarily derive their nutrients from the water as bactivores and by filter-feeding organic debris (Noga 2000). Isolation of these ectocommensal ciliated parasites is typically an indicator of organically polluted waters that may also have high loads of bacteria (Noga 2000). They only become a problem for fish when their numbers increase, leading to a physical impediment to gas exchange required for respiration (Hoffman 1999, Mitchum 1995, Noga 2000). Reduced respiration then contributes to mortality of fish. Like the ectocommensal organisms, infestations

with *Ichthyobodo* and *Chilodonella* are more likely when environmental conditions such as poor water quality are present (Mitchum 1995, Noga 2000).

The ectoparasites isolated from this study, including the prevalent trichodinids and *Ichthyobodo* plus the less prevalent *Chilodonella,* Monogenea (specifically the viviporous *Gyrodactylus*), *Lernaea, Ichthyophthirius multifiliis,* and the digenean *Clinostomum,* are primarily pathogenic in fish (Hoffman 1999, Mitchum 1995, Noga 2000). In fact *Lernaea, I. multifiliis,* and *Clinostomum* require fish as part of their life cycle and can amplify rapidly in closed systems. Even though *Chilodonella*, Monogenea, *Lernaea*, and *I. multifiliis* are very destructive fish parasites they were not very prevalent. The numbers observed in this study did not indicate these parasites were problematic for the Rio Grande silvery minnow. However, *Lernaea* does have potential to cause mortality with only one individual if the infected fish is small (Noga 2000, Roberts 1978). Conversely fish can tolerate a high burden of metacercaria infestation, with the exception of heavy acute infections in small fish such as the *C. marginatum* (yellow grub) isolated in this study (Noga 2000).

Ichthyobodo necator, formerly classified as *Costia necatrix,* is perhaps one of the most important and most destructive external parasites identified. This parasite causes high and acute mortality due to its parasitism of epithelial cells of fish (Markevitch 1963, Noga 2000). Environmental conditions conducive to *I. necator* reproduction lead to rapid amplification that may eventually spread externally over the entire fish (Mitchum 1995, Noga 2000). While the parasite may colonize the epithelium without much visible pathogenesis in healthy fish, *I. necator* typically becomes a problem for warm water species in cooler water conditions when their immune function may be diminished (Egusa 1991, Noga 2000). The parasite cannot survive in temperatures above 30ºC and prefers water temperatures below 25ºC (Noga 2000). As Mitchum (1995) reported previously, *Cryptobia* infestations are often associated with *Ichthyobodo* infections in the same fish, contributing to increased pathogenesis.

In contrast with *Ichthyobodo*'s potential for acute mortality, the prevalent trichodinids isolated in this study often lead to chronic mortality in fish, with a potential of 1% loss per week

(Noga 2000). This percentage may increase with concomitant secondary bacterial infections (Noga 2000), something that often occurs with ectoparasite infections and further contributes to damage of the mucus and epidermal layers. Some species of trichodinids are highly pathogenic and can rapidly cover the entire fish leading to death (Hoffman 1999). In fact Noga (2000) states that *Apiosoma* actually acts as a nidus for bacterial colonization.

The bacteria isolated in this study are likely secondary pathogens or possibly environmental bacteria that contaminated the kidney during sampling in the field. No bacteria isolated during this study were targeted pathogens as listed by the U.S. Fish and Wildlife Service's Aquatic Animal Health Policy (Part 713 FWS 1 Exhibit 2).

The cell lines used for isolating viruses are capable of isolating more than targeted primary viral pathogens as listed by the U.S. Fish and Wildlife Service's Aquatic Animal Health Policy (Part 713 FWS 1 Exhibit 2). The selection of cells for this study were based on their ability to support the growth of viruses of concern from warm and coldwater fish species in the southwest region of the United States. The targeted viruses for this study included: LMBV, IPNV, *Oncorhynchus masou* virus, and infectious hematopoietic necrosis virus. Only one virus, a putative aquareovirus, was isolated in this study. Unlike many bacterial pathogens that will grow in non-selective culture media, viruses are often host specific and must be cultured in permissive cell lines. The one virus isolated in this study is not considered a disease causing agent and is among many other viruses grouped into the aquareoviruses and reoviruses families that have been isolated from free-ranging fish.

7.8 Recommendations for future work

Longer term monitoring is advisable to determine trends in the overall health of the Rio Grande silvery minnow in the middle section of the Rio Grande. This study provides a baseline

of health information that can be used to compare to health and population trends for the Rio Grande silvery minnow in the future and highlights deficiencies in current sampling methods that will allow for refinement in future studies. Development of a standardized parasitology sampling protocol should provide more robust determination of the degree of infection for future studies. Enumeration of the degree of infection will provide a better determination of the impact parasites are having on the Rio Grande silvery minnows. Testing should be expanded to incorporate methods that target specific bacterial pathogens in fish and decrease the effort and expense entailed to identify bacterial isolates that are environmental microflora and not detrimental to fish health.

Drought and environmental contamination from increasing urbanization are likely to put continued strains on Rio Grande silvery minnow populations in this region. Establishing a relevant fish health baseline and continued monitoring of fish health parameters for this species is critical to understanding impacts to recovery efforts of this federally listed endangered fish.

7.9 Literature Cited

Beverly-Burton, M. 1994. Monogenean Diseases. *In* AFS-FHS (American Fisheries Society-Fish Health Section). FHS Blue Book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

Egusa, S. 1991. Infectious diseases of fish. Pauls Press, New Delhi, India.

Ewing, M. S. 2002. Ichthyophthiriasis. *In* American Fisheries Society-Fish Health Section Blue Book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

Hoffman, G. L. 1999. Parasites of North American freshwater fishes. 2nd edition. Cornell University Press, Ithaca, NY-London.

Markevich, A. P. 1963. Parasitic fauna of freshwater fish of the Ukranian S.S.R. Jerusalem: Israel Program for Scientific Translations.

Mitchum, D. L. 1995. Parasites of Fishes in Wyoming. Alternative Enterprises, Cheyenne, WY.

Noga, E. J. 2000. Fish Disease Diagnosis and Treatment. Iowa State University Press, Ames, Iowa.

Roberts, R. J. 1978. Fish Pathology. University Press, Aberdeen, Great Britain.

Taylor, P. W. and A. E. Goodwin. 2002 External Infection by Ciliated Parasites. *In* American Fisheries Society-Fish Health Section Blue Book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

USFWS and AFS-FHS (U.S. Fish and Wildlife Service and American Fisheries Society-Fish Health Section). 2005. Standard procedures for aquatic animal health inspections. *In* American Fisheries Society-Fish Health Section Blue Book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2005 edition. AFS-FHS, Bethesda, Maryland.