Health and Histopathological Evaluation of Rio Grande Silvery Minnow from the Rio Grande, New Mexico

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Abstract: The decline of the federally endangered Rio Grande silvery minnow (RGSM) is associated with physical and hydrologic changes in the Rio Grande system. Decreasing water quality and contaminants may also be factors. This study, as part of a larger study, was designed to evaluate the health of the RGSM in relation to water quality and other environmental stressors in the Rio Grande. Health indicators including somatic indices, gross observation during necropsy, hematology, and histological observations were used to assess the health of wild RGSM seasonally and at several locations along the middle Rio Grande. Both a temporal and spatial relationship was seen. RGSM collected in the summer tended to be less healthy as indicated by: increased macrophage aggregates and granulomas, lowered liver glycogen, and increased spleen size with fewer red blood cells. Whereas silvery minnows from all sites showed impairment, health indicators such as Health Assessment Index (HAI), Splenosomatic Index (SSI), and percentage of fish with 2 or more pathologies showed an upstream to downstream trend, with sites below Albuquerque being less healthy in general. Our results indicate that the RGSM in the reach of critical habitat between Bernalillo, NM and Bosque del Apache National Wildlife Refuge are experiencing chronic stress as evidenced by a suite of health indicators. Given the results from the bioindicators used in this study and the stressors present, it appears that the stress is likely related to poor water and sediment quality (ammonia, metals, bacteria, and possibly PAHs). These results are in agreement and consistent with the known impacts of urban effluents, stormwater runoff, and agricultural return waters.

Keywords: Rio Grande, Rio Grande silvery minnow, health assessment, histopathology

The endangered Rio Grande silvery minnow (*Hybognathus amarus*), once abundant throughout the Rio Grande and Pecos River, is found almost exclusively in a 286-km section of critical habitat in the middle Rio Grande between the Cochiti and Elephant Butte reservoirs. The Rio Grande silvery minnow has been described by Cowley et al. (2006) as a resilient and adaptable species tolerant of low oxygen conditions. It has been determined that it is a benthic herbivore with diatoms comprising an important component of the diet (Magana 2009). Today in the Rio Grande, it is most often found in no- to low-velocity habitats with silted bottoms (USFWS 2007). As an annual pelagophil, it evolved to spawn in spring when Rio Grande discharge increased from headwater snowmelt and seasonal rain events (USFWS 2007). In the contemporary dammed and channelized Rio Grande, spawning is more likely cued on dam releases, and increased run-off from diversion channel flows (Platania and Altenback 1998). Silvery minnow survival in the wild has been determined to be 13 to 25 months, with many dying after only one spawning (USFWS 2007), yet in captivity the RGSM can live 5 to 8 years (Cowley et al. 2006; K. Buhl, pers. comm.).

The demise of Rio Grande silvery minnow populations has been attributed primarily to physical and hydrological changes of the river system (Alò and Turner 2005). However, consistently poor water quality is also believed to be a factor in the decline of this species to the extent that pollution and disease are identified as listing factors in the silvery minnow recovery plan (USFWS 2007). A health assessment of fishes in the Rio Grande conducted in 1997-1998 generalized that study fish from the lower basin (below El Paso) contained greater concentrations of some contaminants and appeared to be less healthy than those from upper basin sites where the silvery minnow is almost exclusively found today (Schmitt et al. 2005). The Elephant Butte Reservoir site from the health assessment (the only site within the silvery minnow critical habitat reach), was considered more contaminated than the site north of critical habitat near the headwaters and also the El Paso site (Schmitt et al. 2005). Elephant Butte

fish had elevated ethoxyresorufin-o-deethylation (EROD) rates and some fish were rated unhealthy and with poor condition factor (Schmitt et al. 2005). More recently, fish sampled between Albuquerque and Elephant Butte were found with lesions, elevated vitellogenin, and slightly elevated EROD levels (Papoulias, unpublished data). Although limited, the existing fish health data and the known water quality information suggest the Rio Grande silvery minnow population could be unhealthy.

Water quality has deteriorated in the middle Rio Grande and adequate flows to support aquatic ecosystems have declined over the past thirty years (Passell et al. 2004, 2005, 2007). The most recent evaluation of water quality in the Middle Rio Grande indicates that quality is insufficient to support warmwater aquatic life. The State of New Mexico CWA Integrated Report (NMED 2008a) identifies low dissolved oxygen, *E.coli*, elevated ammonia, and possibly some heavy metals as reasons for concern. Probable sources of impairment cited include municipal point (e.g., wastewaters) and non-point sources. Such sources are also known to have a plethora of pharmaceuticals and personal care products (Batt et al. 2008; Brown, 2004; Kolpin et al. 2002). In addition, the Rio Grande is heavily used for irrigation (Jackson et al. 2001). Irrigation return waters can increase conductivity, and contain high concentrations of salts, nutrients and trace elements such as arsenic (Norman and Dilley 2002). These potential sources of contaminants together with low flows increase the likelihood that Rio Grande fishes and other aquatic organisms are exposed to stressful and unhealthy conditions.

Continuous exposure to stressors can impair the health of an organism and require it to divert its energy and biological resources away from growth and reproduction and consequently have population-level effects (Schreck et al. 2001). Under chronically stressful conditions fish are more susceptible to pathogens and toxicants which may result in pre-pathological or pathological conditions (Moberg 2000). During fish culture, evaluating fish health at the biochemical, cell, and tissue levels may allow intervention with therapeutic treatments to avoid losses of animals. Assessment and monitoring of the

health of wild populations of fish provides the fisheries biologist with diagnostic information about habitat quality and may indicate specific environmental stressors (de la Torre et al. 2005; Marchand et al. 2009). Monitoring the health of endangered species, especially during reintroduction, restoration and mitigation efforts, is essential to providing scientific information about the success of recovery efforts.

The present study was part of a larger study to evaluate the health of the Rio Grande silvery minnow (RGSM) throughout its designated critical habitat (Lusk 2007). Studies were designed to provide a means to characterize the health status of wild RGSM in relation to water quality and other environmental stressors in the Middle Rio Grande over multiple years, thus capturing variation across seasons. The study included a fish health assessment using somatic indices and systematic observation of fish tissues and organs during necropsy and through histopathology (Goede and Barton 1990; Schmitt et al. 1999; Schmitt and Dethloff 2000; Smith et al. 2002). Therefore, objectives of this portion of the study were to assess the health of the RGSM seasonally at several locations using somatic indices, hematology, and histopathology.

Methods and Procedures

Six sites (Table 1) within the Middle Rio Grande were sampled during 2006-2007 (Year 1) and 2007-2008 (Year 2). Thirty fish were targeted for collection at each site to provide sufficient material for all analyses of the larger study. Site 1 was above the city of Albuquerque, Site 2 was geographically above Albuquerque but below the city's main municipal storm runoff diversion channel outflow, and Sites 3 – 6 were below Albuquerque. Site 1, the furthest upstream site served as a less impacted reference site and Site 3 is immediately downstream of the Albuquerque wastewater treatment plant discharge and other smaller tributaries. Collection efforts were conducted under stable flow conditions. Fish were held in aerated live wells until processed. Fish health assessments were performed in a laboratory within several hours after capture. All collected fish were individually measured for body

weight and total length, followed by visual determination of sex, examination for external and internal abnormalities, and removal and weighting of spleens. Total body weight and spleen weight were used to calculate a Splenosomatic Index (SSI) for each fish that had a recorded spleen measurement; though some were too small. Somatic indices can reflect the status of organ systems, which may change in size due to environmental stressors (Busacker et al. 1990). A Health Assessment Index (HAI) was also calculated as a general indicator of fish health (Adams 1990; Carlander 1969, 1977). Developed and revised by Goede (1988, 1989, 1996), HAI utilizes observations of gross external and internal pathological disorders (see Schmitt and Dethloff 2000 for a complete description). On a sub-sample of RGSM collected liver, spleen, kidney, gonad, and gill were dissected for histopathology.

Opercula of the collected fish were observed for shortening on one or both opercula. For analysis, shortened opercula were assessed as present/absent rather than the degree of shortening to assist in reducing the subjective nature of opercula observation. As part of this study we also evaluated the incidence of shortened opercula in archived RGSM samples from the University of New Mexico, Museum of Southwestern Biology (Accession Number ACC2006-VII:31). Archives contain preserved RGSM from 1938 to 2001. The following cataloged collections of RGSM at the Museum of Southwestern Biology were examined for shortened opercula: 1119, 1120, 1125, 1130, 1151, 1155, 1162, 1171, 3961, 4338, 4384, 5730, 5934, 11589, 13472, 20396, 22671, 30032, 30246, 39103, 45271, 43314, 44627, 48229, and 49969. Right and left opercula of 30 fish from each year for 25 years between 1938 and 2001 were randomly selected, measured for weight and length, and observed for degree of operculum shortening. The degree was noted as slight or severe. Slight shortening is described as visible shortening with flap obviously not covering entire gill with a minimal amount of gill exposed. Severe shortening is described as extreme shortening with the gill exposed and visible to the un-aided eye.

Histopathology

Tissues for histopathology were collected from three individuals at each of the six sites during Year 1 and six individuals at each of the six sites Year 2. Liver, kidney, spleen, gonad, and gill were removed and placed in 10% neutral buffered formalin (NBF). All tissues from an individual were mixed in one vial during Year 1 and this resulted in loss of some of the very small organs (i.e., spleen, kidney, and testes). Therefore, in Year 2, we removed the entire viscera to one vial and placed kidneys and gonads in separate vials. A limited number of opercula were also processed for histology or cleared for staining of bone and cartilage following Kelly and Bryden (1983) for in-depth examination of shortening of the distal margin. Processing for histology consisted of rinsing tissues in two changes of 10 mM HEPES buffer (pH 7.4) and dehydration by immersion in graded aqueous ethanol solutions ranging from 50% to 100%. Gill and opercula were decalicified in Surgipath® Decalcifier II solution prior to processing. This was followed by immersion in xylene and subsequent infiltration with paraffin. The paraffin-embedded samples were sectioned at 5 μm, mounted onto silanized slides, and stored at room temperature until staining. In preparation for histochemical staining, tissue sections were de-waxed with xylene and then rehydrated to water by immersion in graded solutions containing decreasing concentrations of ethanol from 100% to 0%. Tissue samples were stained following the general nuclear stain procedure for hematoxylin and eosin (Luna 1968).

Macrophage aggregates (MAs) are part of the fish's defensive system against foreign matter or infectious disease (Wolke et al. 1985) and have been used as biomarkers of contaminant exposure. Numbers and areas of MAs were assessed on three sections of spleen tissue from each fish and reported per area of spleen. MAs were enumerated on sections of spleen stained with hematoxylin and eosin on an inverted Nikon Diaphot using a 10x objective and Optimas® image analysis software. MAs in some samples were too small to determine area and were only counted.

Light microscopy was used to qualitatively evaluate the tissues for lesions and pathologies. We used RGSM collected from Dexter National Fish Hatchery and Technology Center (Dexter, NM) for a reference set of healthy organs/tissues for qualitative comparisons. Gonads were also assigned sex and stage of reproductive maturity (see Blazer 2002 for description).

Haematology

Analysis of the ratio of leukocytes was made on touch smears of head and trunk kidneys, spleen, and liver for 2 sites in one season. August 8, 2008 six RGSM were caught at Site 1 (relative reference location) and at Site 3. Fish were pithed and organs removed. Each freshly dissected organ was blotted three times onto uncoated microscope slides then preserved by immersion in 95% ethanol for 20 minutes. After air-drying, the slides were later stained using Romanovsky–Giemza stain. Using light microscopy, eight leukocyte types were identified and enumerated according to the classification of Ivanova (1983). Not less than 200 cells were evaluated on each slide.

Statistics

Statistics were computed on the full set of sampled fish for mean length, weight, opercula shortening, SSI and HAI. Weight and length differences between years were tested as well as weight, length, and index differences in combined years to determine site- and month-specific differences. Age and sex can influence size of spleen, therefore length as a surrogate of age was tested for covariance with SSI and sex was included as a second factor with station in an analysis of variance. We tested for site- and month-specific differences in the percent of fish with shortened opercula for each sampling year. A t-test was used to determine if archived fish with shortened opercula differed in size from fish without shortened opercula. Site-specific percent occurrence of the subset of total fish examined for tissue condition, pathology, or fish's sex (determined with histology) was calculated for each sampling

date and means for sites or months were tested for differences. Data were combined for the two years for testing of seasonal and site differences for these measures because few fish were evaluated at each site in a given year. Data were analyzed using SAS v.9.2 software (SAS Institute Inc., Cary, NC, USA). All data were tested for homogeneity of variances (Levene's test) and normality (Shapiro-Wilk statistic). Percentage data were arcsin transformed and SSI data were log-transformed. A general linear model with LSMEANS as the post-hoc comparison test was used to test for site and month differences unless data did not meet assumptions for parametric tests after transformations; then we used the non-parametric Wilcoxon signed-ranks. Macrophage aggregate count and area measures were tested for covariance with estimated fish age prior to analysis of variance among sites. Age was estimated by converting total length to standard length using the formula (SL=1.0182(TL)-10.909) followed by estimation of age from the formula cited by Cowley et al. (2006). The Mantel-Haenszel Chi-Square for trends was used to evaluate trend in percent females upstream to downstream. Leukocytes were analyzed using a t-test and reported as percentage of 200 cells. There are no reported normal health indicators for silvery minnow. Therefore for these studies, fish from Site 1 were considered relative normal and individuals captured at other sites were compared against results from Site 1.

Results

A total of 1,070 RGSM were observed during two years of study. Overall, RGSM collected in Year 1 were larger (70 ± 11 mm Total Length (TL) n = 511, 3.1 ± 1.5 g, n = 516) than those collected in Year 2 (59 ± 11 mm TL n = 554, 1.9 ± 1.0 g, n = 553; $p < 0.001$). There were also significant differences in weights and lengths among months ($p<0.012$) and sites ($p<0.0001$). RGSM were smallest at Sites 1 and 2 and largest in October (Figures 1 and 2). There was no influence of sex on the site- or

month-specific weights or lengths. Neither mean length, mean weight, nor length-weight regression curves were different between males and females (data not shown).

Sex did not influence the effect of site or month on mean SSI values. Length did covary with SSI therefore all SSI analyses were adjusted for length. SSIs were greatest in July and October, and smallest in January and April ($p = 0.0001$). Mean SSIs for Sites 1,2, and 3 were larger than for Sites 4 and 5. Site 1 mean SSI was also larger than Site 6 mean SSI ($p=0.02$) (Figure 3). There was also a significant interaction between site and month ($p<0.0001$). Mean SSIs of fish within Sites 4, 5, and 6 did not significantly differ during any month and in January there were no differences in SSI among sites. Mean SSIs for fish collected at Sites 1 and 2 were greatest in July compared to other months and during July and October at Site 3. At Sites 4 and 5 mean SSIs in October were larger than those in July at these sites. In April, fish at Sites 1 and 2 had similar SSIs but mean site values were larger than for Site 3. In July, mean SSIs of fish at Sites 4 and 5 were smaller than mean SSIs for fish at Sites 1, 2, and 3. In October, mean SSI for fish at Site 3 was larger than that for fish at any other site. At Site 3, Year 2, a single fish with an SSI of 2.89, was recorded.

Rio Grande silvery minnow HAI scores were significantly greater in July compared to other months ($p = 0.05$) for both years combined. Site 3 HAI score was greater than Sites 1 and Site 2 ($p < 0.0491$). Mean HAI scores tended to increase downstream to Site 3 and remain similarly elevated downstream to Site 6 (Figure 4).

Pathology

A total of 573 tissue samples of kidney, liver, spleen, gonad, and gill from a total of 147 fish were submitted for histology from July 2006 to July 2008. As a result of a malfunction with the tissue processing equipment, most of the April 2007 samples and all gill samples from October 2006, January 2007, and April 2007 were damaged and unusable. Few types of histological abnormalities were seen

in gonad, spleen, and liver. A greater percentage of fish had kidney and gill abnormalities. For all tissues, the total percent of fish with a pathological lesion or those with two or more lesions tended to increase from upstream sites to downstream sites.

Gill

Gross gill abnormalities (discoloration, fraying, clubbing, and parasites) were observed in 35% of fish examined during Year 1 and 50% Year 2. Of the 80 gills examined, only three had no lesions and most, irrespective of site, had multiple lesions. Clubbing, curling, and swelling of secondary lamellae were the most common lesions observed. Fused lamellae, lifting of the epithelium and the lamellae, and embolism were also seen.

Opercula

During Year 1, 50% of fish observed had shortened opercula. Year 2, that percentage increased to 89%. Shortened opercula were the most prevalent gross abnormality. During Year 1, this anomaly was overwhelmingly bilateral (83.2 %) and a unilateral abnormality affected only 2.9% right side and 3.1% left side. Year 2 had a lower bilateral frequency of 40.9% with only frequencies of 3.7% right side and 5.8% left side. Year 1 RGSM had increased overall occurrence during April (68%) and July (65%) and reduced overall occurrence during January (38%). Contradictory to Year 1, Year 2 fish had extremely elevated observations of shortened opercula during January (98%); July and October were slightly lower, 86% and 79%, respectively. During Year 1, downstream Site 5 had the highest frequency of opercula shortening for all months sampled. During Year 2, all six sites had high occurrence (> 70%) of shortened opercula. Historic incidence of shortened opercula similarly indicates year-to-year variation, but an increasing temporal trend is evident (Table 2). Archived silvery minnow

with at least one shortened operculum were shorter in total length (p=0.0115, n=249, mean=55.7 mm, range: 21.0-95.0 mm) than RGSM without a shortened operculum (n=390, mean=58.7 mm, range: 22.0-96.0 mm) but there was no difference in weight between these two groups (p=0.6690). Opercula of cleared and stained fish showed differences between a second or third generation RGSM from Dexter, NM with no or only slightly shortened opercula and a wild fish with shortened opercula from Site 2. The distal margin of the opercula in the Dexter, NM RGSM were thicker and appeared to have a higher bone density than the Site 2 RGSM.

Gonads

Overall, 71 of 123 fish analyzed (58%) were females. The percentage of females did not significantly differ between years ($p = 0.3284$) among sites ($p = 0.0931$) or months ($p = 0.3974$), however there was a significant ($p < 0.0001$) trend of progressively more females in samples at sites from upstream to downstream (Figure 5). Based on histological staging of gonads, RGSM appear to be group synchronous batch spawners. Spawning occurred at some time between April and July as we found most females were at Stage IV in April and spent in July. Thirteen percent of the fish, both sexes, had large fat deposits associated with the gonad; occurrence of fat was not seemingly related to month but the majority of these fish were found in Year 1 collections. All but four ovaries (from Sites 2 and 5) with atretic oocytes were also spent. Parasites were only occasionally seen. One testis collected 6 July 2007 had two ovoid structures associated with it that could have been cross-sections of cestodes, cortical alveolar oocytes, or something else (e.g., cross- tissue contamination). A positive identification was not possible.

Liver

Gross liver abnormalites were high for both years. Forty-five percent of fish Year 1 and 59% Year 2 were reported to be discolored or as having a 'mushy' texture. A subset of 147 livers was processed for histopathology. The majority of these livers were low or moderate in glycogen content. There were no site differences but overall, livers of fish caught in July were low in glycogen ($p = 0.0294$). Pathologies were overall low (edema, high fat content, pigments associated with macrophages, granulomas, inflammation, parasites). There was no significant difference among sites or months in incidence of fish having one or more pathologies (Figure 6). Parasites were uncommon all years, but granulomas and MAs were prevalent in July, 2008; hemosiderin and melanin pigments were more common in livers from Year 2 samples. July of Year 1, 53% of the samples had fatty deposits outside of the liver and also fat vacuoles inside of hepatocytes. Sites 2, 3, and 4 all had fish with granulomas but they were most common in Sites 3 and 4 fish; only Site 2 fish also had macrophage aggregates. Nine samples had oocytes associated with the liver. One of these was definitely due to carryover contamination during tissue processing, but from October 2007 forward ovaries were isolated from livers for processing, so this does not explain all observations. The others may be ectopic oocytes, but it is difficult to say with certainty. In some cases, it appears as if the liver tissue partially surrounded the oocytes or they were embedded in fat that was attached to the outside of the liver. It is possible that ovarian tissue had adhered to the outside of the liver and was included in the dissected liver sample.

Spleen

Gross spleen abnormalites (nodules, granular texture, size) decreased from 24% in Year 1 to 11% during Year 2. A subset of 125 spleens was processed for histopathology. In the majority of fish, blood content was either low or moderate and there were no significant site differences whether or not we controlled for month. A greater percentage of RGSM spleens collected in January were perfused

with blood than the other months (p = 0.0092). The incidence of finding fish with a pathological condition (parasite, granuloma, fat, MA) in the spleen was not significantly different among sites (Figure 6). However, Year 1 fish were notably different from Year 2 fish because of the presence of fish with fatty spleens, particularly in July. Twelve percent of the total fish inspected had sparsely pigmented granulomas that may have been caused by bacterial or fungal infection. All granulomas occurred in fish sampled in Year 2. Granulomas were found in fish from all sites, but Site 6 had the most fish with granulomas and Site 5 the fewest. Only one parasite (unidentified) was observed. Macrophage aggregates were most numerous at Site 3 and least abundant at Site 1 but these results were highly variable within a site and not significant among sites (p = 0.1062, no site*month interactions; Table 3). Macrophage aggregates (count and area) were more numerous in July and least in April (p = 0.005), no month*site interactions; data not shown). Estimated age was not a significant covariate with either count or area (n=115, R^2=-0.09, p=0.35 and n=72, R^2=0.04, p=0.76, respectively).

Kidney

Gross kidney abnormalities (size, color, texture, and parasites) were observed in 16% of fish examined during Year 1, increasing slightly to 24% during Year 2. A subset of 117 kidneys was processed for histopathology (pigment, tubular degeneration, regeneration, fat, parasites, and granulomas). In contrast to Year 2, nearly all samples from Year 1 had hemosiderin pigment or melanin or both and a high percentage of fish (36%) had fat associated with the kidneys either surrounding it or infiltrated in the interstitium. Two fish from Year 1 appeared to have ectopic oocytes in the kidney parenchyma, but similarly to the liver this could not be definitively concluded. Several fish (11 total; all sites represented) were collected with kidneys containing many sparsely pigmented granulomas that may have been caused by bacterial or fungal infection. Another fish, collected 6 October 2006 from Site 3, showed production of an inordinate number of segmented leukocytes in the hematopoietic tissue

suggesting that the fish may have had a systemic or other infection. The incidence of finding fish with a pathological condition in the kidney was not significantly different among sites (Figure 6). Ninety-two percent of the fish collected had degenerating kidney tubules (Figure 7). Some regenerating tubules were observed in Year 2 fish.

Leukocytes

Changes in proportion of leucocytes were observable in Site 3 fish relative to Site 1 fish for all investigated organs (Table 4). Hemocytoblasts were reduced in head kidney, but plasma cells and segmented neutrophils were increased. Plasma cells and segmented neutrophils were also increased in trunk kidney but stab neutrophils were reduced. Lymphocytes were fewer in spleen but macrophages and promyelocytes (immature granulocytes) increased. In the liver, lymphocytes increased but neutrophils decreased.

Discussion

Health assessments have proven useful in evaluating an individual fish's integrated response to environmental stressors. In our study, collection of a small number of fish during different seasons for an extended time assured that a range of biotic and abiotic stressors could manifest through our suite of biological health indicators and also allowed us to more easily discern trends. Health of RGSM surveyed over a period of two years was variable but consistently impaired spatially and temporally. The results showed that RGSM health deteriorated from upstream to downstream within the designated critical habitat and that summer and the months with low river discharge were the most stressful for these fish.

Some of the health effects observed in the RGSM may be explained by the suite of environmental stressors present in the waters of the RGSM critical habitat. *E. coli* is rarely a fish

pathogen. It serves as an indicator of the relative level of organic pollution or mammalian fecal waste. The elevated *E. coli* in the Rio Grande reflects the influence that wastewater treatment plants and residential stormwater run-off have on its water quality. Fish chronically exposed to wastewater effluents experience reproductive, endocrine, immune, genotoxic, and nephrotoxic effects (Liney et al. 2005). Chemicals in these waters include polycyclic aromatic hydrocarbons, metals, natural hormones, and pharmaceuticals, all measured in the Rio Grande and many at levels of concern.

The water quality data collected by NMED (2009) and SWQB (2009) during the same period and at the sites sampled in the present study generally support a trend of poorer water quality (i.e., elevated biological oxygen demand (BOD), total phosphorus, and *E. coli*) from upstream to downstream, during summer months, and low flow periods (Table 5). These same studies also document potentially toxic levels of contaminants (primarily metals) in water, sediment, and fish tissue at many of our study sites. Temperature, pH, and ammonia in the NMED (2009) study rarely exceeded water quality criteria, however, reduction of dissolved oxygen at some locations was of concern. Dissolved concentrations of aluminum and copper exceeded water quality standards for chronic and acute effects at most sites (NMED 2009; Table 5). Total concentrations of aluminum, copper, iron, arsenic, cyanide, and fluoride in water, sediments, or both at some sites and in some seasons (NMED 2009; Table 5) were at potentially toxic levels and among elements identified in a 2005 Rio Grande risk assessment as likely contributing to overall environmental stress to RGSM (Marcus et al. 2005). Several sediment PAHs were measured at levels above screening criteria but only at Sites 2 and 3 (NMED 2009). Fish tissues were elevated for zinc at all sites and for total DDT in fish collected between Sites 1 and 2 and between Sites 2 and 3 (NMED 2009). Analysis for pharmaceuticals and personal care products (PPCP) of effluents from seven wastewater treatment plants within our study site found 36 of 54 PPCP analytes (NMED 2009). Total suspended solids (TSS) were high at many sites

and seasonally (Table 5). Although the Rio Grande has historically had periods of both high and low turbidity (Julien et al 2005; Cowley et al. 2009), the contemporary composition of suspended solids may serve as sinks for pathogens and abiotic contaminants by adsorbing to particle surfaces.

Health Indicators

Organo-somatic indices are simple and inexpensive to perform. Although simplistic compared to other biomarkers, health indices and gross observations can be used as general indicators of fish health and support results of biomarkers and environmental contaminant analyses. Indices have been used extensively in fish health and population assessments as indicators of well being of individual organisms (Adams 1990; Goede and Barton 1990). For this study, only the SSI and HAI were evaluated and utilized as general indicators of fish health. Hepatosomatic index was not included because cyprinids have a dispersed liver and weight measurements prove to be inaccurate if not impossible. Furthermore, gonadosomatic index was not included in this study because of the difficulty in dissescting out and accurately weighing the gonad tissue in such a small fish.

The quantitative HAI utilizes a comprehensive necropsy based fish health assessment of observations of gross external and internal pathological disorders (Adams 1990; Adams et al. 1993; Goede 1988, 1989, 1996). HAI can be a valuable method for estimating cumulative stress, and documenting lesions or abnormalities that have advanced to the point of being grossly visible, and those the public tends to be most concerned about (Adams 1990; Adams et al. 1992; Schmitt and Dethloff 2000). Studies have indicated that fish in severely polluted areas exhibit a higher frequency of gross lesions than those in similar, less polluted areas (Schmitt and Dethloff 2000). Relative HAI results showed that fish from Site 1 had the lowest mean HAI score and that HAI scores were highest in July consistent with SSI results and liver and spleen pathological observations.

The SSI has been incorporated into other monitoring protocols evaluating the Mississippi River (Schmitt 2002), Columbia River (Hinck et al. 2004a), Yukon River (Hinck et al. 2004b), Colorado River (Hinck et al. 2006) as well as the Rio Grande Basin (Schmitt (Ed.) 2004 and Schmitt et al. 2005) and was used in the present study. The SSI can be a useful diagnostic because it is both a hematopoietic and an immune system organ and abnormalities could exhibit effects at the whole-organism level (Anderson 1990; Schmitt and Dethloff 2000). Acute or nonspecific stressors such as temperature changes, hypoxia, extreme exercise, and exposure to chemical contaminants may cause the spleen to contract and reduce in size. An enlarged spleen may suggest disease or an immune system disorder due to increased hypertrophy or proliferation of leukocytes (Anderson 1990; Goede and Barton 1990). Chemicals identified as of concern in the Rio Grande can affect the fish immune system (Harris and Bird 2000; Reynaud and Deschaux 2006; Zelikoff, 1993). The differences in leukocyte profiles and increase in macrophage aggregates, between fish from the reference site (Site 1) and the sites below it support an immunotoxic effect of one or a mixture of the chemicals measured.

In general, SSI was low (< 2%) and variable among sites. SSI values increased to their highest levels in July at Sites 1, 2, and 6 then decreased back to January and April levels in October. At Site 3 highest levels were also in July but no return to low levels in October was observed. Mean SSI seasonal pattern and magnitude was more similar among Sites 1, 2, and 6 and among Sites 4 and 5. The SSI results were generally consistent with the observed histopathology. January SSI values were low and more similar among sites and this is when the majority of spleens from all sites were seen to be perfused with blood. When fish are calm, erythrocyte count in the spleen is high but when a fish is stressed red bloods cells are released from the spleen into the blood (Takashima and Hibiya 1995). July SSI was highest at Site 3 and this site in July also was notable for fat associated with the spleen and increased macrophage aggregates.

Macrophage aggregates have been suggested as possible bioindicators of environmental chemical exposure (Wolke 1992). Macrophage aggregates function in fish to protect the body by 1) recycling iron, 2) storing, destroying, or detoxifying effete material, and 3) through a role in immune response (Aguis 1985). A large number of studies have noted increases in macrophage aggregates in fish from polluted waters (reviewed in Schmitt and Dethloff 2000). The formation of MAs may also be influenced by age, diet, temperature and other factors, therefore cause and effect relationships may be difficult to establish (Blazer et al.1987). Blazer et al. (1987) found that stressed fish had 9 MAs per mm^2 liver tissue while healthy fish had 4 MAs per mm^2. Most RGSM in this study had MAs greater than or close to 9 MAs excepting Site 1, which had an average of 3 macrophage aggregates. Although we did not age the fish, length, as an indirect measure of age, was not associated with number or area. Hemosiderin, lipofuscin/ceroid, and melanin are pigments often found within macrophage aggregates. While the pigments themselves do not damage tissue, they may indicate the health of an organism or its exposure to toxic compounds. Hemosiderin is a by-product of hemoglobin breakdown and lipofuscin and ceroid are considered to be lipid pigments produced by the peroxidation of unsaturated fatty acids. Melanin is also found in some macrophage aggregates but more often it is found outside of them. It may be deposited after destruction of melanocytes or melanophores. It is thought that melanin may be important as a bactericide or to decrease the damage caused by the production of free radicals by phagocytic cells (Ellis 1981). These pigments were observed in all the types of tissues analyzed, however hemosiderin/lipofuscin/ceroid were most prevalent in kidney and spleen and melanin in gonads.

Overall, kidneys presented with the greatest diversity of lesions in the greatest number of individuals examined. Kidney histopathologies were consistent with chronic inflammation. Ubiquitous inflammation in kidneys of RGSM cannot be associated with any single stressor and is most likely a

general response to poor water quality (Carmago and Martinez 2007). The change in ratio of leucocytes in the kidneys results from the increased number of plasma cells relative to stab neutrophils in Site 3, suggests a pronounced antibody-mediated response to environmental stress or a reaction to diseases caused by weakening of an organism. The increase in stab neutrophils, is an indicator of inflammatory processes in the organism. The significant decrease of hemocytoblasts shows the exhaustion of the leukopoiesis. There is a repartition of cells due to the decrease of lymphocytes and an increase of macrophages in the spleen. It also suggests inflammatory events in the organism and is indirectly confirmed by an increase of promyelocytes. At the same time an increase in mature forms of granulocytes was not observed indicating early cell death and their following utilization by macrophages. A rather unusual reaction in the liver was observed i.e. lymphocytes and immature form of neutrophils (promyelocytes and myelocytes) increased, but the mature neutrophils decreased. It is possible this is due to lymphoid infiltration of the organ caused by development of autoimmune disease. Similar changes in leukocyte ratios were observed in organs (head and trunk kidney, spleen, but not liver) of common bream (*Abramis brama* L.) from variably polluted areas of Rybinsk reservoir (Lapirova and Zabotkina. in press).

Bacterial pathogens tend to be high in wastewater-dominated streams and this may be reflected by the types of gill lesions observed (Seeley and Weeks-Perkins 1991). The gills are critical sites for ion regulation and respiration. The most common gill lesions (discoloration, fraying, clubbing, curling) were consistent with parasite and bacterial infestations. However, fused lamellae and lifting of the epithelium on the lamellae were also seen. Such lesions have been observed in fish exposed to ammonia concentrations of $\geq$ 0.8 mg/L and chronic exposures to copper (Muhvich et al. 1995; Piya et al. 2009; Spencer et al. 2008). Although all NMED (2009) reported ammonia concentrations were well below this level, Buhl (2002) showed that ammonia and copper together at concentrations found in the

Rio Grande could result in chronic toxicity to Rio Grande silvery minnow. Moreover, tilapia exposed to copper at concentrations found at our Rio Grande sites, exhibited similar gill pathologies (Figueiredo-Fernandes et al. 2007).

Hepatocyte appearance can vary depending on season, nutritional status, age, sex, and pollutant exposure (Ferguson 1989). Hepatic glycogen was most often low in all RGSM which may be related to environmental stress or the end of the spawning season, reduced food resources, or a combination of these stressors. Glycogen is used as an immediate energy source when fish are under acute stress. Reduced glycogen may make them lethargic and less able to respond to predators (Smith 1982). Depressed liver glycogen, particularly in summer months, has been related to exposure to heavy metals (Heath 1995; Levesque et al. 2002: Palermo et al. 2008). Aluminum and copper were the only heavy metals that exceeded NM water quality acute or chronic criteria for dissolved levels at site-specific hardness concentrations. However, total water concentrations of copper, aluminum, and iron were highly elevated across sites (Table 5).

Shortened operculum was the gross abnormality observed at the highest frequency. The anomaly has been present since at least 1938. Abnormalities of the opercular complex affect both wild and cultured fish populations. Possible causative factors include, but are not limited to, vitamin-C deficiency or diet (Dabrowski et al. 1988; Grady et al. 1992b; Koumoundouros et al. 1997), exposure to contaminants (Grady et al. 1992b; Lindesjöö et al. 1994; Olsson et al. 1999), turbulence (Al-Harbi 2001; Backiel et al. 1984; Beraldo et al. 2003), and genetics (Beraldo et al. 2003; Koumoundouros et al. 1997; Tave et al. 1983). The cause of shortened opercula in RGSM is not known, but results of our clearing and staining of a few samples suggest the margins of shortened opercula may not have the same bone density as normal opercula. However, this may be due to age differences as the Dexter RGSM were larger and likely older than the Site 2 RGSM. More same-age animals need to be studied. Available

evidence suggests that this deformity may be a non-inherited congenital defect (Tave et al. 1982; Tave and Handwerker 1994), induced during the embryonic or larval stage (Al-Harbi 2001; Koumoundouros et al. 1997). Although the effects of a shortened operculum on fish function, especially at the gills, are unknown, gill pathologies were reported for nearly all fish in this study. Our finding, from analysis of archived samples, that fish with shortened opercula were also shorter in length may further support a negative effect (on growth) of having a shortened operculum.

Gonads presented with few abnormalities. No evidence of effects of estrogenic exposure (e.g., elevated atresia, intersex) was observed in this study. However, the increase in relative proportion of females from upstream to downstream sites, and the possible finding of a male with testicular oocytes warrants further investigation given that the Rio Grande is dominated by wastewater flows much of the year. Moreover, past findings of vitellogenin induction in fish and >20 pharmaceuticals and personal care product chemicals in waste water treatment plant (WWTP) effluents (NMED 2009) indicates that endocrine active compounds are present. The presence of ectopic oocytes needs to be verified with additional samples. Ectopic oocytes are rare but may result during early development from disruption of migration of the primordial germ cells to the gonad (Kobayashi and Hishida 1985).

Temporal

Fish collected in the first year of sampling were larger than the second year suggesting that the first year collections contained larger individuals from a population that may also contain larger fish.

During the summer months (July-October) flows in the silvery minnow critical habitat are greatly reduced, temperatures are high, and wastewater from the three treatment facilities in the study reach comprises a greater volume of the flow (Kelly et al. 2006; NMED 2008b). Resulting salinity, nitrate and phosphate (Zeglin et al. 2006), total organic carbon, and fecal streptococci concentrations (Langman and Nolan 2005) documented to be high during the summer indicate generally reduced water

quality and may cause stressful conditions as reflected by the silvery minnow histopathology observed in this study. July and October measures of total phosphorus and BOD at the sites in this study tended to be higher than during other months (NMED 2009, Table 5). General health as measured by the HAI and SSI indicated a seasonal pattern of greater stress in summer/fall (July/October). Histopathologies also tended to be more prevalent in fish caught in July: more MAs, and fat in livers and spleens; more granulomas in livers; low liver glycogen; larger spleens lower in red blood cells. These results are indicative of general physiological stress and immune system responses. Consistent with these observations, the greatest number of fish with bacteria was collected during July compared to other months (USFWS 2009) and increased leukocyte proliferation was observed during summer. Gill and kidney pathologies were elevated throughout all months and did not show any seasonal trends.

Spatial

In general, fish at the two sites above Albuquerque were healthier than the fish below Albuquerque, however health in fish at all sites was impaired to some degree. Health assessment results reported here were also consistent with Middle Rio Grande water and sediment quality and tissue residue data collected by the New Mexico Environment Department (NMED 2009) during the study period at sites sampled in this study. Although Rio Grande silvery minnows collected at Site 1 were relatively healthier than fish collected at all other sites, water quality exceedences were reported for chronic Al exposure and *E. coli* levels (NMED 2009). Also, carp tissues, only collected at Sites 1-4, were elevated for Zn at Site 1 and not the other three sites (NMED 2009). Naturally elevated Al concentrations are likely in the RGSM critical habitat as the surrounding mountains are rich in aluminum (NMED 2008b). Of the organochlorines tested, only total DDT was at concentrations of concern only at Site 1 and between Sites 2 and 3 (NMED 2009). In the nearly 20 miles downstream to Site 2, effluents from three wastewater treatment plants (WWTPs) and a flood diversion channel for the

city of Albuquerque enter the Rio Grande. Silvery minnow collected at Site 2 were slightly healthier than RGSM further downstream for some metrics but equivalent to downstream fish for others. Water quality exceedences at this site were reported for chronic Al, and *E. coli* as well as for dissolved oxygen and ammonia (NMED 2009). Chronic and acute Cu levels were exceeded in the river reach just upstream of Site 2 (NMED 2009). Sediment semi-volatile PAHs (polyaromatic hydrocarbons) measured at all sites exceeded NOAA/SQuiRT guidelines primarily at Sites 2-4, and were not detectable at Sites 1 and 6 (NMED 2009). Site 3 is the first site geographically below metropolitan Albuquerque. Effluent from the Albuquerque wastewater treatment plant is the major river inflow. At this site or in the river reach above it to Site 2, water quality exceedences were reported for chronic Al and Cu, acute Cu, *E. coli*, dissolved oxygen, and ammonia (NMED 2009). At Site 3, cyanide, as well as PAHs, was elevated in sediments (NMED 2009). Water quality, sediment contaminant levels, and fish tissue residue results were similar at Sites 3 and 4. A couple of smaller WWTPs enter the river in the nearly 20 miles between these latter sites, and a few more are found in the 50 miles between Sites 4 and 5 and Sites 5 and 6. Only the metals and not PAHs, cyanide, ammonia or pH, were of concern at Site 5 and by Site 6 only chronic Al was in exceedence (NMED 2009). The HAI, SSI, and the percentage of fish with two or more pathologies tended to indicate increasing impairment downstream at or below Site 3. The incidence and severity of pathologies was generally low or variable among sampling dates and numbers of fish sampled were low, and this may have prevented significant site-specific associations. However, liver granulomas were common at Sites 3 and 4, spleen granulomas were frequent at Site 6, and MAs tended to be highest at Site 3 and lowest at Site 1. In addition, the immunological response as evaluated by leukocyte production was greater at Site 3 than Site 1.

Our results indicate that the RGSM in the reach of critical habitat between Bernalillo, NM and Bosque del Apache National Wildlife Refuge are experiencing chronic stress as evidenced by a suite of

health indicators. Given the results from the bioindicators used in this study and the stressors measured by the NMED (2009), it appears that the stress is likely related to constituents of poor water and sediment quality (metals, bacteria, and possibly PAHs). Furthermore, the biology of the RGSM as a benthic feeding fish occupying slow moving waters increases its exposure to chemicals and pathogens associated with sediments. Although the present health study was not designed to identify cause and effect linkages, the health effects we observed in the Rio Grande silvery minnow are in agreement and consistent with the known impacts of urban effluents, stormwater runoff, and agricultural return waters. Recently, RGSM was re-introduced to the Rio Grande near Big Bend National Park (http://www.fws.gov/Endangered/home_stories/silvery_minnows.html). Health monitoring of RGSM along with routine population monitoring in this less polluted section of the Rio Grande and comparison of results with the present and future studies in the middle Rio Grande should serve to increase our understanding of the relative impact of contaminated habitats on Rio Grande silvery minnow recovery.

Disclaimer

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

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Figure 1. Mean total length ± SE of Rio Grande silvery minnow caught during four months (top panel) from 2006-2008 at six sites (bottom panel) in the Rio Grande. Sites or months with the same letter are not significantly different. See Table 2 for locations of sites.

Figure 2. Mean weight ± SE of Rio Grande silvery minnow caught during four months (top panel) from 2006-2008 at six sites (bottom panel) in the Rio Grande. Sites or months with the same letter are not significantly different. See Table 2 for site descriptions.

Figure 3. Mean splenosomatic index (SSI) ± SE of Rio Grande silvery minnow caught during four months (top panel) from 2006-2008 at six sites (bottom panel) in the Rio Grande. Sites or months with the same letter are not significantly different. See Table 2 for site descriptions.

Figure 4. Mean health assessment index (HAI) ± SE of Rio Grande silvery minnow caught during four months (top panel) from 2006-2008 at six sites (bottom panel) in the Rio Grande. Sites or months with the same letter are not significantly different. See Table 2 for site descriptions.

Figure 5. Mean percent ± SE females of Rio Grande silvery minnow collected at each of six sites. Mantel-Haenszel Chi-Square indicated a significant trend in more females in downstream sites ($p < 0.0001$). Numbers on bars indicate the number of sampling events.

Figure 6. Percent of total Rio Grande silvery minnow collected at each of six sites with 1 or $\geq$2 pathologies for kidney (bottom panel), liver (middle panel), spleen (top panel).

Figure 7. Kidney section from a Rio Grande silvery minnow from Dexter National Fish Hatchery (A) showing normal kidney and from a silvery minnow captured at Site 3 showing hyaline droplet degeneration of tubular epithelial cells (arrows). HE stain.

Figure 1.

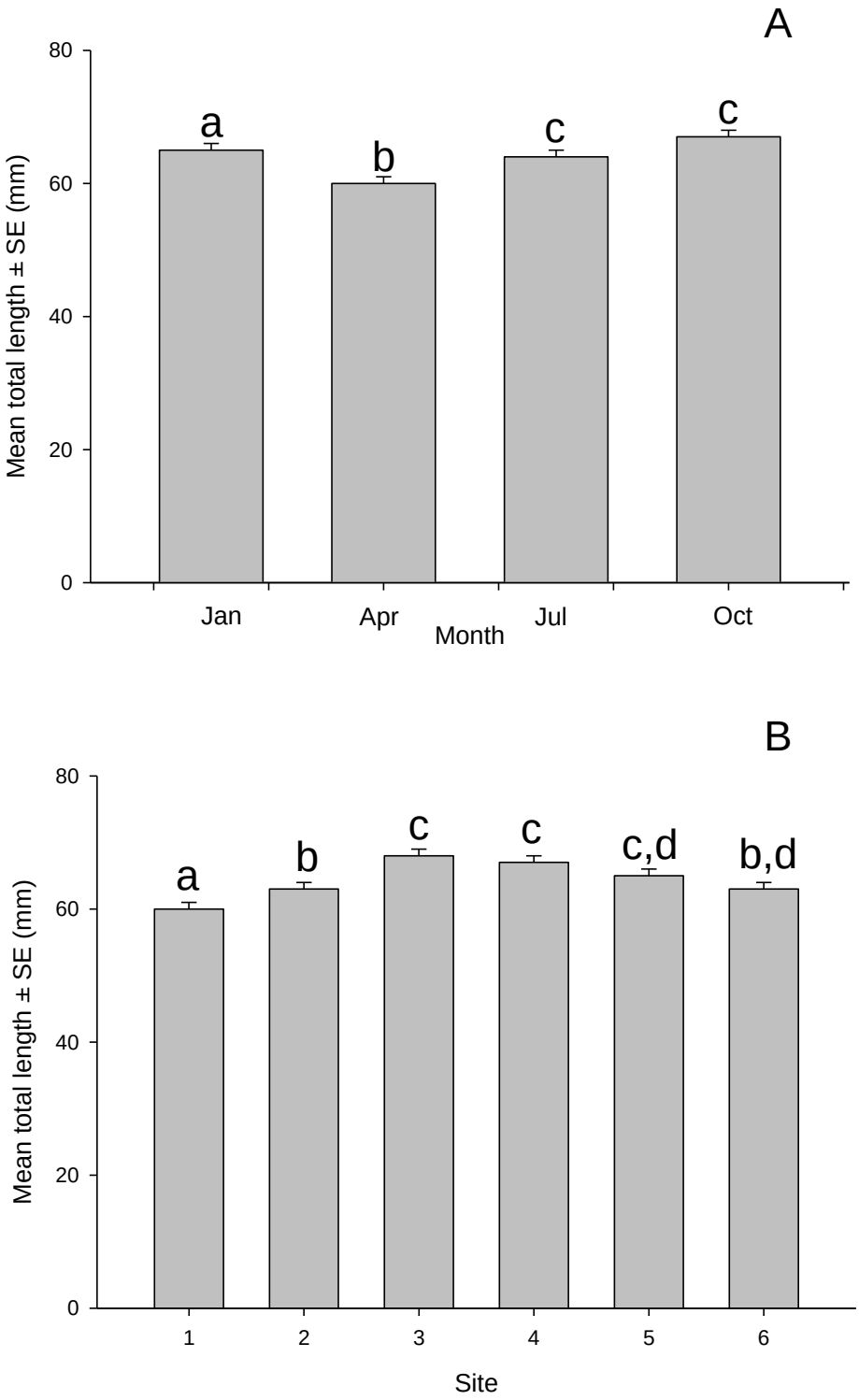

A
80
60
40
20
0
Mean total length ± SE (mm)
a
b
c
c
Jan
Apr
Jul
Oct
Month
B
80
60
40
20
0
Mean total length ± SE (mm)
a
b
c
c
c,d
b,d
1
2
3
4
5
6
Site

Figure 2

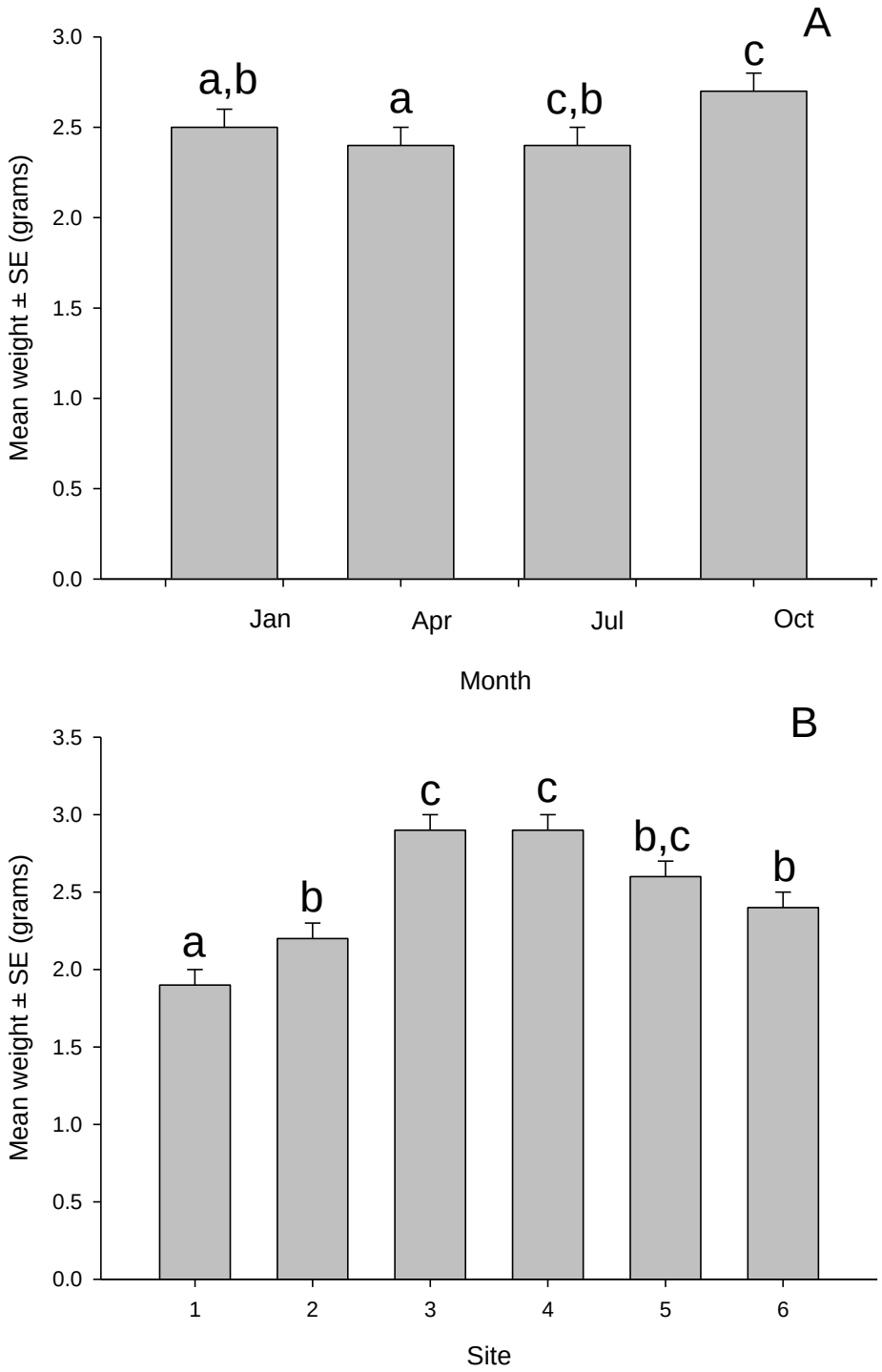

A
a,b
a
c,b
c
Mean weight ± SE (grams)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Jan
Apr
Jul
Oct
Month
B
a
b
c
c
b,c
b
Mean weight ± SE (grams)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
Site

Figure 3

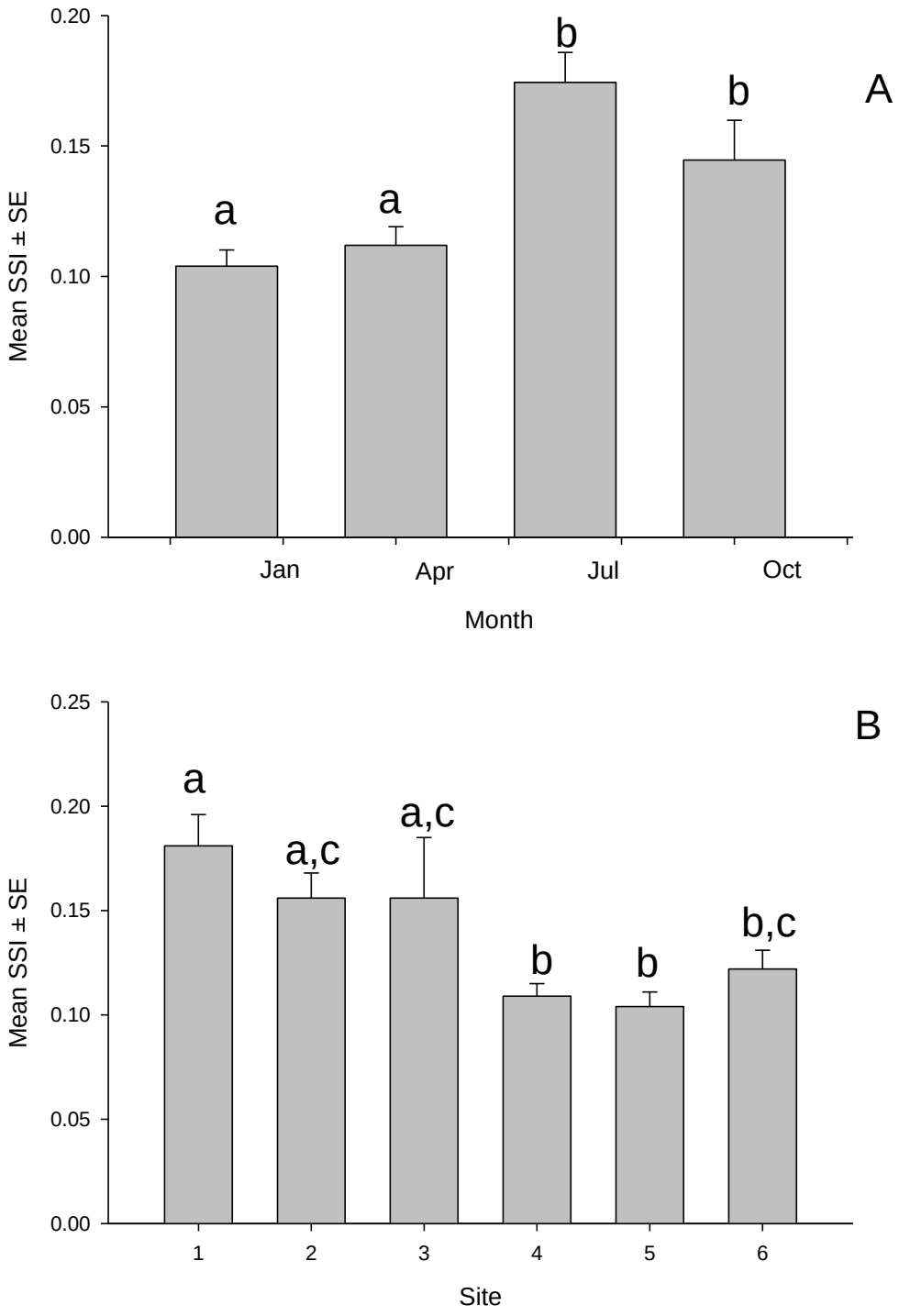

A
Mean SSI ± SE
0.20
0.15
0.10
0.05
0.00
a
a
b
b
Jan
Apr
Jul
Oct
Month
B
Mean SSI ± SE
0.25
0.20
0.15
0.10
0.05
0.00
a
a,c
a,c
b
b
b,c
1
2
3
4
5
6
Site

Figure 4

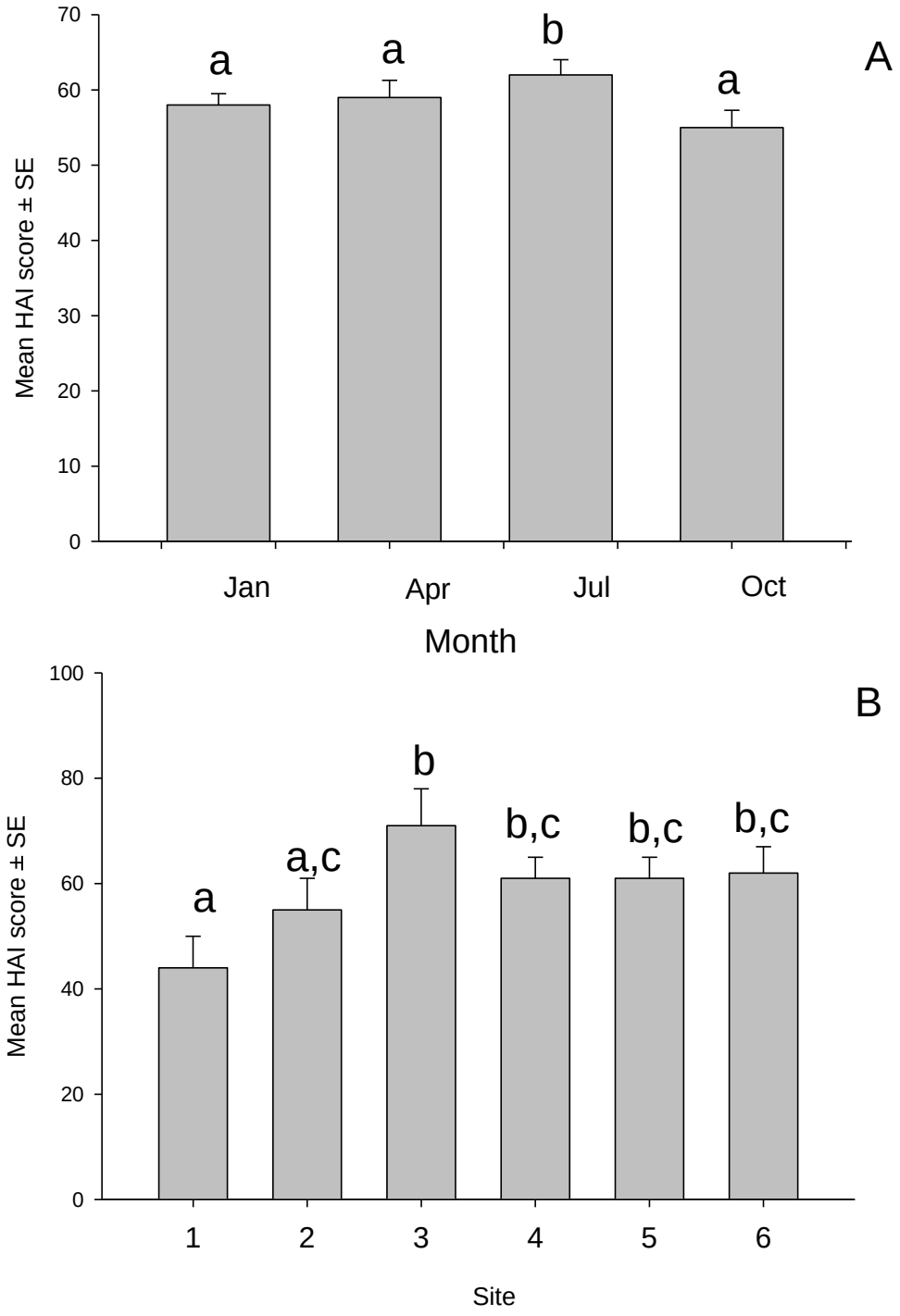

A
Mean HAI score ± SE
70
60
50
40
30
20
10
0
a
a
b
a
Jan
Apr
Jul
Oct
Month
B
Mean HAI score ± SE
100
80
60
40
20
0
a
a,c
b
b,c
b,c
b,c
1
2
3
4
5
6
Site

Figure 5.

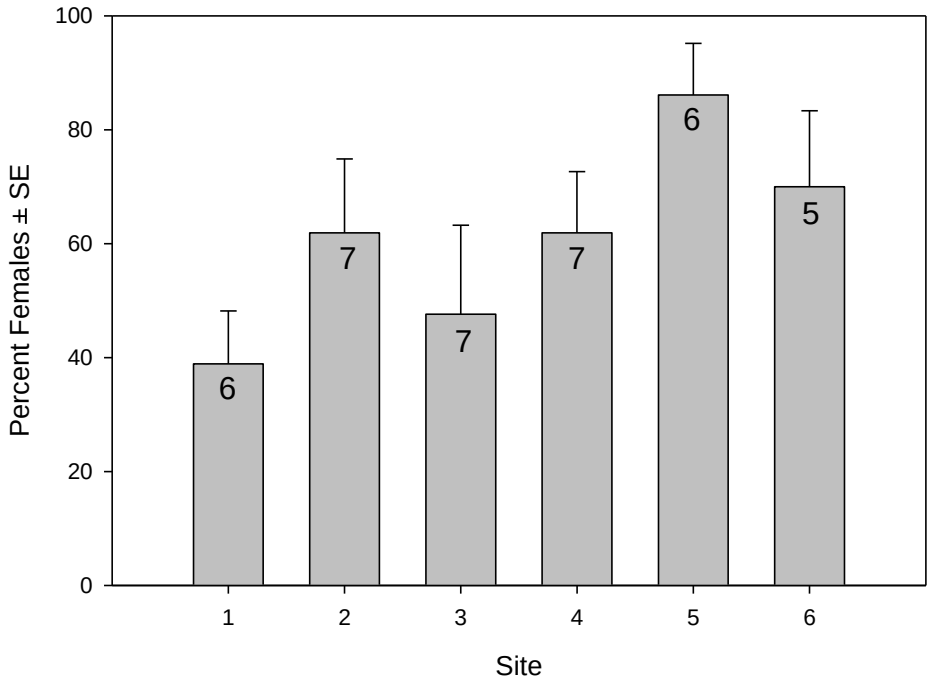
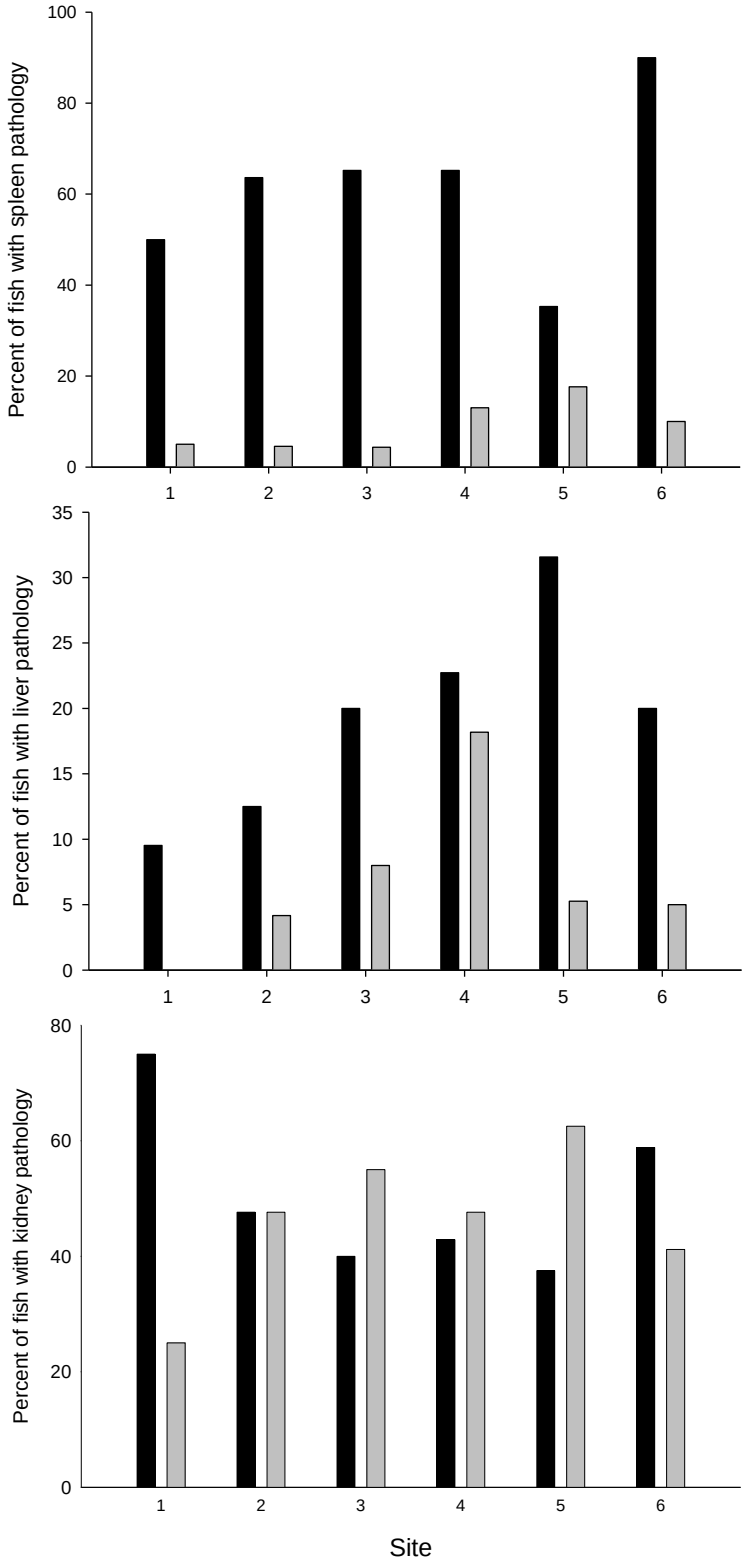


Figure 6.


Percent of fish with spleen pathology
100
80
60
40
20
0
1
2
3
4
5
6
Percent of fish with liver pathology
35
30
25
20
15
10
5
0
1
2
3
4
5
6
Percent of fish with kidney pathology
80
60
40
20
0
1
2
3
4
5
6
Site

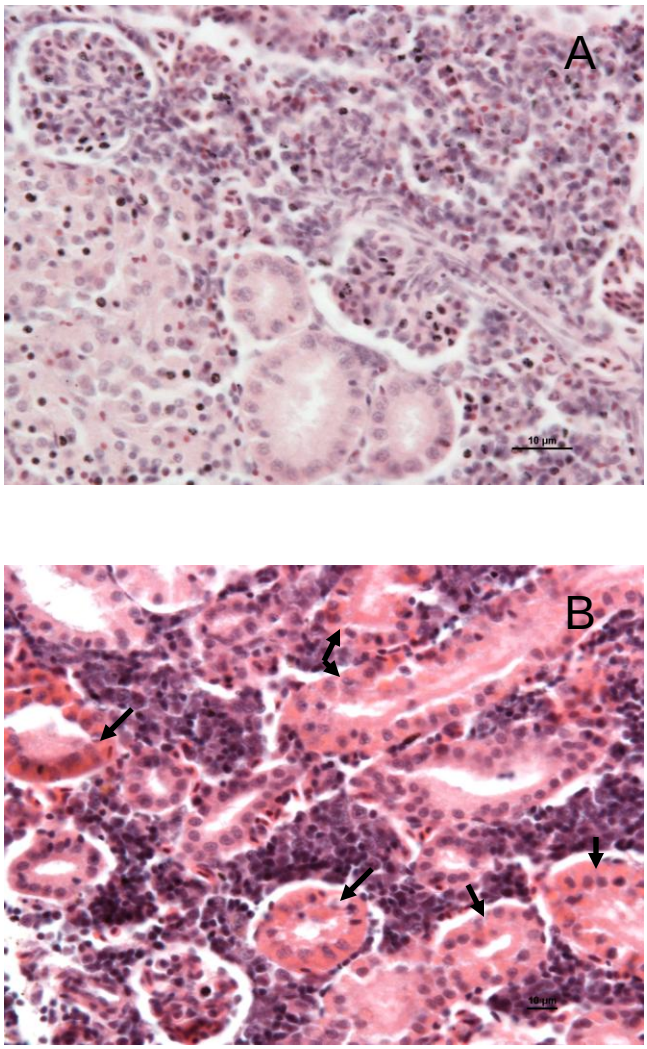



Figure 7.