Water Pollution Studies

Federal Aid Project F-243R-18

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Job Progress Report

Colorado Division of Wildlife

Aquatic Wildlife Research Section

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The results of the research investigations contained in this report represent work of the authors and may or may not have been implemented as Division of Wildlife policy by the Director or the Wildlife Commission.

Table of Contents

List of Tables

List of Figures

State: Colorado Study No. F243R

Title: Water Pollution Studies

Period Covered: July 1, 2010 to June 30, 2011

Project Objective: To develop quantitative chemical and toxicological data on the toxicity of pollutants to aquatic life, investigate water pollution problems in the field, and provide expertise in aquatic chemistry and aquatic toxicology.

STUDY PLAN A: LABORATORY TOXICITY STUDIES

Brief Description: Conduct laboratory-based experiments to test effects of contaminants on aquatic organisms.

Job A.1. Reproductive Toxicity of Endocrine Disrupting Compounds

Job Objective: Determine whether exposure to hormonally active agents results in feminization of rainbow trout, fathead minnows and/or other aquatic organisms. Effects of feminization on reproduction and fecundity will be measured. Concentrations of endocrine disrupting compounds that result in significant feminization will be compared to concentrations observed in wastewater treatment plant effluents and in Colorado streams.

Job A.2. Reproductive Toxicity of Atrazine Herbicide

Job Objective: Measure fecundity and biomarkers of feminization of red shiners exposed to a range of atrazine. Relate concentrations that result in impairment in the laboratory with concentrations observed in Colorado eastern plains streams.

Job A.3. Toxicity of Metals to Fish

Job Objective: Measure acute (96 hour) and chronic (60 day) effects of zinc, copper and/or cadmium exposure on hatching, survival and growth of different life stages of mottled sculpin, longnose dace and/or other species. Results from these experiments will compare toxicity thresholds to USEPA metal criteria to ensure that these species are protected.

Job A.4. Effects of Dietary Exposure of Metals to Fish

Job Objective: Measure the effect of zinc, copper, cadmium and/or selenium from dietary sources on survival and growth of fish in the laboratory. Evaluate the sensitivity of dietary-exposed organisms to waterborne exposure. Relate dietary levels that cause diminished performance in the laboratory with levels found in dietary sources in metal impacted areas such as the upper Arkansas River, Clear Creek and the Eagle River.

Job A.5. Testing and Validation of the Biotic Ligand Model

Job Objective: Determine the ability of the Biotic Ligand Model to estimate acute and chronic toxicity effects of metals on aquatic organisms exposed under multiple water quality conditions.

STUDY PLAN B: TECHNICAL ASSISTANCE

Brief Description: Conducts toxicological experiments as requested from regulators to be incorporated into policy; conducts water chemistry analysis and training for CDOW and other agencies.

Job B.1. Water Quality Assistance to Division of Wildlife Personnel and Other State and Federal Agencies.

Job Objectives: To provide technical assistance and expertise, consultation, evaluation and training in aquatic toxicology and aquatic chemistry to Division of Wildlife and other state and federal personnel as requested. Conduct short or long term experiments to produce toxicity data, or develop site-specific field studies, when such data in the literature are lacking or inadequate. Ultimately, these activities will assist regulatory agencies in the development, implementation, and enforcement of water quality standards needed to protect or enhance the aquatic resources of Colorado.

ACCOMPLISHMENTS

Job A.1. Reproductive Toxicity of Endocrine Disrupting Compounds

The project continued to provide equipment and support for onsite bioassays conducted by personnel at the University of Colorado and Unversity of Denver. The studies' objectives were to detect and quantify estrogenic activity in the city of Boulder wastewater treatment plant effluent after recent treatment plant process upgrades. Estrogenic activity will be compare with tests conducted prior to the upgrades. Assistance was also provided to a Colorado State University study investigating effects of 17α-ethynylestradiol on fathead minnow reproduction in mesocosms.

Job A.2. Reproductive Toxicity of Atrazine Herbicide

No activities during this segment.

Job A.3. Toxicity of Metals to Fish

Cadmium acute and chronic early-life stage toxicity tests were conducted with Rio Grande cutthroat trout. Analyses of water samples collected for measurement of cadmium concentrations have not been completed. Results will be reported next segment. Acute zinc toxicity tests were conducted with Rio Grande cutthroat trout (*Oncorhynchus clarki virginalis*), mountain whitefish (*Prosopium williamsoni*) and flathead chub (*Platygobio gracilis*). Chronic iron early-life stage toxicity tests were conducted with mountain whitefish and brown trout (*Salmo trutta*). Results of the zinc and iron toxicity tests are reported below.

Job A.4. Effects of Dietary Exposure of Metals to Fish

A laboratory culture of the oligochaete, *Lumbriculus variegatus,* is currently being exposed to waterborne cadmium. Lumbriculus will be cultured in cadmium-dosed and cadmium-free water until sufficient masses of organisms are present to be used as food for rainbow trout and/or cutthroat trout fry. Fry will be fed a cadmium-dosed and cadmium-free diet to determine effects of dietary cadmium on survival, growth and accumulation in kidneys and liver. Result of the study will be reported next segment.

Job A.5. Testing and Validation of the Biotic Ligand Model

A fundamental assumption of the biotic ligand model is that the binding affinity and capacity of metals to gills is similar among different taxa. Thus, different tolerances of different species to metals such as zinc are due to different abilities to withstand different amounts of zinc on the gills, measured by LA50. Brook trout and brown trout fingerlings were exposed to a range of concentrations of the stable zinc isotope ^{67}Zn. Accumulation of the stable isotope by the gills was measured in low water hardness over a range of time intervals between 45 minutes and 72 hours. An acute toxicity test was conducted concurrently so that a median lethal accumulation value (LA50) could be calculated. The gill-binding affinity and capacity of brook trout and brown trout will be determined and compared to rainbow trout. Tissue and water samples are currently awaiting analysis by United States Geological Survey (USGS) and will be reported next segment.

Job B.1. Water Quality Assistance to Division of Wildlife Personnel and Other State and Federal Agencies.

Pete Cadmus (MS student) and Dr. Will Clements from Department of Fish, Wildlife and Conservation Biology, Colorado State University continue to collaborate with DOW to determine the dietary effects of metals on aquatic invertebrates. They are also collaborating with DOW on efforts to measure effects of iron and iron precipitates on aquatic life. Benthic invertebrate microcosms were exposed to a range of iron concentrations to study effects on benthic community structure and abundance. Organisms from the test are currently being sorted and identified. Results will be presented next segment.

CDOW participated as Party Status in several Water Quality Control Commission Rulemaking and Administrative Action Hearings, and provided input to WQCC and Stakeholders at regulatory workgroups (nutrient criteria and aquatic life classifications). We continue to serve on BTAG (Biological Technical Assistance Group) committees for the Arkansas River mine site and for the Standard Mine on Coal Creek near Crested Butte, where we provide expertise and data. We represent CDOW on CDPHE's Technical Advisory Committee for mercury contamination in fish tissues. Mercury action limits are being set and protocols for notifying the public of potential health hazards are being developed. We assisted CDOW biologists in coordinating their fish collection with CDPHE chemical analysts to assess risks to anglers at numerous reservoirs around the State. CDOW wrote several letters of support for academic researchers and agencies who are seeking funding to conduct experiments with metals (zinc, cadmium, mercury) and endocrine disrupting compounds.

CDOW worked with the USFWS, BLM, CDPHE, EPA and the Attorney General's Office on inter-agency water quality restoration projects, including Natural Resource Damage (NRD) claims for the upper Arkansas River and the Rocky Mountain Arsenal superfund sites. The NRD program, as detailed in the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), provides an avenue for State, Federal and Tribal governments to pursue monetary damages associated with ecological injury in order to restore habitats and biota after the release of a hazardous substance.

The CDOW continues to invest significant personnel time and resources to the upper Arkansas River NRD case, near Leadville CO, which is facilitated by a Trustee Council of State and Federal Trustees. State Trustees include Colorado Department of Public Health and Environment (CDPHE), Department of Natural Resources (DNR) and the Colorado Department of Law (DOL). Federal Trustees include US Fish and Wildlife Service (USFWS) as the lead, and also Bureau of Reclamation, Bureau of Land Management (BLM).

The Trustee Council and its workgroup for this claim prepared two guiding documents for restoration priorities and expenditures of the ~$20M awarded in the case: the "Upper Arkansas River Watershed Restoration Plan and Environmental Assessment", dated April 24, 2010 (RP/EA) and the "Restoration Monitoring and Outreach Plan for the Upper Arkansas River Watershed", dated June 2010 to address public losses caused by surface water and ground water injuries resulting from releases of substances to and from the California Gulch Superfund Site (see http://www.fws.gov/mountain-prairie/NRDA/LeadvilleColo/CaliforniaGulch.htm). CDOW contributed a significant amount of concepts and text to these documents. Additional projects that CDOW supports which are related to the restoration and CERCLA activities include the Union Creek Stream Stabilization Project, Sugarloaf Mountain Mining District BMP Performance Monitoring Project, The Lake Fork of the Arkansas River Watershed Plan, and Flow Related Physical Habitat for Brown Trout in the Lake Fork of the Arkansas River near Leadville, Colorado. Tangential remediation and restoration projects are being conducted in the river under CERCLA Superfund authority, involving the CDPHE and EPA. CDOW assists with data collection, consolidation and analysis for this project.

CDOW is responsible for habitat restoration and acquisition activities in the river corridor, including in-stream, floodplain and upland habitats, which are NRD activities prioritized for spending in the RP/EA . CDOW's restoration project has an associated monitoring activity schedule outlined in the Trustee Monitoring and Outreach Plan document to insure that measurable restoration success is documented. Critical activities by CDOW in this fiscal year included conceptual design, engineered plans for construction activities, budget development and logistical planning with stakeholders. Many of the restoration activities and baseline monitoring activities are scheduled to begin in 2011, and post-restoration monitoring will begin in 2012. Construction of in-stream habitat for brown trout is expected to take up to a decade to complete for the entire 11 mile reach. The state Trustees, via CDPHE and CDOW plus numerous sub-contracts, will be responsible for restoration activities on properties with public fishing and recreational access. The Federal Trustees will fund restoration activities on private land, with intermediary contract assistance from the Sangre de Cristo Resource, Conservation and Development agency (RC&D). For the next 5-10 years, many miles of river will be under construction simultaneously, with the potential to impact numerous landowners and stakeholders actions. In addition, multiple projects will be underway in the same river reaches, including survey and development, construction and baseline biotic and water quality sampling.

To insure the success and continued public support of CERCLA and NRD projects in the upper Arkansas river, CDOW plays an important role with technical and public relations coordination among Trustee Council agencies (USFWS, BOR, BLM, CDPHE, DNR), technical government agencies and universities/colleges (EPA, USGS, USFS, CSU, CMC, etc) , quasi-government agencies (Conservation districts), participating non-government entities (e.g. Trout Unlimited, LACOSI), local government representatives (county Commissioners, Representatives and Senators) and participating landowners (private lands, easement holders, City of Aurora, State Parks, etc.). We attend numerous meetings to share ideas and to present technical plans, and also offer field assistance with other stakeholders who are collecting data related to the NRD case in the Upper Arkansas.

The Native Aquatic Species Restoration Facility (NASRF) in Alamosa has experienced poor survival of plains minnows eggs (*Hybognathus placitus*) and boreal toad tadpoles (*Bufo boreas boreas*). Unknown water quality problems were suspected as a possible cause. The Aquatic Toxicology Laboratory agreed to hatch and rear eggs of plains minnows and boreal toad tadpoles in its water source and compare with results at NASRF. Plains minnows eggs transported to Ft Collins were mostly unfertilized or otherwise not viable. Fry hatched <24 hrs later and had high survival. Boreal toad egg masses were split between the toxicology lab and NASRF. Mortality of boreal toad eggs were generally low (<10%) for both facilities except for one egg mass that had low survival (ca. 50% at both facilities) due to fungus infection. Water quality was eliminated as a likely cause of poor survival of plains minnows or boreal toad tadpoles.

Acute Toxicity of Zinc to Rio Grande Cutthroat (*Oncorhynchus clarki virginalis*), Mountain Whitefish (*Prosopium williamsoni*) and Flathead Chub (*Platygobio gracilis*)

INTRODUCTION

Discovery of the Colorado Mineral Belt led to extensive mining of the Rocky Mountains in the late 19th and early 20th centuries. There are an estimated 23,000 abandoned mines in Colorado, many of which leach waste minerals affecting aquatic life. Zinc is often present in high concentrations in acid mine drainage and affects aquatic life in an estimated 900 miles of streams in Colorado (CDPHE 2010). Colorado's zinc standards for protection of aquatic life are based on national water quality criteria derived from toxicity data for many aquatic species (USEPA 1987, USEPA 1996). National criteria are intended to be conservatively protective of all or almost all bodies of water (Stephan et al. 1985) which assumes that species sensitivity in the national database is representative of untested species. Most of the toxicity data used to develop national criteria are from species that are easy to culture and maintain in the laboratory. Species native to Colorado Rocky Mountains are poorly represented in the toxicity data set. As such, it is uncertain whether national criteria for zinc are protecting the native aquatic species exposed to mining waste in Colorado. Mottled sculpin, a native to Colorado, have recently been found to be very sensitive to zinc and may not be protected by national zinc water quality criteria (Woodling et al. 2002, Brinkman and Woodling 2005, Besser et al. 2007).

National criteria that protect all or almost all waters of the nation may sometimes result in overprotection for certain bodies of water. Recognizing this, USEPA provides guidance for development of site-specific standards to reflect regional or local aquatic communities and water quality characteristics (USEPA 1994). The Recalculation Procedure has been used in Colorado to develop site-specific standards and is based on local aquatic communities. The Recalculation Procedure calculates site-specific standards using the same methodology as national criteria but uses a modified subset of the toxicity database based on species that occur or are expected to occur at the site. If no data are available for a species present at the site, toxicity data from related or surrogate species are used. Again, toxicity data for Colorado native species are lacking and therefore the toxicity data of surrogate species may not well represent untested native species. The objective of this study was to measure acute zinc toxicity to Rio Grande cutthroat *Oncorhynchus clarki virginalis*), mountain whitefish (*Prosopium williamsoni*) and flathead chub (*Platygobio gracilis*) to evaluate protectiveness of existing zinc water quality standards for these native species and for use in development of site-specific water quality standards.

MATERIAL and METHODS

Organisms

Rio Grande cutthroat trout were obtained as eyed eggs from the Colorado Division of Wildlife Pitkin Hatchery (Source: Haypress Lake Mineral County Colorado). Eggs were incubated and raised in Ft. Collins dechlorinated municipal tap water (hardness near 50 mg/L as $CaCO_3$, temperature 12°C, pH 7.5). After swimup, fry were fed starter salmon chow (Rangen,

Inc. Buhl, ID) supplemented with brine shrimp nauplii. Cutthroat fry were 44 days post-swimup (0.287g) at the start of the test and were fasted for 48 hours prior to exposure.

Mountain whitefish eggs were collected from wild adults during a spawning run up Mad Creek (tributary to the Elk River) near Steamboat Springs, Colorado. Individual families were created by crossing each female with two males each to ensure viable milt was available for fertilization. Fertilization rates were enhanced by maintaining anhydrous conditions during the process, with conception occurring in the ovarian fluid bath. Fertilized eggs from six females were combined and then hardened for an additional 30 min. in water from the brood source before making the 3.5 hour journey in a 3.8 L cooler to the aquatic toxicology lab where they were then treated with 1600 mg/L formalin to control fungus (Piper et al. 1982). Eggs were incubated in Ft. Collins dechlorinated municipal tap water (hardness near 50 mg/L as $CaCO_3$, temperature 7°C, pH 6.8-7.4). After hatching, water temperature was adjusted to 13.5°C and maintained at this temperature until the start of the test. Mountain whitefish fry were fed brine shrimp nauplii and a 1:1 mixture of freeze-dried brine shrimp and bloodworms sieved through <500 μm screen. Fry were 30 days post-hatch (0.061g) at the start of the test and were fasted for 48 hours prior to exposure.

Flathead chubs were collected from Fountain Creek (El Paso County) and transported to the lab in an aerated cooler. Flathead chub fry readily consumed frozen bloodworms. Fry were maintained at test water quality conditions for 14 days prior to the start of toxicity tests. Fry were fasted for 48 hours prior to initiation of toxicity tests.

Toxicity Test

A continuous-flow serial diluter (Benoit et al. 1982) delivered exposure concentrations. The diluter was constructed of Teflon, polyethylene, and polypropylene components. Nalgene food-grade vinyl tubing delivered test solutions to exposure chambers. Test solutions overflowed from the exposure chambers into a water bath maintained at test temperature using a recirculating chiller (VWR model 1175MD). A stock solution was prepared by dissolving a calculated amount of zinc sulfate salt in deionized water ($ZnSO_4 \cdot 7H_2O$ Mallincrodt). Stock solutions were delivered to the diluter via a peristaltic pump at a rate of 2.0 mls/min. Diluters delivered five concentrations with a 50% dilution ratio and a control. For the tests with cutthroat trout and mountain whitefish, a flow splitter allocated each concentration equally among four replicate exposure chambers at a rate of 40 ml/min for each 2.8 L polypropylene chamber. For the flathead chub tests, a flow splitter allocated each concentration equally among three replicate exposure chambers at a rate of 60 ml/min for each 18L glass aquarium. Ambient fluorescent lighting provided a 16-h/8-h light-dark photoperiod. Diluters and toxicant flow rates were monitored daily to ensure proper operation. At the start of exposure, ten cutthroat and whitefish fry and six flathead chub were randomly allocated to each exposure chamber. Fry were not fed during the test.

Water quality parameters were measured at 0, 48, and 96 hrs in all treatment levels within a replicate. Different replicates were selected each sampling. Hardness and alkalinity were determined according to Standard Methods (APHA 1998). A Thermo Orion 635 meter was used

to measure pH and conductivity. Dissolved oxygen was measured using an Orion 1230 dissolved oxygen meter. The conductivity, pH and dissolved oxygen meters were calibrated prior to each use.

Water samples for zinc were collected at 0, 48, and 96 hrs. Samples for zinc and analysis were passed through a 0.45µm filter and immediately preserved with high purity nitric acid to pH <2. Chambers with no survivors remaining were not sampled. Metal concentrations were measured using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard from an outside source (High Purity Standards, Charleston SC). Chloride and sulfate concentrations were measured with a flow-injection analyzer (QuikChem 8000; Lachat Instruments, Loveland CO) using USEPA methods 325.1 and 375.4, respectively. Sample splits were collected and spikes prepared at each sampling event to verify reproducibility and analytical recovery. Ninety six hour median lethal concentrations (LC_{50}) were estimated from measured zinc concentrations using the Trimmed Spearman-Karber technique with automatic trim (Hamilton et al. 1977, 1978).

RESULTS and DISCUSSION

Relative standard deviation of sample splits was 0.9% (0-3.5%). Mean recovery of sample spikes was 103% (96.8-110%). Recovery of external quality assurance standards was 99.3% (96.0-103%). Water quality characteristic were consistent within each toxicity test as evidenced by relatively low standard deviations of measurements (Table 1). Dissolved organic carbon (DOC) was not measured in this study but in previous studies at our lab using the same water sources, DOC concentrations were found to be low (average 1.7 mg/L, range 0.8-4.0 mg/L, N=27).

Measured dissolved zinc concentrations were near target levels and were constant during the Rio Grande cutthroat toxicity test (Table 2). All fry died at zinc concentrations ≥ 267 µg/L. No morality occurred at concentrations ≤76 µg/L. The 96 hour median lethal concentration (LC50) was 142 µg/L with a 95% confidence interval 128-157 µg/L. Given the importance of cutthroat trout in Rocky Mountain streams, surprisingly few zinc toxicity tests have been published. An unspecified strain of cutthroat trout strain had a 670 µg/L 14 day zinc LC_{50} obtained by diluting zinc-contaminated river water with well water (Nehring and Goettl 1974). Another study with an unspecified strain of cutthroat trout reported a 96 hr LC_{50} of 90 µg/L (Rabe and Sappington 1970). These toxicity values were not used in derivation of zinc criteria because water quality characteristics varied too much during the test or were not reported. The LC50 from the current study is consistent with previous cutthroat trout toxicity tests conducted by this project (Figure 1). The effect of hardness on toxicity values exhibits the characteristic linear relationship on a log scale.

The mountain whitefish toxicity test had constant measured zinc concentrations that were near target values (Table 3). All mountain whitefish fry died at zinc concentrations ≥ 823 µg/L.

No morality occurred at concentrations ≤223 µg/L. The 96 hour median lethal concentration (LC50) was 357 µg/L with a 95% confidence interval 327-390 µg/L. No published zinc toxicity data are available for whitefish. Median lethal concentrations from toxicity tests previously conducted by this project are 427 µg/L and 477µg/L (Brinkman and Vieira 2008).

Measured zinc concentrations during the flathead chub test were near target concentrations and were constant throughout the test (Table 3). All flathead chub died at 7230 µg/L. No morality occurred at concentrations ≤881µg/L. The 96 hour median lethal concentration (LC50) was 2593 µg/L with a 95% confidence interval 2150-3127 µg/L.

Rio Grande cutthroat trout were most sensitive to zinc followed by mountain whitefish, then flathead chub. In general, salmonids tend to be sensitive to metals relative to other fishes. The national water quality criterion for zinc at 50 mg/L water hardness is 67 µg/L (USEPA 1999). The current Colorado table value standard for zinc at 50 mg/L hardness is 79 µg/L. Both values appear to be protective of cutthroat trout, mountain whitefish and flathead chub.

Planktonic crustaceans are generally not present in riverine systems resulting in their deletion from recalculated site-specific water quality standards. Deletion of metal-sensitive planktonic crustaceans such as Daphnia and Ceriodaphnia will typically increase recalculated site-specific zinc standards in riverine systems. In those instances, cutthroat trout and mountain whitefish will become among the most sensitive species and will potentially significantly impact the final acute value for zinc.

REFERENCES

Benoit, D.A., V.R. Mattson, and D.C. Olsen. 1982. A Continuous Flow Mini-diluter System for Toxicity Testing. Water Research. 16:457-464.

Besser J.M., Mebane C.A., Mount D.R., Ivey C.D., Kunz J.L., Greer I.E., May T.W., Ingersoll C.G. (2007) Sensitivity of mottled sculpin (*Cottus bairdi*) and rainbow trout (*Onchorhynchus mykiss*) to acute and chronic toxicity of cadmium, copper, and zinc. Environmental Toxicology and Chemistry 26(8): 1657-1665

Brinkman, S.F. and D.L. Hansen. 2004. Federal Aid in Fish and Wildlife Restoration. Job Progress Report F-243R-11. Colorado Division of Wildlife, Fort Collins, CO, USA.

Brinkman, S.F. and N. Vieira. 2008. Federal Aid in Fish and Wildlife Restoration. Job Progress Report F-243R-15. Colorado Division of Wildlife, Fort Collins, CO, USA.

Brinkman, S.F. and N. Vieira. 2010. Federal Aid in Fish and Wildlife Restoration. Job Progress Report F-243R-17. Colorado Division of Wildlife, Fort Collins, CO, USA.

Brinkman S.F., Woodling J.D. (2005) Zinc toxicity to the mottled sculpin (*Cottus bairdi*) in high-hardness water. Environmental Toxicology and Chemistry 24:1515-1517

CDPHE. 2008. Status of water quality in Colorado – 2008. The update to the 2002, 2004, and 2006 305(b) reports.

Davies, P.H., S.F. Brinkman and D. Hansen. 2000. Federal Aid in Fish and Wildlife Restoration. Job Progress Report F-243R-7. Colorado Division of Wildlife, Fort Collins, CO, USA.

Hamilton M.A, R.C. Russo, R.V. Thurston (1977) Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11(7): 714-719.

Hamilton M.A, R.C. Russo, R.V. Thurston (1978) Correction. Environ. Sci. Technol. 12:417.

Nehring R.B., Goettl J.P. (1974) Acute toxicity of a zinc contaminated stream to four species of salmonids. Bull Environ Contam Toxicol 12(4):464-469.

Rabe R.W., Sappington C.W. (1970) Biological productivity of the Coeur d'Alene Rivers as related to water quality. Project A-024-Ida. Water Resources Research Institute, Moscow, ID.

U.S. Environmental Protection Agency. 1983. Methods for the chemical analysis of water and wastes. EPA-600/4-79-020.Washington, DC.

U.S. Environmental Protection Agency 1987. Ambient Water Quality for Zinc - 1987. U. S. Environmental Protection Agency EPA-440/5-87-003.

U.S. Environmental Protection Agency 1996. Water Quality Criteria Documents for Protection of Aquatic Life in Ambient Water - 1987. U. S. Environmental Protection Agency EPA-820-B-96-001.

U.S. Environmental Protection Agency 1999. National Recommended Water Quality Criteria. U. S. Environmental Protection Agency. EPA#:822/Z-99-001 YEAR: 1999.

Woodling J.D., Brinkman S.F., Albeke S.A. (2002) Acute and chronic toxicity of zinc to the mottled sculpin (*Cottus bairdi*). Environmental Toxicology and Chemistry 21:1922-1926

Table 1. Mean water quality measurements of exposure water of zinc toxicity tests with Rio Grande cutthroat trout, mountain whitefish and flathead chub fry. Standard deviations are in parentheses.

	Rio Grande Cutthroat trout	Mountain Whitefish	Flathead Chub
Hardness* (mg/L)	41.7 (0.3)	43.2 (0.5)	52.4 (1.0)
Alkalinity (mg/L)	31.4 (1.1)	35.8 (1.5)	37.2 (0.9)
pH (S.U.)	6.85 (0.10)	7.33 (0.10)	7.05 (0.20)
Temperature (ºC)	12.3 (0.1)	13.7 (0.1)	15.1 (0.2)
Conductivity (µS/cm)	83.0 (2.1)	91.0 (1.1)	112 (6)
DO (mg/L)	8.85 (0.08)	9.03 (0.6)	8.25 (1.00)
Calcium (mg/L)	14.6 (0.1)	15.2 (0.2)	17.7 (0.4)
Magnesium (mg/L)	1.2 (0.0)	1.3 (0.0)	2.0 (0.0)
Sodium (mg/L)	3.0 (0.1)	3.4 (0.1)	3.5 (0.1)
Potassium (mg/L)	0.5 (0.0)	0.6 (0.0)	0.6 (0.0)
Chloride (mg/L)	4.5 (0.8)	3.2 (0.1)	5.3 (0.0)
Sulfate (mg/L)	11.7 (0.2)	9.5 (0.2)	11.9 (0.1)

*Calculated from calcium and magnesium ion concentrations.

Table 2. Mean measured dissolved zinc concentrations (µg/L) and associated 96 hr mortality (%) of Rio Grande cutthroat trout fry. Standard deviations are in parentheses.

Target Concentration	0	62	125	250	500	1000
Measured [Zn] (µg/L)	<10 (2)	76 (8)	139 (1)	267 (3)	512 (4)	963 (6)
96 hr Mortality (%)	0 (0)	0 (0)	57.5 (21.2)	100 (0)	100 (0)	100 (0)

96 hour median lethal concentration (95% confidence limits) = 142 µg/L (128-157 µg/L)

Table 3. Mean measured dissolved zinc concentrations (µg/L) and associated 96 hr mortality (%) of mountain whitefish fry. Standard deviations are in parentheses.

Target Concentration	0	100	200	400	800	1600
Measured [Zn] (µg/L)	<10 (1)	120 (2)	223 (1)	426 (4)	823 (6)	1607 (23)
96 hr Mortality (%)	0 (0)	0 (0)	0 (0)	77.5 (18.9)	100 (0)	100 (0)

96 hour median lethal concentration (95% confidence limits) = 357 µg/L (327-390 µg/L)

Table 4. Mean measured dissolved zinc concentrations (µg/L) and associated 96 hr mortality (%) of flathead chub fry. Standard deviations are in parentheses.

Target Concentration	0	500	1000	2000	4000	8000
Measured [Zn] (µg/L)	<10 (2)	440 (30)	881 (51)	1791 (64)	3517 (85)	7230 (423)
96 hr Mortality (%)	0 (0)	0 (0)	0 (0)	16.7 (28.9)	77.8 (25.4)	100 (0)

96 hour median lethal concentration (95% confidence limits) = 2593 µg/L (2150-3127 µg/L).

Chronic Toxicity of Iron Hydroxide to Early Life Stages of Brown Trout and Mountain Whitefish

Over 955 miles of streams and rivers in Colorado are adversely heavily affected by iron (Fe) (Colorado State Department of Public Health and Environment 2010). Iron leaches into streams from natural sources as well as from mining activities in the Mineral Belt of the Rocky Mountains. Iron is not soluble in oxygenated, neutral pH waters precipitating out of solution as ferric hydroxide and ferric oxide precipitates. As such, Fe is generally considered less toxic than soluble metals such as cadmium, copper and zinc. Though the chemical toxicity of iron precipitates may be low to target organs such as fish gills, the precipitates can otherwise adversely affect aquatic life through increased turbidity, reduced primary production and of interstitial space in the benthic zones and smothering of bottom-dwelling invertebrates, plants and incubating fish eggs (USEPA 1976, Davies and Goettl 1977, DeNicol et al. 2002, McKnight et al. 1984, Vuori 1995, Linton et al. 2007). Precipitated iron also physically clogs fish gills and leads to gill damage that may cause respiratory impairment (Peuranen et al. 1994, Dalzell et al 1999).

The Colorado chronic iron table value standard is 1.0 mg/L (total recoverable). The value is primarily based on field observations that determined that trout and other fishes were not present in an iron-polluted Colorado stream until dilution or loss of iron from the water column resulted in a concentration less than 1.0 mg/L (USEPA 1976). A search of the literature found very few chronic toxicity tests have been conducted. There are insufficient data from a limited range of taxa to derive a water quality criterion using established guidelines (USEPA 1985). The objective of this study is to measure chronic toxicity of iron to early-life stage brown trout and mountain whitefish. It is expected that early-life stages will be most sensitive to iron. Specifically, iron precipitates are expected to coat and smother the incubation of eggs.

MATERIALS and METHODS

Test methods followed guidance provided by ASTM method E1241, *Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes* (ASTM 1997). Green (freshly fertilized) eggs were collected from wild spawning adults. Brown trout eggs were collected as part of annual Colorado Division of Wildlife spawning operations at North Delaney Buttes reservoir. Mountain whitefish adults in spawning condition were collected from Mad Creek, Colorado. Eggs were stripped and fertilized in the field, placed in a cooler, and transported to the Colorado Division of Wildlife Aquatic Toxicology Laboratory in Ft. Collins. Upon arrival at the laboratory, eggs were treated with 1600 ppm formalin for 15 minutes to control fungus (Piper et al. 1982).

A continuous-flow diluter (Benoit et al. 1982) constructed of teflon, polyethylene and polypropylene components delivered five exposure levels of iron hydroxide and an exposure control. Source water was dechlorinated Ft. Collins municipal tap water. Target iron concentrations were 4000, 2000, 1000, 500, and 0 µg/L. Iron stock solution was prepared by dissolving ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, Mallinkrodt analytical reagent grade) with

sufficient NaOH (1:3 stoichiometry) to neutralize acidic conditions caused by precipitation of ferric hydroxide. The stock solution was pumped to the diluter with a peristaltic pump at a rate of 2 mLs/min. A flow splitter allocated each iron concentration equally at 30 mLs/min to each of six replicate 7.5 L glass aquaria. Exposure solutions were delivered via food-grade vinyl tubing to egg incubation cups constructed of 1000 µm nylon screen affixed to PVC pipe segments (53 mm I.D. X 75 mm) with aquarium-grade silicone adhesive. Each incubation cup was suspended in a 7 L glass aquarium with a standpipe that allowed the exposure solution to overflow into a temperature-controlled water bath. Thirty eggs were distributed to each incubation cup. Treatments were arranged so that each species were exposed to three replicates of each iron concentration. Treatments were randomized in complete blocks. Ambient fluorescent light (16h:8h photoperiod) provided illumination. Diluter operation and toxicant flow rate were monitored daily. Temperature of dilution water and water bath was initially 7°C and then increased to 12°C after hatch of whitefish. Temperatures were adjusted from low during egg incubation to warmer post-hatch for three reasons: 1. Mountain whitefish eggs do not survive temperatures >8°C (Rajagopal 1979, Brinkman and Vieira 2009), 2. The egg stage was expected to be a sensitive life stage and lower incubation temperatures would extend exposure times, 3. lower temperatures during egg incubation is a more natural temperature regime for fall spawners such as mountain whitefish and brown trout.

Incubation cups were inspected daily for egg mortality and hatch. The first twelve brown trout eggs and first fifteen whitefish eggs to hatch were carefully transferred from the incubation cup to the aquarium using a glass tube. Remaining eggs in the incubation cups were monitored for hatching and removed once hatching was completed. Thus, hatching success for each species was based on thirty embryos in each incubation cup while fry survival and growth was based on twelve and fifteen fry transferred to the aquarium for brown trout and whitefish, respectively. After absorption of the yolk-sac, brown trout fry were fed starter trout chow (Rangen soft-moist) 5X/ day with an automatic feeder at rate 5% BW/day. Whitefish fry were fed <24 hour brine shrimp nauplii 3X/day (1X or 2X /day on weekends and holidays) at an estimated rate of 20% BW/day. Aquariums and diluter compartments were aerated to help keep iron precipitates suspended in the water column.

Water quality characteristics were measured weekly in all aquariums within a replicate. A different replicate was selected each week. Alkalinity was determined according to Standard Methods (APHA 1998). Dissolved oxygen and pH measured with electronic meter (Oakton Model 300) calibrated prior to each use. Conductivity was measured with an YSI model 35 conductance meter.

Water samples for iron analyses were collected weekly from each exposure level with surviving fry. Grab samples for total iron were collected in 2 oz HDPE bottles (Nalgene), immediately preserved with high purity nitric acid (JT Baker) to pH <2. Filtered samples for dissolved iron analyses were passed through a 0.45 µm filter (Acrodisc) and stored in trace metal grade 15 mL centrifuge tubes and preserved with high purity nitric acid (JT Baker) to pH <2. Iron concentrations were measured using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the

calibration curve verified through analyses of external quality assurance samples (High Purity). Sample splits and spikes were collected at each sampling event to verify analytical reproducibility and recovery.

Statistical Analysis

Statistical analyses of data were conducted using Toxstat version 3.5 software (West, Inc. 1996). Analysis of variance (ANOVA) was used to test toxicity endpoints which included hatching success, swim-up survival, mean weights of surviving fry, and biomass of surviving fish at test termination. Hatching success and survival data were arcsine square root transformed prior to ANOVA. Normality and homogeneity of variances were tested using Chi-square and Bartlett's test, respectively. Treatment means were compared to the control using William's one-tailed test (Williams 1971, Williams 1972). The highest measured iron concentration not associated with a treatment effect (e.g. decreased survival, decreased body weight) was designated as the no-observed-effect concentration (NOEC). The lowest measured iron concentration of cadmium associated with a treatment effect was designated as the lowest-observed-effect concentration (LOEC). Chronic values were calculated as the geometric mean of the LOEC and NOEC. The inhibition concentration (IC20), the concentration estimated to cause a 20% reduction in organism performance compared with the control (USEPA 1993), was calculated using the combined weight of surviving organisms from each treatment (biomass or standing crop).

RESULTS

Dissolution of ferric chloride and the subsequent precipitation of ferric hydroxide release acidic protons according to reaction:

$$FeCl_3 + 6H_2O \rightarrow Fe(OH)_3 + 3H^+ + Cl^-$$

Mixing a stock solution of ferric chloride with dilution water from the diluter would alter pH and alkalinity and confound interpretation of toxicity results. To prevent changes in pH and alkalinity, sodium hydroxide was added to the stock solution in a 3:1 stoichiometric ratio in order to neutralize the acid formed by the precipitation of ferric hydroxide. As a result, measured alkalinity and pH were similar among the iron exposure levels (Table 5). Neutralization with sodium hydroxide resulted in a slight increase in conductivity associated with iron exposure levels. Conductivity increased from 74.8 µS/cm in the control to 90.8 µS/cm in the highest iron concentration. Mean temperatures were 7.5-7.6°C during egg incubation and 12.2-12.4 °C post-hatch.

Measured iron concentrations were more variable than with soluble metals, such as cadmium, copper and zinc, due to the particulate nature of iron. The stock solution, diluter compartments and aquariums were aerated in an attempt to keep iron precipitates in the water column. Differences in aeration from one aquarium to another increased variation of measured iron concentrations in grab samples as did fish movements during sample collection which stirred up iron precipitates that settled in the bottom of the aquaria.

Significant adverse effects of iron on brown trout embryos, larvae and fry were not detected at iron concentrations used in the test (Table 6). Hatching success was 81% in the control exposure and ranged between 61-75% in the iron exposure treatments. Brown trout fry survival was good and >94% in all iron concentrations. The small reduction in brown trout fry growth and biomass at the highest iron concentration was not significant ($P>0.3$). A chronic Fe value for brown trout early-life stages could not be calculated but would exceed 5146 µg/L. An IC20 could not be calculated for brown trout.

Iron did not significantly affect mountain whitefish hatching or fry survival at iron concentrations ≤5146 µg/L, the highest concentration of the test (Table 7). Growth and biomass of mountain whitefish fry was significantly reduced in iron concentrations ≥1329µg/L. but not significantly reduced at an iron concentration 658µg/L. The lowest observed effect concentration (LOEC) and no observed concentration (NOEC) were 1329 and 658 µg/L, respectively. The chronic Fe value for mountain whitefish based on growth and biomass was 935 µg/L. The IC20 was 1550 µg/L.

DISCUSSION

Elevated iron concentrations from acid mine drainage are strongly associated with low pH in a phenomenon known as acid rock drainage (ARD) which has been called the greatest water quality issue in the Western United States (Mineral Policy Center 1997). Introducing iron and allowing the pH to change would have been a more environmentally realistic exposure scenario. However, it was decided in the present tests to neutralize the stock solution to isolate the effects of iron from the effect of lowered pH and any possible interaction.

Contrary to expectations, hatching of brown trout and mountain whitefish eggs were not significantly affected by iron precipitates at iron concentrations as high as 5000 µg/L. Iron precipitates reportedly smother incubating fish eggs (Smith 1973, USEPA 1976, Goettl and Davies 1977). Complete mortality occurred in rainbow trout eggs exposed to 2,200 and 3,400 µg/L during the egg and sac fry stage attributed to smothering of the eggs and coating of the gills after hatch (Goettl and Davies 1977). Smith et al. (1973) observed reduced hatching of fathead minnow eggs incubated in lime-neutralized iron hydroxide at 1500 µg/L, the lowest concentration tested. The reported impacts of iron on incubating fish eggs contrast with results from Smith et al. (1976) who did not observe any effect of lime-neutralized iron hydroxide on brook trout or coho salmon eggs at iron concentrations up to 12,000 µg/L.

In our tests, ferric precipitates were not lethal to incubating eggs or fry. However, we did observe a sublethal effect in the reduction of mountain whitefish fry growth. No significant effect of iron on growth of brown trout was detected. This is consistent with the notion that iron in oxygenated pH-neutral waters will exert an effect based on physical rather than chemical toxicity. Thus its effects are more likely to be chronic, sublethal and subtle. Examples of such effects include increased turbidity, reduced primary production, reduction of interstitial space in the benthic zones and smothering of bottom-dwelling invertebrates, plants and incubating fish eggs

(USEPA 1976, Davies and Goettl 1977, DeNicol et al. 2002, McKnight et al. 1984, Vuori 1995, Linton et al. 2007). Precipitated iron also physically clogs fish gills and leads to gill damage that may cause respiratory impairment (Peuranen et al. 1994, Dalzell et al 1999). Respiratory impairment can be expected to reduce the critical swimming speed of fish and should be the topic of additional research.

Table 5. Water quality characteristic of brown trout and mountain whitefish early-life stage toxicity tests in each of the iron exposure levels.

Target Iron Concentration	0	625	1250	2500	5000
Acid Soluble Fe concentration (µg/L)	<50 (14)	658 (133)	1329 (176)	2438 (225)	5146 (872)
Dissolved Fe Concentration (µg/L)	<50 (16)	<50 (35)	<50 (21)	<50 (18)	<50 (13)
Alkalinity (mg/L)	33.8 (1.1)	33.9 (0.9)	33.6 (1.2)	33.8 (1.1)	33.5 (1.3)
pH (SU)	7.48 (0.10)	7.50 (0.09)	7.47 (0.07)	7.49 (0.07)	7.49 (0.09)
Incubation Temperature (°C)	7.6 (0.2)	7.5 (0.2)	7.6 (0.2)	7.6 (0.3)	7.5 (0.2)
Post-hatch Temperature (°C)	12.3 (0.2	12.4 (0.2)	12.3 (0.2)	12.3 (0.2)	12.2 (0.2)
Conductivity (µS/cm)	74.8 (1.3)	76.3 (1.6)	79.1 (1.2)	81.7 (2.6)	90.8 (1.8)
Dissolved Oxygen (mg/L)	9.50 (0.42)	9.46 (0.52)	9.46 (0.48)	9.49 (0.49)	9.48 (0.49)

Table 6. Mean measured acid soluble Fe concentrations, dissolved iron concentrations and associated hatch of brown trout eggs. Standard deviations are in parentheses.

Target Iron Concentration	0	625	1250	2500	5000
Acid Soluble Fe concentration	<50 (14)	658 (133)	1329 (176)	2438 (225)	5146 (872)
Dissolved Fe Concentration (µg/L)	<50 (16)	<50 (35)	<50 (21)	<50 (18)	<50 (13)
Brown Trout hatch (%)	81.1 (9.6)	64.4 (8.4)	61.1 (12.6)	65.6 (9.6)	74.4 (8.4)
Brown Trout Fry Survival (%)	97.2 (4.8)	100 (0)	97.2 (4.8)	94.4 (9.6)	97.2 (4.8)
Brown trout weight at termination (g)	0.277 (0.017)	0.277 (0.167)	0.271 (0.0148)	0.285 (0.0135)	0.248 (0.042)
Brown Trout biomass at termination (g)	3.437 (0.288)	3.327 (0.200)	3.161 (0.307)	3.229 (0.344)	2.894 (0.380)

Table 7. Mean measured acid soluble Fe concentrations, dissolved iron concentrations and associated hatch of mountain whitefish eggs. Standard deviations are in parentheses.

Target Iron Concentration	0	625	1250	2500	5000
Acid Soluble Fe concentration	<50 (14)	658 (133)	1329 (176)	2438 (225)	5146 (872)
Dissolved Fe Concentration (µg/L)	<50 (16)	<50 (35)	<50 (21)	<50 (18)	<50 (13)
Mountain Whitefish Hatch (%)	86.7 (11.5)	85.6 (1.9)	90.0 (5.8)	82.2 (5.1)	88.9 (1.9)
Mountain Whitefish Fry Survival (%)	84.4 (7.7)	97.8 (3.8)	86.7 (6.7)	82.2 (15.4)	66.7 (17.6)
Mountain Whitefish Fry Weight (g)	0.159 (0.012)	0.141 (0.011)	0.118* (0.007)	0.124 * (0.012)	0.099 * (0.012)
Mountain Whitefish biomass at termination (g)	2.001 (0.056)	2.067 (0.172)	1.532* (0.093)	1.544* (0.451)	0.980* (0.252)

REFERENCES

APHA (1998) Standard Methods for the Examination of Water and Wastewater, 16th edn. American Public Health Association, American Water Works Association, and Water Pollution Control Federation. Washington, D.C.

Benoit, D.A., V.R. Mattson, and D.C. Olsen. 1982. A Continuous Flow Mini-diluter System for Toxicity Testing. Water Research. 16:457-464.

Brinkman S.F. and D.L. Hansen. 2007. Toxicity of cadmium to early life stages of brown trout (*Salmo trutta*) at multiple water hardness. Environmental Toxicology and Chemistry 26(8):1666-1671.

Brinkman, S.F. and N. Vieira. 2009. Federal Aid in Fish and Wildlife Restoration. Job Progress Report F-243R-16. Colorado Division of Wildlife, Fort Collins, CO, USA.

Chapman G.A. 1978. Toxicities of cadmium, copper, and zinc to four juvenile stages of Chinook salmon and steelhead. Trans Am Fish Soc 107:841-847.

CHPHE (2010) Colorado Department of Public Health and Environment. 2010 Status of water quality in Colorado – 2010. The update to the 2008 305(b) report.

Davies, P. H., S. F. Brinkman and D. L. Hansen. 2002. Federal Aid in Fish and Wildlife Restoration. Job Progress Report F-243R-9. Colorado Division of Wildlife, Fort Collins, CO, USA.

Goettl J.P. and P.H. Davies. 1977. Federal Aid in Fish and Wildlife Restoration. Job Progress Report F-33-R-12. Colorado Division of Wildlife, Fort Collins, CO, USA.

Mineral Policy Center (1997). Golden dreams, poisoned streams. Washington, D.C., USA.

Piper, R.G., I.B. McElwain, L.E. Orme, J.P. McCraren, L.G. Fowler, and J.R. Leonard. 1982. *Fish Hatchery Management.* U.S. Fish and Wildlife Service, Department of the Interior, Washington, D.C.

Rajagopal, P.K. 1979. The embryonic development and the thermal effects on the development of the mountain whitefish, *Prospium williamsoni* (Girard). Journal of Fish Biology 15:153-158.

Smith E.J., J.L. Sykora. 1976 Developmental effects of lime-neutralized iron hydroxide suspensions on brook trout and coho salmon. Transactions of the American Fisheries Society 2:308-312.

Smith E.J., J.L. Sykora, and M.A. Shapiro. 1973 Effect of neutralized iron hydroxide suspensions on survival, growth and reproduction of the fathead minnow (*Pimephales promelas*). Journal of the Fisheries Research Board of Canada 30 (8): 1147-1160.

USEPA 1976. Quality Criteria for Water. PB263-943.

USEPA. 1985. Guidelines for deriving numerical standards for the protection of aquatic organisms and their uses. PB85-227049. Washington, DC USA.

USEPA. 1993. A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (Icp) Approach (Version 2.0). Norberg-King, T.J. NETAC Technical Report 03-93. U.S. Environmental Protection Agency, Duluth, MN.

West Inc. 1996. Toxstat Version 3.5. Westen EcoSytems Technology, Cheyenne WY.

Effect of Temperature on Hatch, Survival and Growth of Rio Grande Cutthroat Trout (*Oncorhynchus clarki virginalis*)

INTRODUCTION

Temperature fundamentally affects distribution of fishes. States develop temperature standards based on studies that evaluate negative effects of temperatures on growth and survival outside of species' optimal temperature range. Colorado's cold water temperature standards were developed based on protection of economically and recreationally important cutthroat trout using temperature tolerance data from Bonneville, Lahontan, Snake River, Westslope, and Yellowstone subspecies (Todd et al. 2008). Cutthroat trout subspecies can vary in their responses to temperature (Vigg and Koch 1980, Wagner et al. 2001, Myrick 2008). Temperatures for optimum growth rates for cutthroat trout subspecies are reported as 13.6°C for Westslope (Bear et al. 2007), 14.7°C for Yellowstone ((Myrick 2008), 14.5°C for Snake River (Myrick 2008), and 15.3-16.4°C for Colorado River (Brandt 2009). An apparent trend is for optimum growth temperature to increase as distribution moves south. Rio Grande cutthroat has the southerly-most distribution of cutthroat trout subspecies (Behnke 1992). If the trend of increasing optimum growth temperature with southerly distribution is valid, Rio Grande cutthroat trout may be expected to have a higher temperature threshold than northerly subspecies. An understanding of temperature tolerances is important for management, and selection of sites for reintroduction as part of a recovery program.

A series of tests were conducted to measure effect of temperature on Rio Grande cutthroat trout eggs and fry. An egg incubation test investigated effect of temperature on hatching success. This was followed with a test to determine the effect of constant temperatures on Rio Grande cutthroat trout fry growth and survival. Optimum temperature for growth and the upper incipient lethal temperature (UUILT) were determined using the Acclimated Chronic Exposure (ACE) methodology (Selong et al. 2001, Bear et al. 2007). ACE measures effects of temperature on growth and survival over a long time period (60d) thus providing a more sensitive measure of temperature effects than most shorter-term experiments (Selong et al. 2001, Bear et al. 2007). An additional experiment was conducted to measure the effect of small and large diel temperature cycles on fry growth and survival.

METHODS

Eggs were collected from mature ripe Rio Grande cutthroat from Haypress Lake (Mineral County CO, USA), fertilized and water hardened in the field as part of Colorado Division of Wildlife annual egg collection operations. Eggs were transported in a 7.5 L water cooler to the Colorado Division of Wildlife Aquatic Toxicology Laboratory in Fort Collins (approximately 5 hours). Upon arrival in the lab, eggs were treated with 1600 ppm formalin for fifteen minutes (Piper et al. 1982). An egg incubation temperature test was started with twenty eggs randomly distributed to incubation cups constructed from 1000 micron mesh nylon screen affixed to PVC pipe sections with aquarium-grade silicone adhesive. Incubation cups were suspended in 7.6 L

glass aquaria and received 40 mls/min dechlorinated Ft Collins municipal tap water from an aerated temperature-controlled head tank. Water temperatures in each of eight head tanks were controlled using temperature programmers (Series 16B, Love Controls, Michigan City IN) and aquarium heaters and delivered water flow to three replicate aquaria. The temperature in all aquaria when the eggs were allocated was initially 12°C and then adjusted to target temperature over a 24 hr period. Target temperatures of the eight head tanks were set at 6-20°C at 2°C intervals. Aquaria were randomized in a block design. Temperature data loggers (Onset, Bourne MA) in each egg incubation cup recorded water temperatures at 1 hour intervals. Egg mortality and hatching were monitored and recorded daily.

Remaining eggs were incubated for later use in fry growth and survival studies. Eggs were placed in incubation trays that received dechlorinated Ft Collins municipal tap water (average temperature 13°C). Eggs eyed up after 13 days, hatched 7 days later and fry resorbed the yolk sac (swimup) 14 days after hatch. Fry were fed starter trout chow supplemented with live <24hr brine shrimp nauplii (Argent Chemical Laboratories, Redmond WA).

The effect of temperature on fry growth and survival was measured using the Acclimated Chronic Exposure (ACE) method (Selong et al. 2001, Bear et al. 2007). Twenty fry, (14d post swim-up, mean weight 0.184g), were randomly distributed into 30 7.6 L glass aquaria which received 50 mls/min of water flow from aerated temperature-controlled head tanks. Each of ten head tank temperatures was controlled using temperature programmers and aquarium heaters and delivered flow to three replicate aquaria. The temperatures of the head tanks were all initially set at 14°C then adjusted to target temperatures of at a rate of 1°C/day. Target temperatures spanned 10-28°C with 2°C intervals. Temperature adjustments were staggered so that all treatments achieved the target temperature on the same day. Temperature data loggers recorded water temperatures at 1 hour intervals in each aquarium. Five fry were subsampled after the tanks had attained the target temperature (day 0) and again at 20, 40, and 60 days. Subsampled fry were terminally anesthetized with MS222, blotted dry with a paper towel and weighed to 0.001g. The number of fry subsampled from each tank was adjusted as necessary for mortality so that the same number of fry remained in each aquarium after each subsampling event. Fry were fed soft-moist trout starter (Rangen, Buhl ID) five times per day using automatic feeders (Fishmate). Trout chow was supplemented with live <24hr brine shrimp naupalii (Argent Chemical Laboratories, Redmond WA). Presence of excess uneaten food was noted daily and feeding rates adjusted to ensure fry were fed in excess of satiation. Aquaria were cleaned daily with a siphon to remove feces and uneaten food.

The effects of diel temperature fluctuations were assessed by subjecting fry to three temperature regimes: constant temperature (20°C), small daily fluctuation (18-22°C) and large daily fluctuation (15-25°C). The minimum and maximum of each cycle occurred at approximately 06:00 and 18:00 each day, respectively. Temperatures in each of three head tanks were controlled using temperature programmers and aquarium heaters. Each head tank delivered 90 mLs/min to three replicate 19 L aquaria. Fry were 110 days post-hatch at the start of the test and had been acclimated to 20°C for 7 days. Twenty one fry were randomly distributed when aquaria temperatures were 20°C (+- 0.9°C) and on the rising limb of the temperature cycle. Fry were fed and aquaria cleaned as described above. Seven fry were subsampled from each

aquarium after 10, 20, and 30 days. The number of fry subsampled from each tank was adjusted for mortality. Subsampled fry were terminally anesthetized with MS222, blotted dry with a paper towel and weighed to 0.001g. Loggers recorded water temperatures at 10 minute intervals in each aquarium.

Fry and egg mortality data and hatch rates were arc-sine squareroot transformed and analyzed by ANOVA. Mortality rates at the different temperatures were compared using Ryan's Q means test (α=0.05). Growth rate (mg/fish/d) for each aquarium was determined from linear regression of mean fry weight as a function of time. Growth rates were plotted against mean measured temperatures and a second order polynomial regression line fitted to the data. The ultimate upper incipient lethal temperature (UUILT) was calculated based on the estimated median lethal temperature (LT50) using the trimmed Spearman-Karber method (Hamilton et al. 1977, 1978).

RESULTS

The temperature programmer for the 28°C head tank failed during the acclimation phase of the constant temperature fry growth test and was discarded as a treatment. Otherwise, no other complications occurred. Temperatures were stable during the egg incubation and fry growth tests. The standard deviation of temperatures within each aquarium averaged 0.27°C (range 0.05-1.19°C). The average daily temperature fluctuation of aquaria was 0.33°C (range 0.13-0.94°C). The diel temperatures exhibited a consistent cycle (Figure 2). Average temperatures were 19.80°C for the constant temperature, 19.83°C for the small cycle temperature regime and 19.80°C for the large cycle temperature regime. Average daily temperature range was 17.91-21.78°C for the small cycle and 14.64-24.94°C for the large cycle.

Hatch success was 46-70% in the temperature range 6-16°C but was significantly reduced at 18°C and 20°C (Figure 2). A single egg out of 60 hatched at 20°C but the sac fry died the following day.

In the constant-temperature fry test, mortality was minimal (≤5%) during the temperature acclimation period and was not different among temperatures. Survival was high (87-100%) at temperatures 10-22°C after 60 days (Figure 3). All fry died at temperatures ≥24°C. Temperature-related mortality occurred shortly after reaching target temperatures (Figure 4). All fry died within 5 days after reaching 26°C and within 15 days at 24°C. The ultimate upper incipient lethal concentration (UUILT) rapidly decreased initially with time before leveling off at a constant value (Figure 4). The UUILT decreased from 25.69 on day three, to 24.65 C on day seven, to 22.60 on day fifteen. No other mortality occurred after fifteen days and the UUILT remained unchanged until the end of the test on day 60. Though survival was not affected at 22°C, severe scoliosis was observed in about 50% of subsampled fry after 40 days which increased to about 75% of fry after 60 days (Figure 5).

Fry growth was linear over the 60 days at constant temperature. Coefficient of determination for change in mean weight with time in individual aquariums ranged from 0.912 to 0.999. Growth rates increased with temperature from 10°C to 15°C then declined at temperatures > 15°C (Figure 6). Growth rates ranged from a low of 5.3 mg/d at 21.9°C to a high of 43 mg/d at 15.0°C. Estimated temperature for optimum growth of Rio Grande cutthroat fry is 15.33°C.

No significant difference in fry survival in the cyclical temperature test was detected. Fry survival was 92%, 97%, and 100% in the constant, small cycle and large cycle temperature regimes, respectively. Average growth rates of fry in the cyclical temperature test were 60.5, 58.5 and 43.9 mg/d in the constant, small cycle and large cycle temperature regimes, respectively. Growth rates of fry were not statistically different among the different temperature regimes ($p>0.28$).

DISCUSSION

The UUILT determinations in fishes have been traditionally based on 7-d test periods (e.g., Brett 1952; Dickerson and Vinyard 1999; Johnstone and Rahel 2003). The 7-day UUILT for Rio Grande cutthroat trout fry in the present study was 22.60°C. This value is in the range of published lethal thermal limits for other strains of cutthroat trout which include 22.3-22.6°C for Lahontan cutthroat trout (in water similar to that used in the present study) (Vigg and Koch 1980), 24.2°C for Bonneville cutthroat trout (Johnstone and Rahel 2003) and 24.1°C for westslope cutthroat trout (Bear et al. 2007). Seasonally high temperatures typically last for longer than 7 days. Longer term temperature experiments may be needed to detect chronic effects of elevated temperatures that are not detected by short-term exposures (Bear et al. 2007). For example, the 30-d UUILT for westslope cutthroat trout was 4.5°C lower at 30 days compared to 7 days (Bear et al. 2007).

Extending the duration of temperature tests also enables detection of sublethal responses. We observed high incidence of scoliosis in fry at 22°C after 40 and 60 days. Spinal deformities in laboratory-raised fishes have previously been associated with elevated rearing temperatures (Fitzsimmons and Perutz 2006, Schultz and Bonar 2009).

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REFERENCES

Bear E.A., T.E. McMahon, and A.V. Zale. 2007. Comparative thermal requirements of westslope cutthroat trout and rainbow trout: Implications for species interactions and development of thermal protection standards. Transactions of the American Fisheries Society 136:1113-1121.

Brandt, M.M. 2009. Optimal starter diets and culture conditions for Colorado River cutthroat trout (*Oncorhyncus clarkii pleuriticus*). Masters' Thesis. Colorado State University.

Brett, J. R. 1952. Temperature tolerance in young Pacific salmon, genus Oncorhynchus. Journal of the Fisheries Research Board of Canada 9:265–322.

Fitzsimmons , S.D. and M. Perutz. 2006. Effects of egg incubation temperature on survival, prevalence and types of malformations in vertebral column of Atlantic cod (*Gadus morhue*) larvae. Bull. Eur. Ass. Fish Pathol. 26(2):80-86.

Hamilton M.A, R.C. Russo, R.V. Thurston (1977) Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11(7): 714-719.

Hamilton M.A, R.C. Russo, R.V. Thurston (1978) Correction. Environ. Sci. Technol. 12:417.

Johnstone, H.C. and F.J. Rahel. 2003. Assessing temperature tolerance of Bonneville cutthroat trout based on constant and cycling thermal regimes. Transactions of American Fisheries Society 132:92-99.

Myrick, C.A. 2008. Development and evaluation of starter diets and culture conditions for 3 subspecies of cutthroat trout and Gila trout. Western Regional Aquaculture Center Annual Progress Report, Colorado State University, Fort Collins Colorado.

Piper, R.G., I.B. McElwain, L.E. Orme, J.P. McCraren, L.G. Fowler, and J.R. Leonard. 1982. *Fish Hatchery Management.* U.S. Fish and Wildlife Service, Department of the Interior, Washington, D.C.

Selong, J. H., T. E. McMahon, A. V. Zale, and F. T. Barrows. 2001. Effect of temperature on growth and survival of bull trout, with application of an improved method for determining thermal tolerance in fishes. Transactions of the American Fisheries Society 130:1026–1037.

Schultz A.A., and S.A. Bonar. 2009. Growth and survival of larval and juvenile gila chub at different temperatures. North American Journal of Aquaculture 71:1-5.

Stonecypher R.W., W.A. Hubert, W.A. Gern. 1994. Effect of reduced incubation temperatures on survival of trout embryos. Progressive Fish Culturist 56: 180-184.

Todd, A.S., M.A. Coleman, A.M. Konawal, M.K. May, S. Johnson, N.K.M. Vieira and J.F. Saunders. 2008. Development of new water temperature criteria to protect Colorado's fisheries. Fisheries 33(9):433-433

Vigg S.C. and D.L. Koch 1980. Upper lethal temperature range of Lahontan cutthroat trout in waters of different ionic concentration. Transactions of the American Fisheries Society 109:339-339.

Wangila, B.C.C., T.A. Dick. 1988. Influence of genotype and temperature on the relationship between specific growth rate and size of rainbow trout. Transactions of the American Fisheries Society 117:560-564.

Zale, A. V. 1984. Applied aspects of the thermal biology, ecology, and life history of the blue tilapia *Tilapia aureau* (Pisces: Cichlidae). Doctoral dissertation. University of Florida, Gainesville.

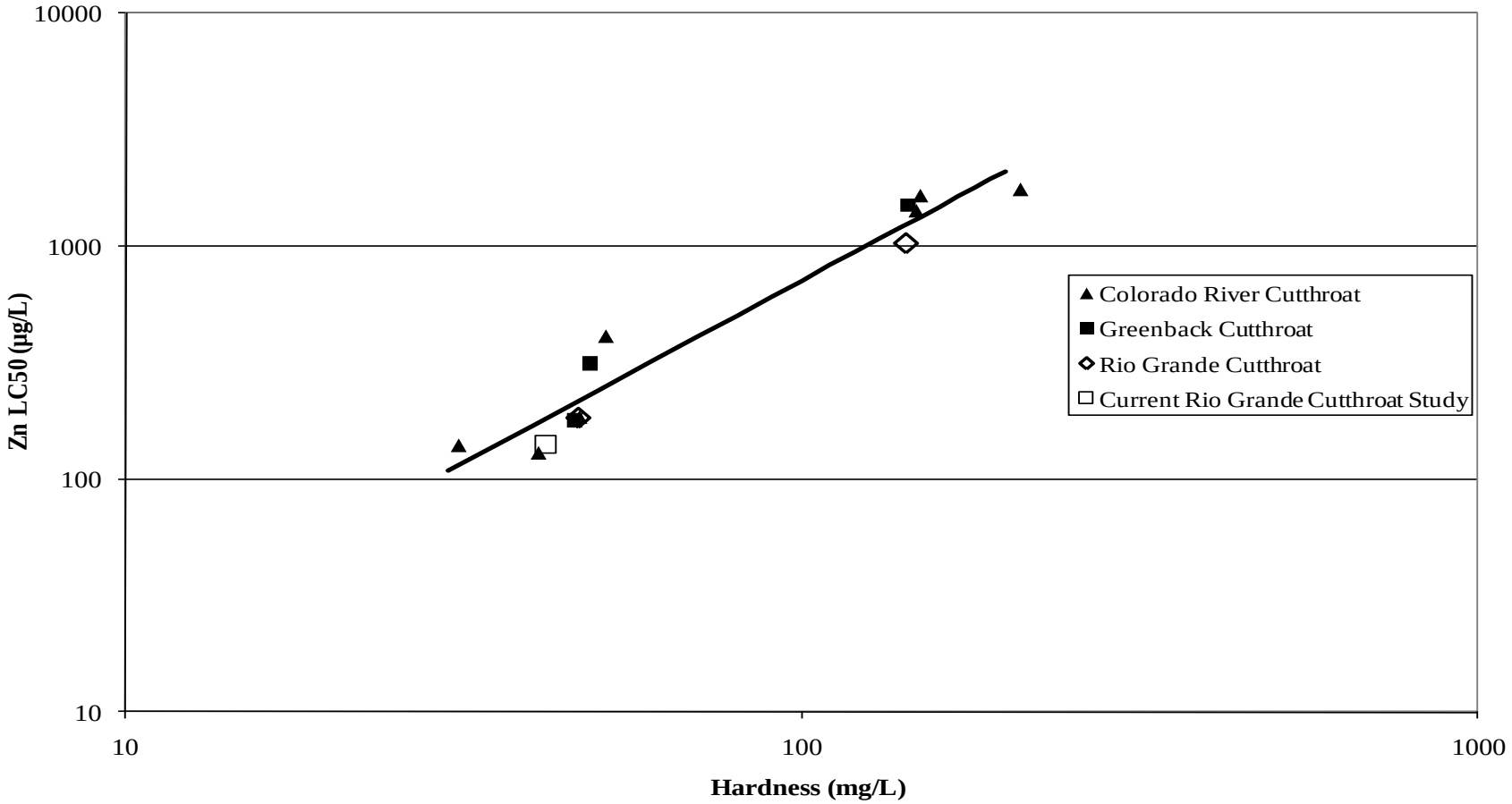

Figure 1. 96 hour zinc median lethal concentrations (LC50) (µg/L) at different water hardnesses (mg/L) for Colorado River, Greenback and Rio Grande cutthroat trout. Data are compiled from Davies et al. 2000, Brinkman and Hansen 2004, Brinkman and Vieira 2008, Brinkman and Vieira 2010.

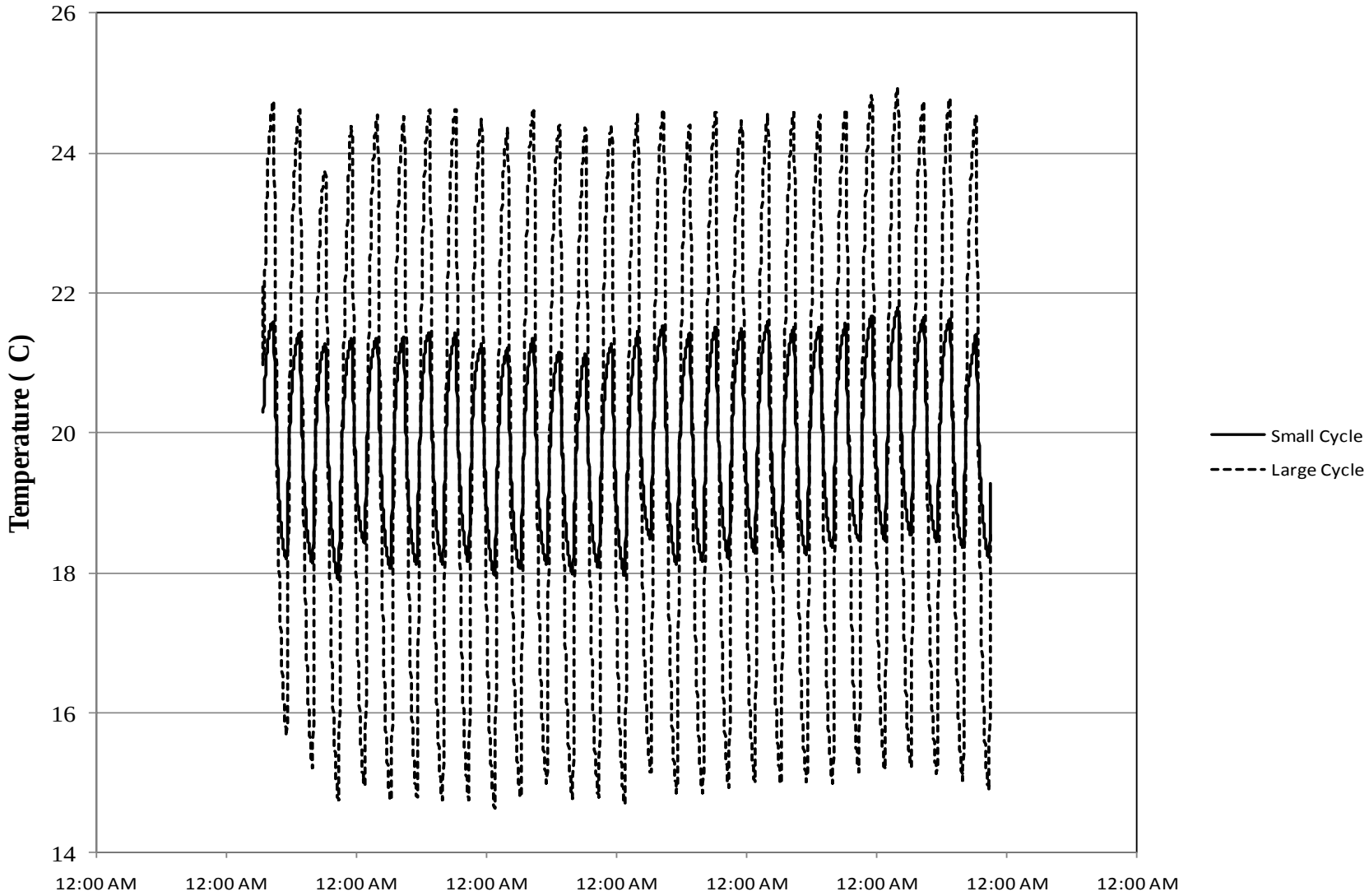

Figure 2. Mean hourly temperatures (°C) in aquariums containing Rio Grande cutthroat fry subjected to large and small temperature cycles.

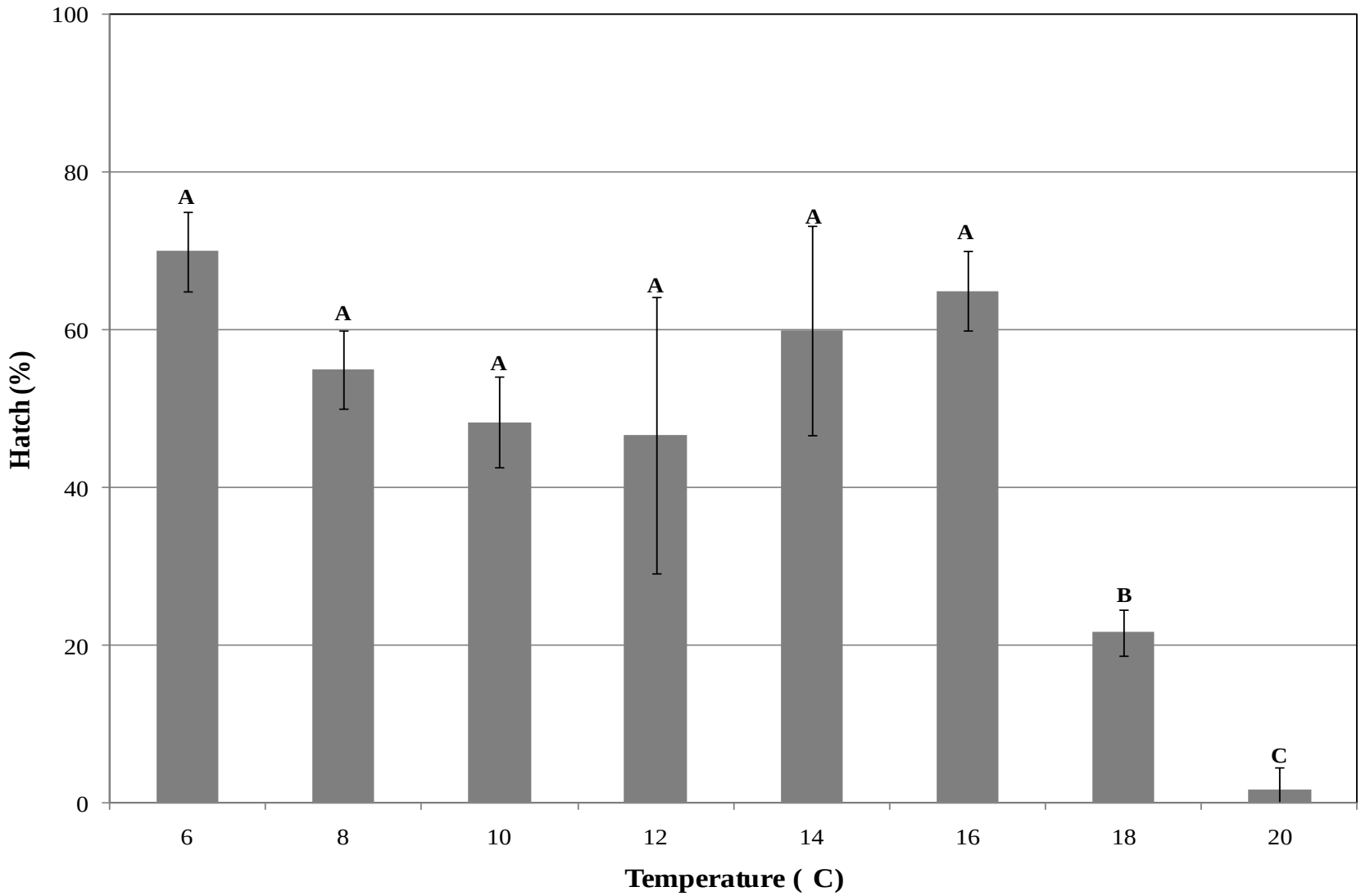

Figure 3. Hatch success (%) of Rio Grande cutthroat trout eggs incubated at temperatures from 6-20ºC. Error bars represent standard deviation of replicate aquaria. Different letters denote significant difference ($p<0.05$)

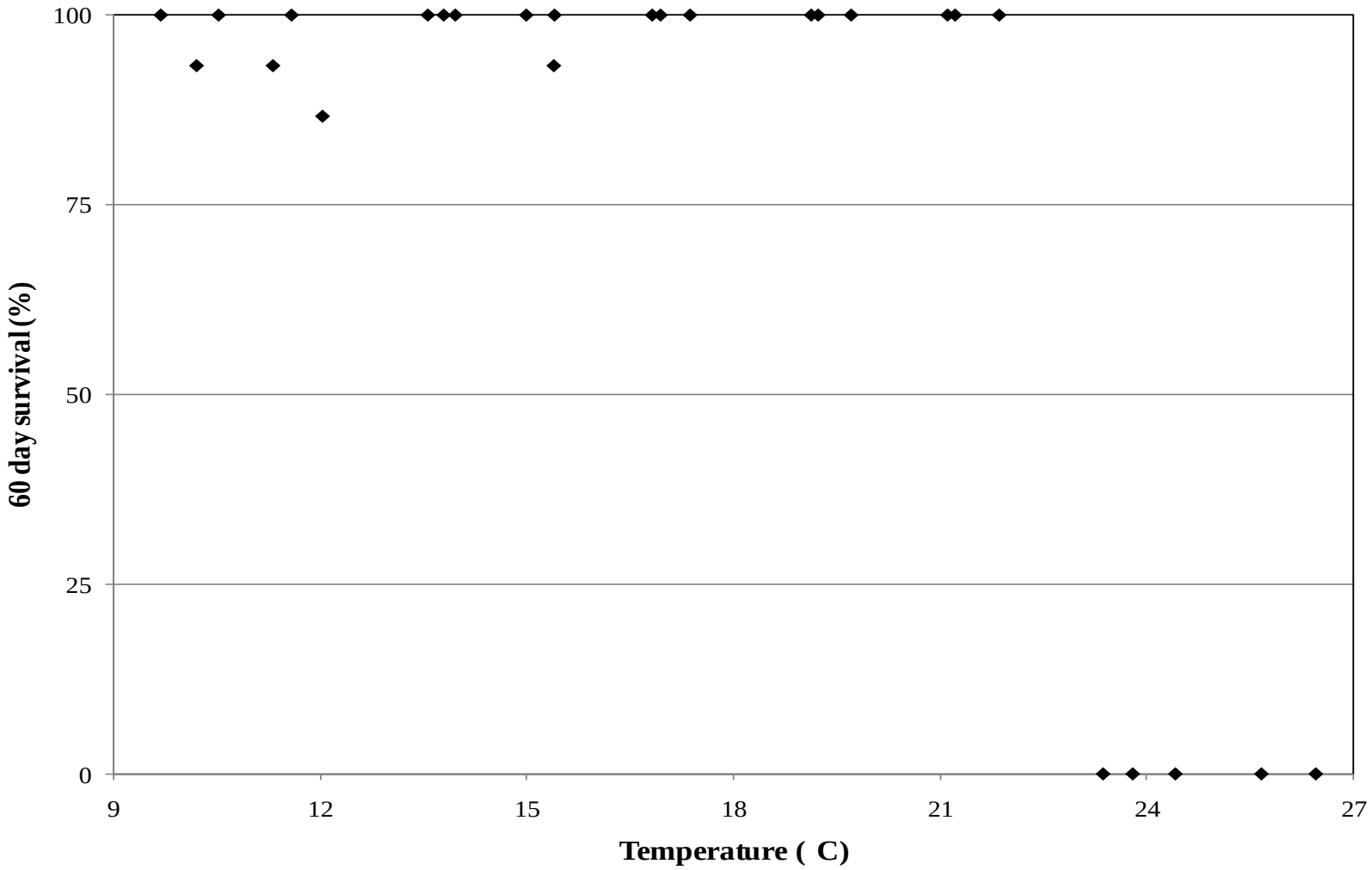

Figure 4. Survival of Rio Grande cutthroat fry held at constant temperatures for 60 days.

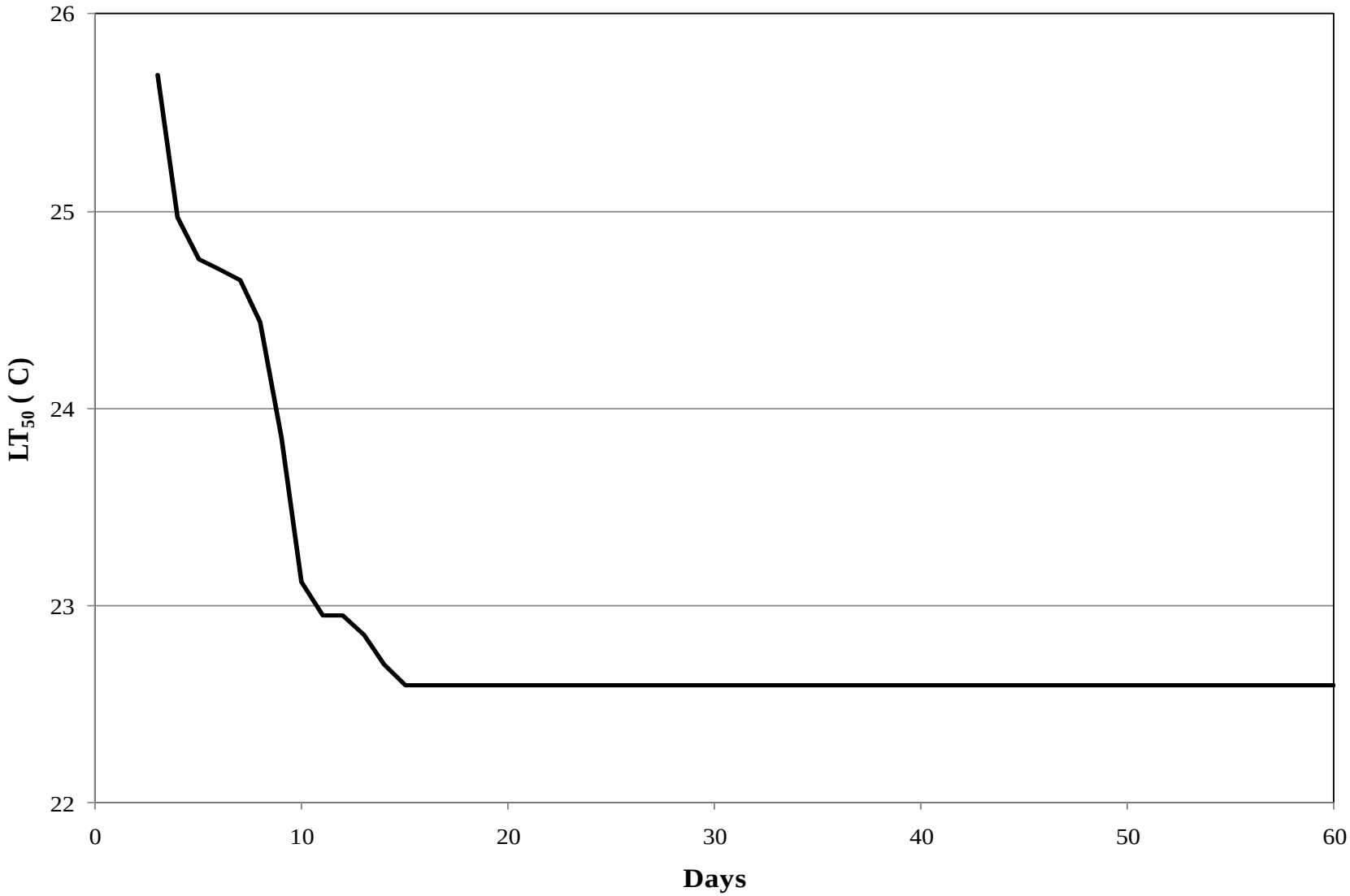

Figure 5. Median lethal temperature (LT50) of Rio Grande cutthroat trout held at constant temperatures over course of 60 days.

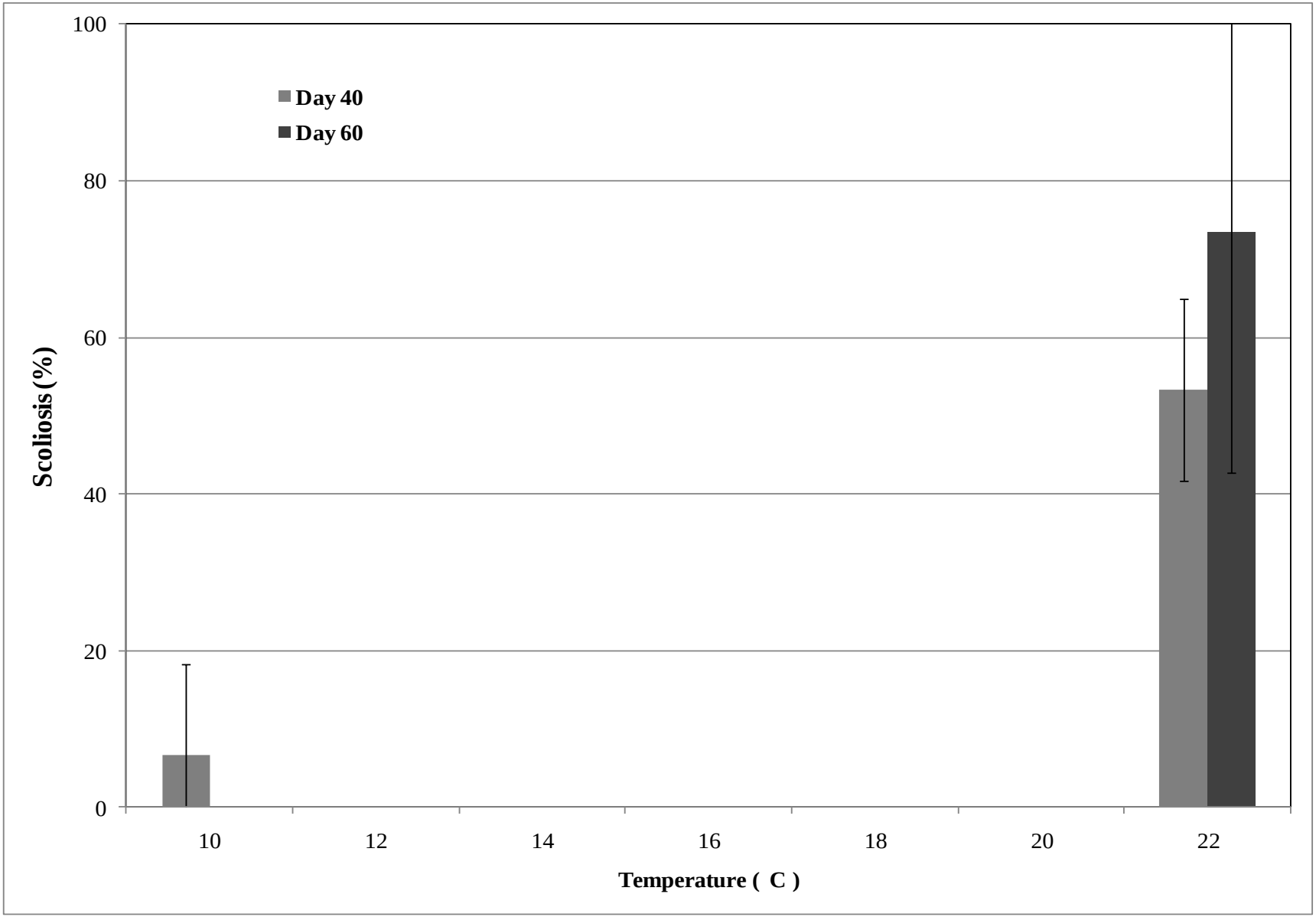

Figure 6. Incidence of scoliosis in Rio Grande cutthroat fry after 40 and 60 days at held at constant temperatures from 10-22°C

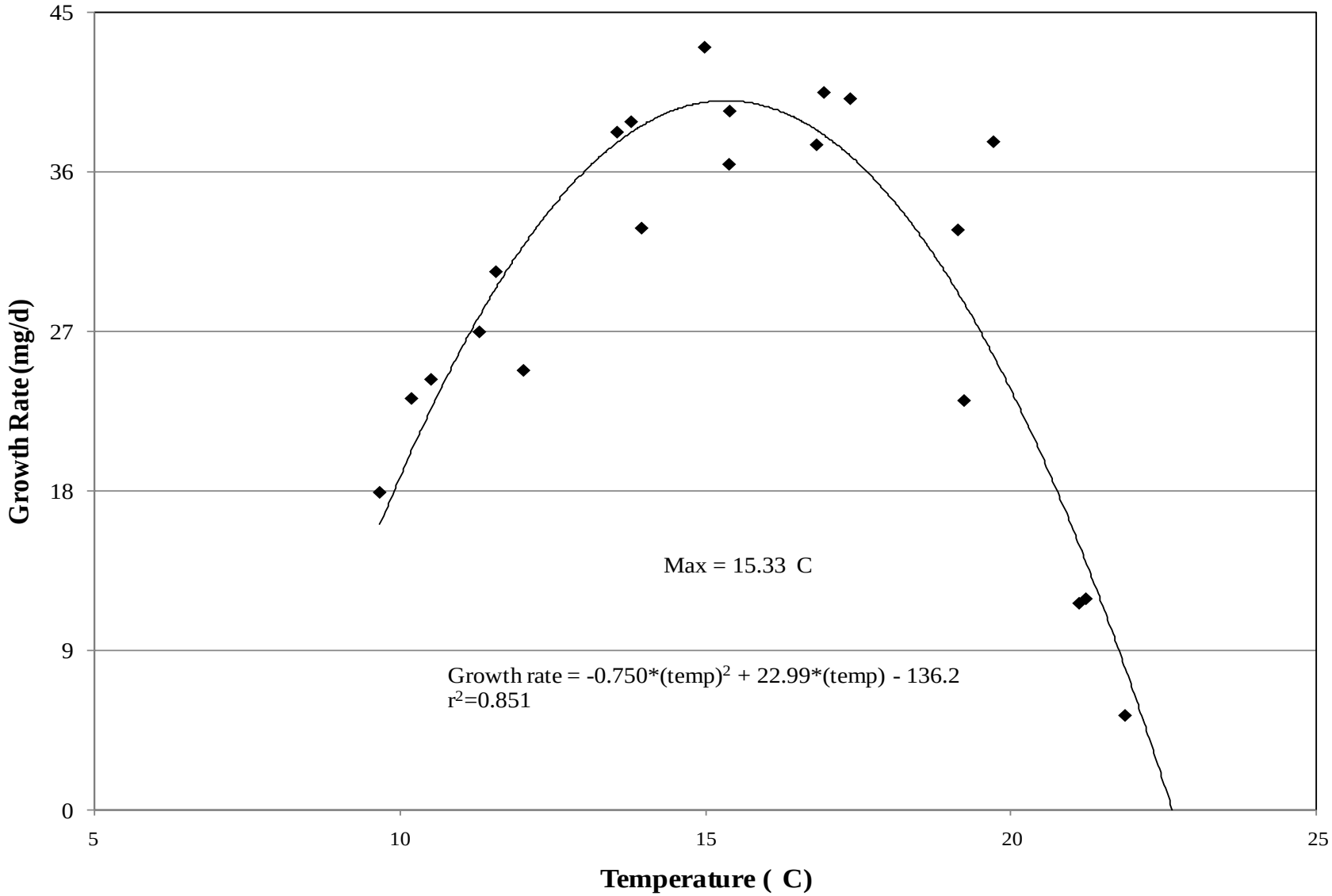

Figure 7. Growth rates of Rio Grande cutthroat fry (mg/d) at different temperatures for 60 days.