

**Biological Assessment of Green Sturgeon in the Sacramento-San  
Joaquin Watershed, Phases 3-4**

**Anadromous Fish Recovery Program, Agreement # 11332-1-G005**

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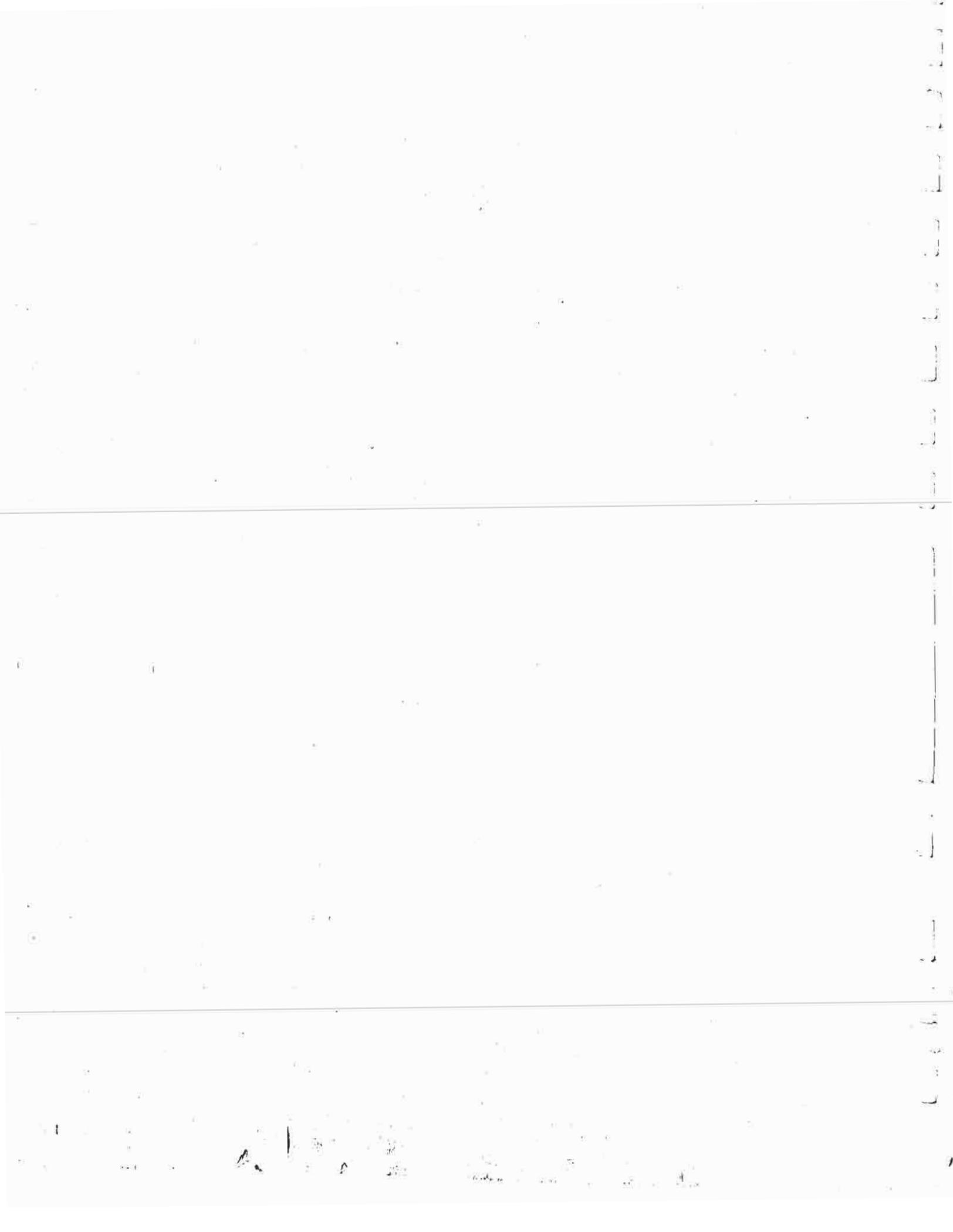
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**Final Report**

June, 2004



**Biological Assessment of Green Sturgeon in the Sacramento-San Joaquin  
Watershed, Phases 3-4**

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**Task 1, Green Sturgeon Environmental Tolerance Limits and Behavioral Tendencies,  
Stress Responses and Swimming Performance**

Joseph J. Cech, Jr., Ryan B. Mayfield, Scott E. Lankford, Peter J. Allen

The objectives of Task 2 were to: 1. elucidate the responses, preferences, and tolerance limits of juvenile green sturgeon (GS) to temperature, salinity, and water velocity; and 2. characterize GS stress responses and effects of stress. This report includes results of experiments on these two objectives plus information on the acid-base responses of GS associated with metabolic loading (i.e., with metabolic and other acids, simulating severe, exercise-related stress). The Yurok Tribe's Fishery Program (led by Dave Hillemeier and Mike Belchick) assisted in broodstock capture for captive breeding and Prof. S. Doroshov's laboratory group (J. Van Eenennaam, and J. Linares-Casenave) at UC Davis provided newly hatched GS for our studies. Overall, work was conducted in collaboration with three other laboratories at UC Davis (S. Doroshov, T. Adams, I. Werner), one at San Francisco State University (C. Crocker), and the California Department of Fish and Game Bay-Delta Laboratory (N. Kogut).

**Responses, Preferences, and Tolerance Limits of Juvenile GS to Temperature and Salinity**

Physiological ecology experiments showed that temperature is a significant factor on GS bioenergetic variables. The M.S. thesis work of Ryan Mayfield showed that food consumption rates, growth rates, and food conversion efficiencies all increased when temperature increased from 11 to 15°C at both 50% and 100% rations. However, these variables did not increase as temperature increased further to 19°C. In contrast, metabolic (oxygen consumption) rates increased mainly as temperature increased from 19 to 24°C. GS activity and gill ventilatory frequency increased with each temperature increase, over the 11 - 24°C range. Fish acclimated to 24°C preferred significantly higher temperatures, compared with those acclimated to lower temperatures, whereas yearling GS swimming performance significantly decreased at 24°C, compared with the lower temperatures. Overall, temperature-related increases in food consumption rate were allocated more to growth at temperatures <15°C and proportionately more to maintenance metabolism at temperatures >15°C, while volitional activity increased throughout the temperature range. These results are now in press in *Transactions of the American Fisheries Society* in a paper entitled, "Temperature Effects on Green Sturgeon Bioenergetics" (Mayfield and Cech 2004). Mr. Mayfield completed his M.S. requirements in Ecology at UC Davis and is employed as a Fisheries Biologist, currently, with the California Department of Fish and Game, Bay-Delta Laboratory in Stockton. In addition, a paper entitled "Routine Metabolism of Larval Green Sturgeon (*Acipenser medirostris* Ayres)," by Gisbert, Cech, and Doroshov, was published in *Fish Physiology and Biochemistry*, vol 25, pp.195-200.

Subsequent work on GS growth was conducted at higher temperatures ( $>19^{\circ}\text{C}$ ), as part of Mr. Peter Allen's dissertation requirements at UC Davis. Although initial studies showed that growth decreased at  $24^{\circ}\text{C}$ , compared with the fish at  $19^{\circ}\text{C}$ , these results may have been affected by confinement stress. In this experiment, individual sturgeon were confined in plastic containers containing continuous water flows and gentle aeration. This design was necessary, unfortunately, due to a recent fire in one of our CABA (Center for Aquatic Biology and Aquaculture) laboratory buildings. In a subsequent experiment (in the renovated laboratory building), both GS reared at  $24^{\circ}\text{C}$  and those reared at a cycling (between  $19^{\circ}\text{C}$  at 05:00 and  $24^{\circ}\text{C}$  at 17:00) temperature regime grew faster than those reared at  $19^{\circ}\text{C}$ . These fish were reared in groups of 20 fish in 50-liter, flow-through tanks with aeration. These results were presented at the 2003 Annual Meeting of the American Fisheries Society (Quebec City), and are being prepared for publication.

Mr. Allen also conducted studies on the outmigration of age-0, juvenile GS, including their tolerance for hypertonic environments (salinity tolerance experiments), seawater adaptation (including the sampling of GS for related hormones [cortisol, thyroid hormones] and enzymes [ $\text{Na}^+$ ,  $\text{K}^+$ -ATPase]), flow preference, and swimming performance. Experiments were designed to map changes coincident with development of these young GS. Fish gradually increase their salinity tolerance with increased body size and age. Short-term (72 h, at UC Davis) and long-term (28 d at the UC Bodega Marine Laboratory) GS seawater exposures were achieved (with 100% survival) at ca. 30 cm total length (TL) and at age  $< 7$  mo. GS plasma samples are being analyzed for electrolytes, osmolality, and hormones levels, while GS gill and gut tissues samples are being analyzed for  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase enzyme activity. Also, preliminary studies of fixed gill tissue samples, to track chloride cell location and abundance (density) from both freshwater-acclimated GS and from GS subjected to short-term and long-term salinity exposures, are ongoing. A poster on these preliminary findings was presented at the 2003 Annual Meeting of the American Fisheries Society (Quebec City), and a manuscript is being prepared for publication.

Experiments on salinity acclimation and metabolism of juvenile GS were completed to measure the metabolic "cost" of the juvenile GS inhabiting marine environments. Metabolic rates, including salinity acclimation at different salinity levels, were determined by measuring oxygen consumption rates (flow-through respirometry) in freshwater, brackish, and seawater salinities at UC Davis and at the UC Bodega Marine Laboratory. Measurements were followed by taking plasma and tissue samples to assess osmoregulatory abilities of these age-0 and age-1 GS. Analyses of the oxygen consumption rate and plasma electrolytes data have been completed and analysis of tissue  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase samples started. Four students helped us in this project: Brandon Jang, an accelerated high school student as a part of the UC Davis Young Scholars Program; Norm Ponferrada, a student at Modesto Junior College participating in the UC Bridges Program; and Stephanie Cole and Mary Nicholl, both undergraduates at UC Davis. A manuscript describing these findings is being prepared for publication.

Peter Allen, along with UC Davis undergraduate student, Brian Hodge, measured the swimming performance of juvenile GS throughout their seawater-tolerance,

developmental period. They placed individual GS into a 15-cm diameter, recirculating swim tunnel and started their swimming at a low speed ( $15 \text{ cm s}^{-1}$ ). The water velocity was increased by  $10 \text{ cm s}^{-1}$  every 20 min, until the fish had fatigued (swept against back screen and unable to stay off of screen). The critical swimming velocity ( $U_{\text{crit}}$ ) was determined by the length of time swum at the fatigue velocity. Interestingly, GS  $U_{\text{crit}}$  increased with size (and age) as they (simultaneously) developed 100% seawater tolerance. In contrast, once they reached the size (i.e., 30 cm TL) at which 100% seawater tolerance was achieved,  $U_{\text{crit}}$  decreased with increasing GS size. This pattern is strikingly similar to that of many juvenile salmonid fishes, a group separated from the sturgeons by ca.  $2 \times 10^8$  years of evolutionary history, during their parr-smolt transformation. These results will be presented at the 2004 Annual Meeting of the American Fisheries Society (Madison), and a manuscript is being prepared for publication. Mr. Allen will complete his Ph.D. requirements in 2005.

### **GS Stress Responses and Effects of Stress on Juvenile GS**

Dr. Scott Lankford, who completed his Ph.D. requirements in Molecular, Cellular, and Integrative Physiology at UC Davis in 2004, studied GS' stress responses and effects of stress. We completed an interesting study, with Prof. Tom Adams, on the influences of time of day and temperature on the modification of the physiological stress response in GS. These studies showed that GS exhibited greater stress responses (i.e., plasma cortisol and lactate concentrations) during nocturnal, as compared with diurnal, hours. Also, increased temperatures ( $19^\circ\text{C}$ ) accelerated these stress responses, compared with those at  $11^\circ\text{C}$ . These results were presented at the International Congress on the Biology of Fishes meeting (Vancouver, B.C., Canada), and at the American Physiological Society meeting (San Diego) in 2002. These results are now published in *Comparative Biochemistry and Physiology* in a paper entitled, "Time of Day and Water Temperature Modify the Physiological Stress Response in Green Sturgeon, *Acipenser medirostris*" (Lankford et al. 2003).

Another of Dr. Lankford's dissertation chapters concerned effects of chronic stress on GS. Collaborating with Prof. Adams and (undergraduate student) Ryan Miller, we exposed yearling GS to 2 of 3 stressors  $\text{d}^{-1}$  for 28 days to simulate chronic stress conditions. The 3 stresses were: reduction of tank water level to the dorsal surface of the GS, confinement of GS in a net in their holding tank, or chasing the GS underwater with a small net. Both the chronically stressed and the control GS gained some weight over the 28-d period, but the chronically stressed GS showed an elevated "maintenance" metabolic rate, compared with controls. Even though the active metabolic rates (and the  $U_{\text{crit}}$  values) were not statistically distinguishable between the two groups, the elevated maintenance rate decreased the scope for activity in the chronically stressed GS. A decreased scope for activity indicates that the fish is less able to deal with additional energy demands and still be active, grow, and reproduce at the same levels. Presentations of this material were made at the Federation of Affiliated Societies for Experimental Biology (FASEB) meeting and the Western Division and California-Nevada Chapter Annual Meeting of the American Fisheries Society, which were both in San Diego

(2003). The following manuscript has been provisionally accepted for publication in *Physiological and Biochemical Zoology*, pending our revisions: Lankford, S.E., T.E. Adams, R.A. Miller, and J.J. Cech, Jr. "The Cost of Chronic Stress: Metabolic Scope for Activity, Critical Swimming Velocity and Liver Glycogen Levels in Sturgeon."

Another of Dr. Lankford's dissertation chapters concerned experiments designed to further understand the environmental modification of the GS stress response at the interrenal tissue level. Adrenocorticotrophic hormone (ACTH) is a pituitary hormone that, upon a chemical signal from the hypothalamus in the brain perceiving a stressor, circulates to the interrenal for cortisol production and release. Production of anti-green sturgeon adrenocorticotrophic hormone (gsACTH) antibodies was achieved in host animals, allowing a functional blocking of the cortisol. Antibodies produced were found to be highly specific, and infusion (passive immunization) with the antibodies produced a significantly decreased cortisol response. In the future, for example, these antibodies could serve to elucidate cortisol's role in the observed maintenance metabolic rate increase and metabolic scope for activity decrease in GS exposed to chronic stress, or to develop a reliable GS biomarker for chronic stress.

On a related project, we investigated the effects of metabolic (i.e., lactic) and mineral (hydrochloric) acids on the stress responses (i.e., plasma lactate and plasma cortisol concentrations) of juvenile GS. The two acids produce similar transient pulses in plasma lactate concentrations (returning to resting levels by 1.5 – 2 h), but the lactic acid infusion stimulates a more sustained peak in plasma cortisol, similar to that found in air-exposed GS by Lankford et al. (2003). These results were published in *Comparative Biochemistry and Physiology* (Warren et al. 2004).

## List of Publications from Task 1 Activities

### Publications

Gisbert, E., J.J. Cech Jr. and S.I. Doroshov. 2001. Routine metabolism of larval sturgeon (*Acipenser medirostris* Ayres). *Fish Physiology and Biochemistry* 25: 195-200.

Lankford, S.E., T.E. Adams and J.J. Cech Jr. 2003. Time of day and water temperature modify the physiological stress response in green sturgeon, (*Acipenser medirostris*). *Comparative Biochemistry and Physiology Part A* 135: 291-302.

Warren, D. E., S. Matsumoto, J. M. Roessig and J.J. Cech Jr. 2003. Cortisol response of green sturgeon to acid-infusion stress. *Comparative Biochemistry and Physiology Part A* 137: 611-618.

Mayfield, R.B. and J.J. Cech Jr. 2004. Temperature effects on green sturgeon bioenergetics. *Transactions of the American Fisheries Society* 113: 959-968.

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### Manuscript in Review

Lankford, S.E., T.E. Adams, R.A. Adams and J.J. Cech Jr. The cost of chronic stress: metabolic scope for activity, critical swimming velocity and liver glycogen levels in sturgeon. (Submitted to *Physiological and Biochemical Zoology*).

### Abstracts

Mayfield, R.B. and J.J. Cech, Jr. 2001. Green sturgeon bioenergetics responses to temperature. 2001. 4<sup>th</sup> International Symposium on Sturgeon (Oshkosh).

Lankford, S.E. and J.J. Cech Jr. 2001. Time of day and temperature modify the stress response of the green sturgeon, (*Acipenser medirostris*). 4<sup>th</sup> International Symposium on Sturgeon (Oshkosh).

Lankford, S.E. and J.J. Cech Jr. 2001. Modification of the physiological stress response of green sturgeon, (*Acipenser medirostris*): The influence of time of day and temperature. *The Physiologist* 45(4):352

Allen, P., J.J. Cech Jr., A. Vlazny, S. Doroshov, J. Van Eenennaam and D. Hillemeier. 2002. Warm water-induced growth depression on juvenile green sturgeon. 36<sup>th</sup> annual Meeting of the California-Nevada Chapter of the American Fisheries Society (Tahoe City).

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Lankford, S.E., T.E. Adams and J.J. Cech Jr. 2002. Modifications of the physiological stress response in green sturgeon, (*Acipenser medirostris*), by of time of day and temperature. Annual Meeting of the California-Nevada and Humboldt Chapters of the American Fisheries Society (Santa Rosa).

Allen, P., J.J. Cech Jr., S. Cole and M. Nicholl. 2003. Effects of elevated and fluctuating temperature on the growth of juvenile green sturgeon, (*Acipenser medirostris*). 133<sup>rd</sup> Annual Meeting of the American Fisheries Society (Quebec City).

Allen, P., J.J. Cech Jr., S. Cole and M. Nicholl. 2003. Salinity tolerance in developing juvenile green sturgeon, (*Acipenser medirostris*). 133<sup>rd</sup> Annual Meeting of the American Fisheries Society (Quebec City).

Allen, P., J.J. Cech Jr., M. Nicholl and S. Cole and. 2003. Growth in juvenile green sturgeon (*Acipenser medirostris*). Annual Meeting of the Western Division of the American Fisheries Society (San Diego).

Lankford, S.E., T.E. Adams and J.J. Cech Jr. 2003. Productive Pacific ecosystems. Annual Meeting of the Western Division of the American Fisheries Society (San Diego).

Allen, P., J.J. Cech Jr., S. Cole, M. Nicholl, and B. Hodge. 2003. Ontogeny of salinity tolerance in juvenile green sturgeon, (*Acipenser medirostris*). Annual Meeting of the Western Division of the American Fisheries Society (San Diego).

Lankford, S.E., J.J. Cech Jr. and T.E. Adams. 2003. Swimming Performance, "standard" metabolic rate, and metabolic scope for activity as measures of chronic stress in green sturgeon, (*Acipenser medirostris*). Experimental Biology (San Diego).

Miller, R.A., J.J. Cech Jr. and S E. Lankford. 2003. Metabolic and hormonal responses to changing flow velocities in green sturgeon, (*Acipenser medirostris*, 14<sup>th</sup> Annual Undergraduate Conference (Davis).

Allen, P., B. Hodge and J.J. Cech Jr. 2004. The effects of size on juvenile green sturgeon (*Acipenser medirostris*). Annual Meeting of the California-Nevada and Humboldt Chapters of the American Fisheries Society (Redding).

Lankford, S.E., T.E. Adams and J.J. Cech Jr. 2003. Investigations into the effects of chronic stress on swimming performance, standard metabolic rate and metabolic scope for activity in green sturgeon, (*Acipenser medirostris*). CALFED Science Conference (Sacramento).



## Routine metabolism of larval green sturgeon (*Acipenser medirostris* Ayres)

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**Key words:** green sturgeon, larvae, metabolic rate, respirometry, routine metabolism

### Abstract

Routine metabolic rates of green sturgeon (*Acipenser medirostris*) from hatching to 31 days post hatch (dph) were determined under normoxic conditions. During the endogenous feeding stage that comprised period from hatching to 15 dph, the oxygen consumption rate ( $\text{MO}_2$ ,  $\mu\text{g O}_2 \text{ larva}^{-1} \text{ h}^{-1}$ ) increased 5-fold before yolk reserves became exhausted and  $\text{MO}_2$  rates steady. The allometric relationships between  $\text{MO}_2$  and body mass had mass exponents greater than 1.0 ( $b = 1.64 \pm 0.21$ ) and equal to 1.0 ( $b = 1.04 \pm 0.07$ ) during the endogenous and exogenous feeding phases, respectively. The magnitude and changes of  $\text{MO}_2$  rates in green sturgeon larvae reflected their early ontogeny, especially during the endogenous feeding phase when the increase in metabolic rates was associated with organogenesis, acquisition of organ functions, and the conversion of yolk sac into new tissues.

### Introduction

The anadromous green sturgeon is considered a rare or vulnerable species in the United States and Canada (Birnstein 1993) and an endangered species in Russia (Artyukhin and Andronov 1990). Published information on this species is very limited, and we are currently investigating life history and environmental physiology of green sturgeon, using artificial spawning techniques and cultured fish (Van Eenennaam et al. 2001).

The only known spawning populations of green sturgeon in North America are in the Klamath, Rogue and Sacramento Rivers, all of which are affected by water projects (Moyle et al. 1994). Changes in flow of rivers controlled by dams, especially during the reproductive season of green sturgeon (late spring, Van Eenennaam et al. 2001), may be detrimental for reproduction, in regards to both water quality (temperature and dissolved oxygen) and availability of food resources for green sturgeon larvae. Khakimullin (1987) reported that Siberian sturgeon (*A. baerii*) larvae during their endogenous feeding phase could not avoid

hypoxic areas, which directly affected their survival. There is significant information in Russian literature (reviews of Klyashtorin 1982; Gershanovich et al. 1987) on the respiratory metabolism of juvenile sturgeon and paddlefish, but only limited information exists on the oxygen consumption of larval stages (Khakimullin 1984; Dabrowski and Kaushik 1986).

The objective of this study was to measure the routine respiratory metabolic rate of green sturgeon larvae from hatching to onset of metamorphosis under normoxic conditions. Knowledge of oxygen consumption patterns during the larval development (including endogenous and exogenous feeding phases) will provide basic information for further studies on green sturgeon larval physiology and for the projects aiming to protect reproductive habitat of green sturgeon.

### Materials and methods

#### *Fish supply and maintenance*

Larvae were obtained by induced spawning of one female and male green sturgeon that were caught

by gillnets and held in cages in the Klamath River (Weitchpec, California). Fish were induced to ovulate and spermiate by injections of gonadotropin releasing hormone as previously described (Van Eenennaam et al. 2001). Ovulated eggs were fertilized *in vitro* (fertilization rate 78% at early cleavage) and, after bathing in silt to eliminate adhesiveness, were transported to the Center of Aquatic Biology and Aquaculture (CABA, UC Davis) and incubated in MacDonald jars at 13–15 °C for seven days before hatching. Five hundred hatched larvae were transferred into a circular fiberglass tanks (120 l) supplied with well water in a temperature-controlled recirculation system. Fish were held at a constant temperature of 16 °C and exposed to 12L:12D artificial photoperiod. Food was presented at 12 days post hatch (dph) and larvae were fed with a semi-moist commercial diet (Silver Cup, Nelson & Sons, Inc., Murray, UT) mixed with live chopped *Tubifex* worms. Once first feeding was detected between 14 and 16 dph, larvae were fed *ad libitum* and the excess of uneaten feed and feces were removed daily. Prior to the measurement of routine metabolic rates, larvae were fasted for 16 h in a separate tank in order to avoid a postprandial increase in oxygen consumption (Dabrowski et al. 1987).

#### Respirometry

Measurements of oxygen partial pressure ( $PO_2$ ) were made using an  $O_2$  analyzer (PHM71/MK2 Radiometer, Copenhagen). Static-type respirometers (glass syringes, approximate volume: 60 ml; Micro-Mate Interchangeable, Popper & Sons, Inc. USA) were used to measure routine metabolic rates of fish (Fry 1971). The advantages of using this type of respirometer with fish larvae are described in Marty et al. (1990). Water samples were injected directly from the respirometer into a thermostatted chamber that contained the calibrated  $O_2$  electrode. Twelve assays were carried out each day from 09:00 to 13:00 h in order to measure the oxygen consumption rates of larvae. For each experiment, six respirometers were situated in an aerated, temperature-controlled ( $16 \pm 0.1$  °C) fiberglass water bath similar to general rearing tanks. Two respirometers were used as a blank (no fish) to correct for potential microbial oxygen consumption. In all experiments, microbial oxygen consumption was negligible.

Prior to each experiment, fish were quickly transferred from the tank to the respirometers with a dip net in order to minimize handling stress, and were

allowed 1-h acclimation before oxygen consumption measurements. Preliminary assays indicated that this time was fully adequate for the complete acclimation of fish (metabolic plateau) to the respirometer. After acclimation, the initial  $PO_2$  (> 80% air saturation) was measured by injecting 5 ml of water (remaining water volume in the respirometer was 50 ml) directly into the oxygen electrode chamber. The static-type respirometers were left undisturbed on the bottom of the experimental water bath for one hour. At the end of each experiment, water in the respirometer was gently mixed by slowly inverting the syringe, and 5 ml were injected into the oxygen electrode chamber. Sample  $PO_2$ s (mm Hg) were converted to oxygen concentrations (mg  $O_2$ /l) by using the nomogram of Green and Carrit (1967). Oxygen concentration at the end of each experiment was > 60% air saturation and never reached the limits of optimal range (52–58% air saturation) known in larvae of other species, such as Siberian sturgeon and stellate sturgeon, *A. stellatus* (Lozinov 1956 quoted by Khakimullin 1987).

Oxygen consumption (including mass-specific) rates were calculated from the rate of decrease in water of  $PO_2$  in the respirometer, respirometer volume, time between initial and final measurements, number and total mass of larvae in each respirometer (Barrionuevo and Burggren 1999). The number of larvae placed in each respirometer decreased with increasing age and size to avoid confining-stress effects on oxygen consumption rates (five larvae from hatching to 7 dph, three from 8 to 23 dph, two from 24 to 28 dph, and one from 29 to 31 dph). After each series of metabolic measurements, fish were anaesthetized with tricaine methanesulphonate (MS 222) and wet body weight (BW) measured to the nearest 0.01 mg using an electronic balance. For comparing green sturgeon routine oxygen consumption rates ( $MO_2$ ) with those already reported in other species, data for endogenous and exogenous feeding phases were expressed as allometric functions,  $MO_2$  ( $\mu g O_2 \text{larva}^{-1} \text{h}^{-1}$ ) =  $a BW^b$ , where BW is a wet weight of larva (Rombough 1988a). The relationship between the age and the oxygen consumption rate was determined by fitting linear regression using SigmaStat 2.0 (Jandel Scientific). The accepted significance level was  $P < 0.05$ . Data in text is mean  $\pm$  SE.

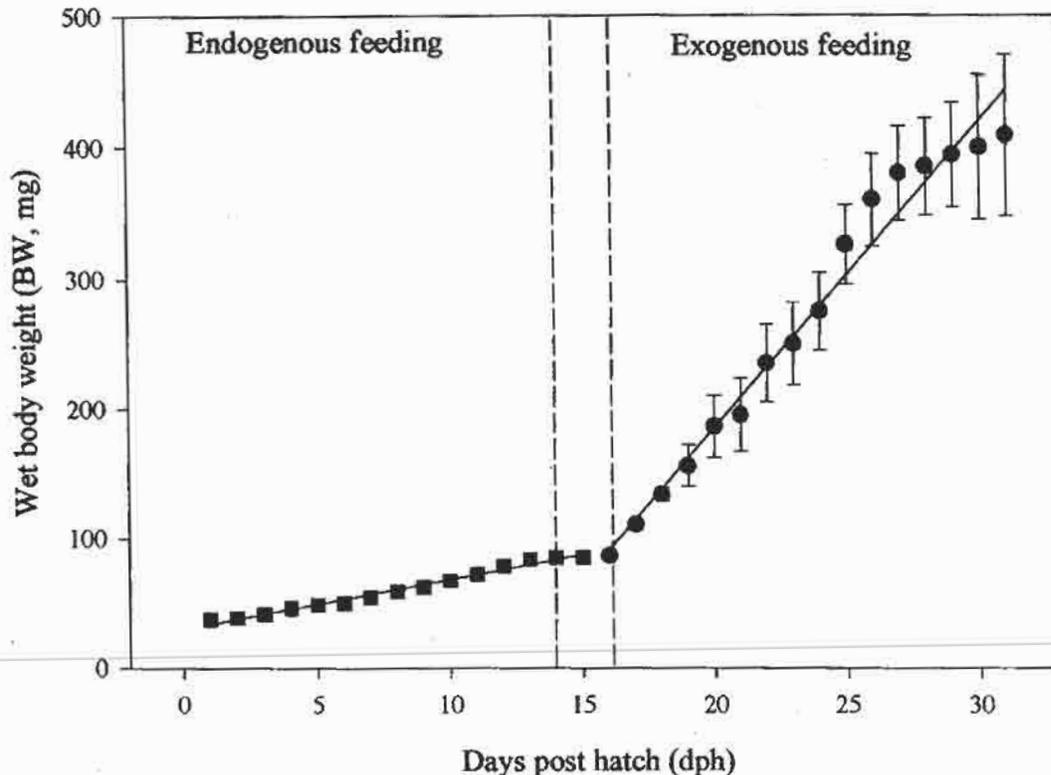


Figure 1. Wet body weight (mean  $\pm$  SD,  $n = 10$ ) of green sturgeon larvae during 31 days post hatch. (■) Yolk-sac larvae; (●) *ad libitum*-fed larvae. Dashed line represents the period of transition to exogenous feeding. The SD bars during the endogenous feeding phase are not visible due to their small value.

## Results

Growth in wet weight of green sturgeon larvae could be divided in two different phases according to linear regression results (Figure 1): a lecithotrophic (yolk feeding) phase between hatching and onset of exogenous feeding at 15 dph ( $BW \text{ mg} = 30.0 + 3.8 \text{ dph}$ ,  $r^2 = 0.98$ ) and an exogenous feeding phase between 16 and 31 dph ( $BW \text{ mg} = -285.0 + 23.6 \text{ dph}$ ,  $r^2 = 0.97$ ).

During the endogenous feeding phase, the  $MO_2$  ( $\mu\text{g O}_2 \text{ larva}^{-1} \text{ h}^{-1}$ ) increased 5-fold, from  $8.7 \mu\text{g O}_2 \text{ larva}^{-1} \text{ h}^{-1}$  ( $0.233 \mu\text{g O}_2 \text{ mg}^{-1} \text{ h}^{-1}$ ) at hatching to  $44.9 \mu\text{g O}_2 \text{ larva}^{-1} \text{ h}^{-1}$  ( $0.529 \mu\text{g O}_2 \text{ mg}^{-1} \text{ h}^{-1}$ ) at 14 dph (Figure 2). The large increase in  $MO_2$  occurred from hatching to 10 dph ( $MO_2 \mu\text{g O}_2 \text{ larva}^{-1} \text{ h}^{-1} = 3.4 + 4.34 \text{ dph}$ ;  $r^2 = 0.99$ ), with a plateau at the end of yolk resorption (11–15 dph). During the exogenous feeding phase, the  $MO_2$  increased from  $48.3 \mu\text{g O}_2 \text{ larva}^{-1} \text{ h}^{-1}$  ( $0.567 \mu\text{g O}_2 \text{ mg}^{-1} \text{ h}^{-1}$ ) at 15 dph to  $155.3 \mu\text{g O}_2 \text{ larva}^{-1} \text{ h}^{-1}$  ( $0.451 \mu\text{g O}_2 \text{ mg}^{-1} \text{ h}^{-1}$ ) at 28 dph, and reached

$322.7 \mu\text{g O}_2 \text{ larva}^{-1} \text{ h}^{-1}$  ( $0.723 \mu\text{g O}_2 \text{ mg}^{-1} \text{ h}^{-1}$ ) at 31 dph (Figure 2). The allometric relationships between the individual  $MO_2$  and body mass revealed increased rate of oxygen consumption ( $b = 1.64 \pm 0.21$ ) during the endogenous feeding phase, and close to the unity regression coefficient ( $b = 1.04 \pm 0.07$ ) during the exogenous feeding larvae (Figure 3).

## Discussion

Green sturgeon produce large yolky eggs, investing greater amount of energy for nourishment of the embryo, compared to other acipenserids (Van Eenennaam et al. 2001; Deng et al. 2002). At hatching, most functional systems of green sturgeon larvae are incomplete; consequently, significant organogenesis and acquisition of organ functions occur during the larval development (Deng et al. 2002). Similar to modern teleosts, sturgeon embryo utilizes ca. 20% of yolk proteins before hatching, conserving yolk nutrients for continuing organogenesis and as a source of energy

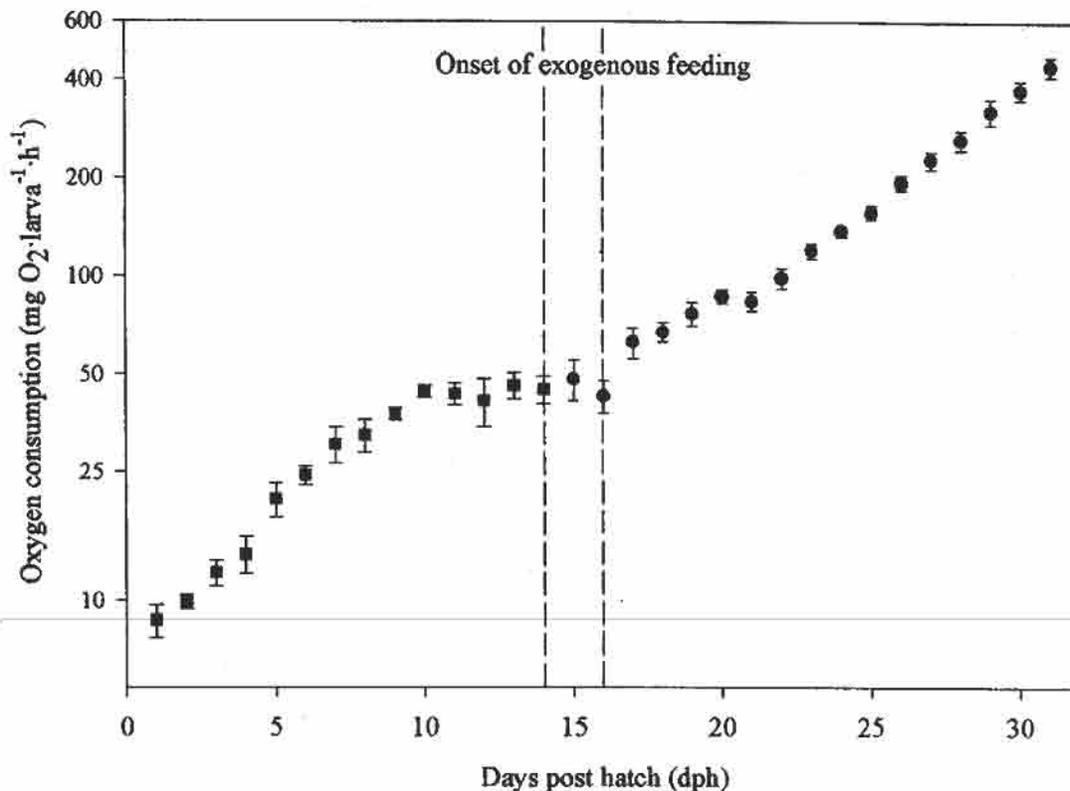


Figure 2. Larval oxygen consumption rates of green sturgeon during the endogenous (■) and exogenous (●) feeding phases. Dashed line represents the period of transition to exogenous feeding. Symbols are mean  $\pm$  SE from 10 respirometry measurements.

during the larval stage (Wang et al. 1987). Organogenesis and replacement of embryonic structures with the functional adult organs in lecithotrophic phase of sturgeon larval development (Schmalhausen 1991; Gilbert 1999) may be reflected in larval energetics and metabolism. During the endogenous feeding phase, the  $MO_2$  of green sturgeon larvae increased 5-fold before yolk sac reserves became limiting and metabolic rates steady. The large increase in  $MO_2$  rate from 5 to 10 dph coincided with the disappearance of embryonic vascular system in the posterior region of endodermal yolk sac (involved in cutaneous respiration and nutrient transport), and with the onset of branchial respiration (rhythmic movements of mandible and operculum). Similar developmental changes in the  $MO_2$  during endogenous feeding were reported in Siberian sturgeon (Dabrowski and Kaushik 1986) and salmonids (Lukina 1983; Rombough 1988b; Rombough and Ure 1991), while in common carp the transition from cutaneous to branchial respiration resulted in a decrease in  $MO_2$  rates (Kamler 1972). Similar to Siberian sturgeon (Dabrowski and Kaushik 1986),

the increase in metabolic rate of green sturgeon during the endogenous feeding stage is likely associated with the conversion of yolk proteins into new larval tissue and acquisition of definitive organ functions (e.g., gill respiration, digestive system development).

In feeding larvae, the  $MO_2$  increased, after a short-term plateau, to the end of rearing. The almost two-fold increase in  $MO_2$  observed between 29 and 31 dph could be associated with metamorphic events (Kamler 1992) or with the confining effect (fish size greatly increased at this age) of the respirometer (Cech et al. 1985). In addition, we used a single individual in the respirometer during 29–31 dph, and it has been shown that  $MO_2$  is lower when measured in the small groups (vs. individual fish) of larval and juvenile sturgeon (Khakimullin 1988) and paddlefish, *Polydon spatula* (Gershanovich et al. 1987).

When expressing the  $MO_2$  as an allometric function ( $MO_2 = aBW^b$ ) for the entire experimental period, the mass exponent  $b$  was close to the unity ( $b = 1.14 \pm 0.1$ ), but developmental differences between endogenous and exogenous feeding phases were ob-

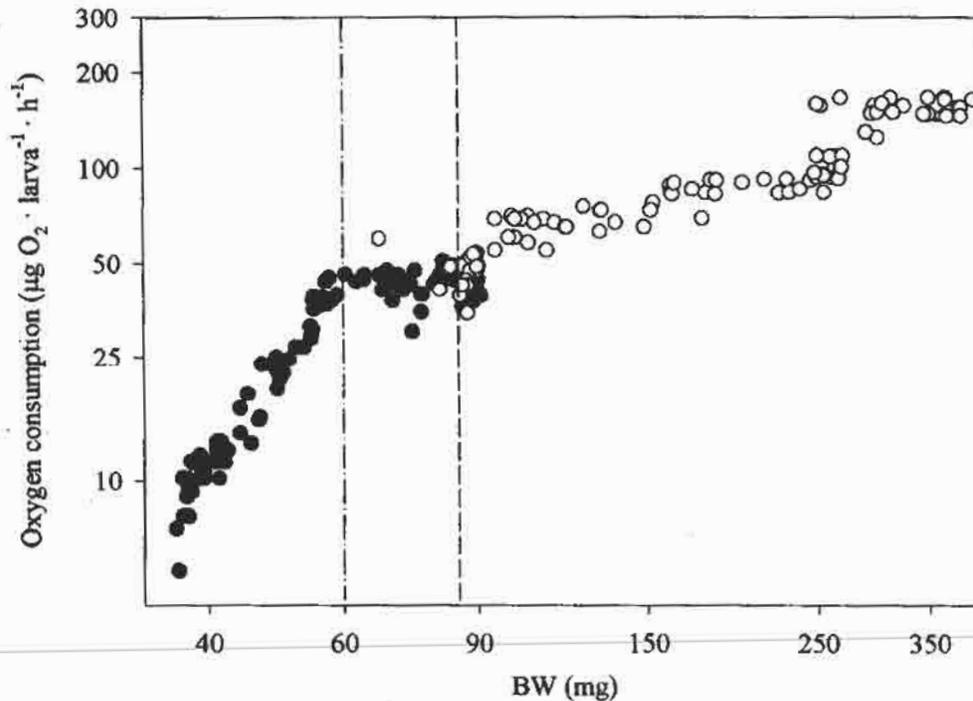


Figure 3. Relationship between oxygen consumption rates and wet body weight during the endogenous (●) ( $MO_2 = 0.04 (0.03)BW^{1.64(0.21)}$ ;  $r^2 = 0.87$ ;  $P < 0.001$ ;  $n = 140$ ) and exogenous (○) ( $MO_2 = 0.40 (0.15)BW^{1.04(0.07)}$ ;  $r^2 = 0.97$ ;  $P < 0.001$ ;  $n = 140$ ) feeding phases of green sturgeon larvae (see text for details). The dashed-dotted line shows the average weight ( $60.0 \pm 2.1$  mg) of the complete transition to branchial respiration (9–10 dph); dashed line represents the average weight ( $85.6 \pm 1.7$  mg) at the onset of exogenous feeding (14–15 dph).

scured (Rombough 1988a). In the separate equations, mass exponent was greater than 1.0 ( $b = 1.64 \pm 0.2$ ) in the endogenous feeding phase and did not differ from 1.0 ( $b = 1.04 \pm 0.1$ ) in the exogenous feeding phase. Khakimullin (1984) reported similar difference between two phases (mass exponents 1.31 and 0.85 for endogenous and exogenous feeding phases, respectively) in larval development of Siberian sturgeon. Gershanovich et al. (1987) reported mass exponents ranging 0.79–0.98 for feeding larvae and juveniles of several species of sturgeon and paddlefish, which is similar to our results for exogenous feeding phase. Rombough (1988a) reviewed the allometric relationships in more than 25 teleosts, and concluded that high mass exponents could be a reflection of favorable, for the respiratory exchange, surface to volume ratios in the early life stages. However, metabolic mass exponents were seldom the same for different species, because they are affected by the experimental design, fish activity and environmental factors such as temperature.

In conclusion, the magnitude and changes of  $MO_2$  rates in green sturgeon larvae reflected their early

ontogeny, especially during the endogenous feeding phase when the increase in metabolic rates seemed to be associated with morphogenesis and the conversion of endogenous nutrients into new tissues. During the exogenous feeding phase, routine metabolic rates increased in a direct proportion to the increase in larval weight as indicated by the metabolic mass exponent equal to unity.

#### Acknowledgements

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## Time of day and water temperature modify the physiological stress response in green sturgeon, *Acipenser medirostris*

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### Abstract

The effects of time of day and water temperature on the acute physiological stress response were investigated in young-of-the-year green sturgeon (*Acipenser medirostris*). The response to a 1-min air-emersion stressor was assessed during the day (08.00 h) and at night (20.00 h), as well as after acclimation to either 11 °C or 19 °C. Blood samples were collected prior to stress and at several times after exposure to the stressor, and plasma concentrations of cortisol, lactate, and glucose were determined. The magnitudes of cortisol (19.1 ng ml<sup>-1</sup> vs. 4.9 ng ml<sup>-1</sup>) and lactate (190.6 mg l<sup>-1</sup> vs. 166.7 mg l<sup>-1</sup>) were significantly higher in fish stressed at night when compared with the day. There were no significant differences in glucose levels between time periods. Although, acclimation temperature did not affect peak cortisol concentrations (56.7 and 50.3 ng ml<sup>-1</sup> at 11 °C and 19 °C, respectively), the duration of the response was significantly extended at 11 °C. Post-stressor lactate increases were similar between temperature groups, but at 11 °C post-stressor glucose levels were significantly increased through 6 h, suggesting stressor-induced glycogenolysis and gluconeogenesis or decreased glucose utilization. These data demonstrate that the physiological stress response in green sturgeon is modified by both time of day and temperature.

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**Keywords:** Cortisol; Diel cycle; Fish; Gluconeogenesis; Lactate; Stress; Sturgeon; Temperature

### 1. Introduction

The physiology of fish and other ectothermic animals displays a higher level of environmental plasticity than that of endothermic animals. In order to thrive, ectotherms have developed physiological and behavioral adaptations that allow them to occupy specific environmental niches. However, recent changes in aquatic environments, such as water diversion, habitat destruction and toxicant additions have outpaced the process of genetic

adaptation. These alterations, and the rate at which they occur, necessitate more intensive management of wild populations and, perhaps, supplementation of wild stocks with hatchery-reared fish. In order to properly manage wild fishes and successfully culture endangered or economically important fishes, we must first understand the influences that relevant environmental variables such as the diel (light/dark) cycle and environmental temperature have on the physiological responses of these fishes.

The physiological stress response is a highly integrated neuroendocrine-systemic process involving both behavioral and physiological elements (Donaldson, 1981). The hypothalamo-pitu-

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itary–adrenal axis (HPA) in mammals and the hypothalamo–pituitary–interrenal axis (HPI) in fishes are functional homologs (Carragher and Sumpter, 1990) and represent one of the most highly conserved components of the endocrine stress response in vertebrates. Perception of a stressor initiates a neuroendocrine cascade beginning with the release of corticotropin-releasing hormone (CRH) from the hypothalamus. This hypothalamic peptide triggers the synthesis and release of adrenocorticotropin hormone (ACTH) from the corticotrope cells of the anterior pituitary. Adrenal and interrenal tissues express high affinity receptors for ACTH and, once activated, initiate de novo synthesis of corticosteroids (Wendelaar Bonga, 1997; Barton et al., 2002).

Glucocorticoids are widely regarded as a primary component and reliable measure of the non-specific, physiological acute stress response in most vertebrates. Cortisol is the major glucocorticoid synthesized in fishes (Idler and Truscott, 1972) and is known to regulate several secondary components of the stress response, including glucose mobilization due to cortisol-dependent enhancement of gluconeogenesis (Vijayan et al., 1997; Mommsen et al., 1999). Cortisol-dependent up-regulation of glucose synthesis during times of stress promotes the replenishment of hepatic glycogen stores used during the acute phase and helps to maintain metabolic resources during prolonged or chronic stress. Cortisol also suppresses growth rate, reproductive function, and the immune response during acute and chronic stress (Maule et al., 1988; VanWeerd and Komen, 1998; Haddy and Pankhurst, 1999). The attenuation of these processes during stress is postulated to permit reallocation of the free glucose toward vital functions necessary for survival (Moberg, 1985). Increases in plasma lactate levels are also associated with acute stress and are believed to result from increased anaerobic metabolism in fast glycolytic white muscle fibers during 'burst' swimming (Driedzic and Hochachka, 1978).

The effects of the diel cycle on the HPI axis in fish have received little attention. Circadian control of cortisol secretion in fishes is suggested by reports that basal levels of cortisol reach a peak in the late night or early hours of the morning. However, considerable species-specific differences may exist (Pickering and Pottinger, 1983; Davis et al., 1984; Nichols and Weisbart, 1984; Belanger et al., 2001). The effect of temperature on the

cortisol response in fish has been more rigorously examined, but the findings are somewhat contradictory. The acute response to abrupt temperature fluctuations typically elicits a short-term elevation in plasma cortisol, with cortisol concentrations returning to resting levels within 1–15 days in teleosts depending on temperature range and species (Rotlant et al., 2000). However, there is also evidence of both attenuated and augmented basal levels of cortisol, as well as a reduced or enhanced response to stress in teleosts and chondrosteans acclimated to cooler temperatures (Umminger and Gist, 1973; Strange, 1980; Davis et al., 1984; Barton and Schreck, 1987; Cataldi et al., 1998), which further suggest seasonal and species-specific differences. Davis and Parker (1990) suggested that an optimal temperature range exists in striped bass and that exposure to temperatures above or below this range accentuates the stress response.

The green sturgeon (*Acipenser medirostris*) is native to North America's Pacific Northwest regions and is currently considered a 'Species of Special Concern' in California due to significant reduction in its distribution. One of the few remaining spawning grounds left for green sturgeon is the Klamath/Trinity river system in Northern California. This system has been heavily modified in the past 125 years resulting in significantly altered habitat, water flow regimes, and temperature profiles. These changes in the Klamath/Trinity drainage were initiated by hydraulic mining practices, which raised the riverbeds destroying suitable spawning habitat. More recently, the massive diversion of water for crop irrigation and human consumption has compounded and accentuated these detrimental changes to this delicate ecosystem. Construction of dams (Lufkin, 1991) to precisely control river flow has caused river temperatures to fluctuate 3–5 °C daily during the summer with mean river temperatures warming from approximately 8 °C in the winter to >25 °C during late summer (Dave Hillemeier, Yurok Tribal Fisheries Department, Klamath, CA, personal communication). Our aim was to define the stress response in green sturgeon and elucidate modifications of this response by environmentally relevant variables. We measured the physiological changes (plasma cortisol, lactate and glucose) that accompany the stress response in this chondrosteans fish and investigated the modification of the stress response by time of day and temperature. We

hypothesized that the stress response would be augmented at night in this nocturnally active species and that acclimation to warmer temperatures would result in an increased response to stress.

## 2. Materials and methods

### 2.1. Source and care of fish

Fish used to study the modification of the stress response by time of day and temperature were young-of-the-year (YOY) green sturgeon weighing 415–650 g and 700–1125 g, respectively. Experiment 1 (time of day effects) was conducted approximately 4 months before experiment 2 (temperature effects), accounting for the weight difference. All fish were obtained from artificially spawned, wild-caught brood stock from the Klamath river (Van Eenennaam et al., 2001) and maintained in outdoor flow-through tanks supplied with 19 °C well-water under a natural photoperiod (38.6 N, 121.7 W) at the Center for Aquatic Biology and Aquaculture (CABA), University of California, Davis. Fish were fed Silver Cup™ extruded non-floating trout chow diet at the rate of 1.5% body weight day<sup>-1</sup> via a 24-h belt feeder. Two weeks prior to the experiment, fish were randomly selected and transferred into indoor experimental tanks located in a facility equipped with skylights to maintain a natural photoperiod. All other fish care requirements in the indoor tanks were maintained in the same manner as the outdoor holding tanks. In all experiments feeders were emptied 12 h prior to sampling to insure all fish were in a post-absorptive metabolic state.

### 2.2. Experiment 1: effects of diel light/dark cycle on the stress response

Groups of eight fish were transferred into 1.5-m-diameter indoor tanks ( $n=6$ ) and allowed to acclimate for 2 weeks. Black polyethylene sheets (2.5 m high) were placed between the tanks 48 h before the experiment to allow investigators to approach individual tanks without disturbing adjacent tanks. Additional barriers were placed above the tanks during the 20.00 h sampling period to shield the tanks from low-level, indirect light necessary for sampling. Each tank was randomly assigned a sampling period of 0 (pre-response control), 15, 30, 60 or 120 min (digital timers) post-stressor. Fish in non-control tanks were

stressed with a 1-min air emersion stressor in a net. This experiment involved two separate groups of fish ( $n=48$  group<sup>-1</sup>), which were staggered by 2 weeks to provide naive fish for the measurement of the stress response at both 08.00 and 20.00 h due to limited tank resources.

At the designated post-stress time, fish were netted and quickly transferred into a buffered tricaine methanesulfonate (MS 222; 500 mg l<sup>-1</sup> and 6 g l<sup>-1</sup> NaCl) anesthetic bath. Fish were removed from the anesthetic bath when body undulations ceased (approx. 1–1.5 min) and blood samples were collected via the caudal vein with heparinized syringes. All samples were collected before plasma cortisol increases due to the sampling techniques were detectable (approx. 5 min; Barton et al., 2002). Indeed, the low baseline levels in resting green sturgeon indicate that measured cortisol concentrations reflected the experimental variables of time of day and temperature, and not sampling techniques. Blood was kept on ice (approx. 15 min) until centrifugation at 2500 rev. min<sup>-1</sup> and the plasma removed and stored at -80 °C in cryovials until later assayed for cortisol, lactate, and glucose. Cortisol was measured in triplicate by a radioimmunoassay (RIA; Daley et al., 1999), which was verified for spike recovery and parallelism using green sturgeon plasma. The intra-assay CV was  $\leq 9\%$  and the inter-assay CV was  $\leq 13\%$ . Lactate and glucose were measured in triplicate with a polarographic analyzer (YSI 2700 Select, Yellow Springs Instruments Incorporated, Yellow Springs, OH).

### 2.3. Experiment 2: effect of temperature on the stress response

Individual fish ( $n=16$ ) were transferred into 90-l indoor tanks and held at 19 °C ( $n=8$ ), or slowly cooled by 1 °C day<sup>-1</sup> to 11 °C ( $n=8$ ) and allowed 2 weeks to acclimate to the new temperature. To facilitate collection of multiple blood samples without disturbance, fish were cannulated (caudal vasculature) according to the procedure detailed by Crocker and Cech (1998). Briefly, fish were initially anaesthetized in a buffered MS 222 (500 mg l<sup>-1</sup> and 6 g l<sup>-1</sup> NaCl) bath and then transferred to a surgery table where the gills were continuously irrigated with a similar anesthetic bath containing 150 mg l<sup>-1</sup> MS 222. Using a modified 17-G Touhy needle as a guide, polyethylene tubing (Intramedic Clay-Adams PE-50, Bec-

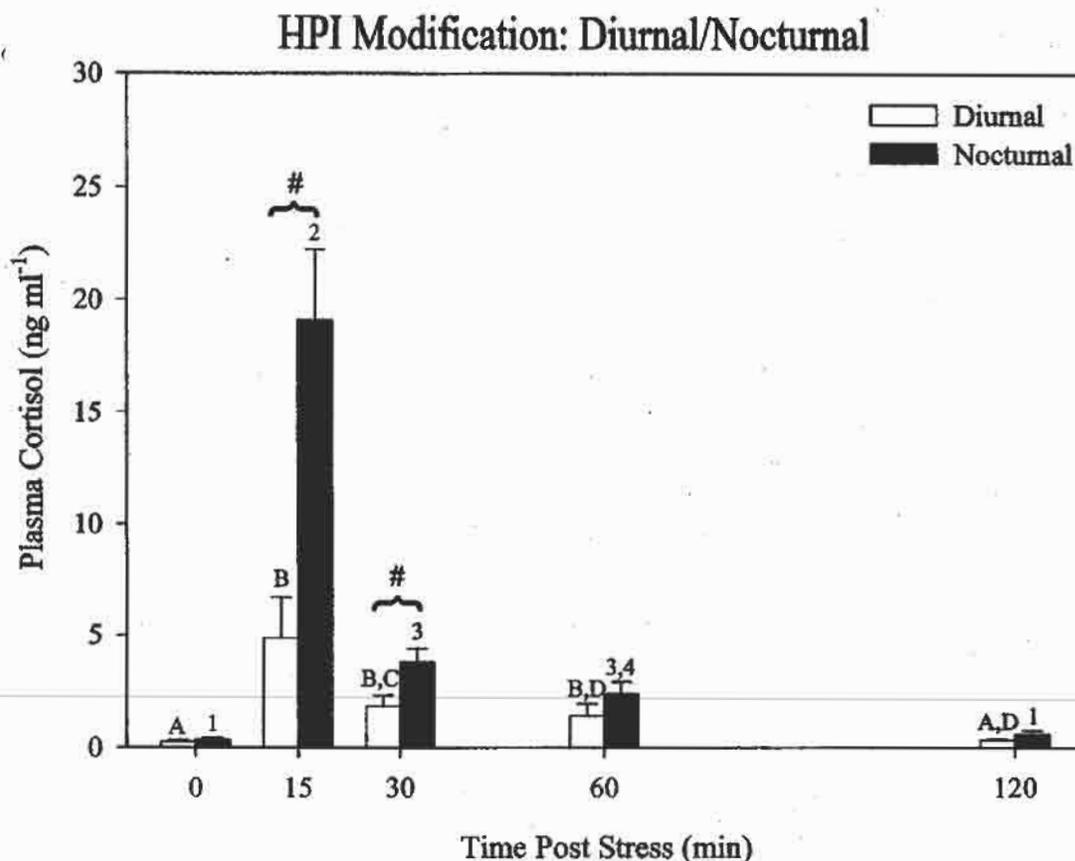


Fig. 1. Plasma cortisol responses to a 1-min air emersion in green sturgeon stressed at 08.00 h (open bars) or 20.00 h (black bars). Values are means  $\pm$  S.E. ( $n=7-8$ ) and data were log transformed when necessary. Bars with same symbol, within a single treatment, are not significantly different (one-way AVOVA with Tukey's multiple comparison test;  $P<0.05$ ); # denotes a significant difference between diel groups (two-way ANOVA;  $P<0.05$ ).

ton Dickson and Company, Sparks, MD) was inserted into the caudal vasculature and sutured (silk) in place. Cannulae were flushed with heparinized saline (50 units  $\text{ml}^{-1}$ ) and tied off after sampling so that the fish could swim freely. The entire cannulation procedure was completed in approximately 10 min. After a 2-day recovery period, all fish were stressed by a 1-min air-emersion in a net and blood was sampled at 0, 10, 20, 30, 60, 120 and 360 min post-stressor. Plasma was collected and assayed as described.

#### 2.4. Statistical analysis

Data in experiment 1 were tested for statistical significance using one-way analyses of variance (ANOVA); while data in experiment 2 were analyzed using one-way repeated measures ANOVA.

Differences between the treatment groups in both experiments were analyzed by two-way ANOVA, with time post-stressor and either time of day or temperature being the factors. To make comparisons among post-stressor time periods within a treatment group in both experiments, Tukey's multiple comparison method was conducted at the  $P<0.05$  level. Inverse or log transformations were used when normality or equal variance assumptions were not met.

### 3. Results

#### 3.1. Experiment 1: effects of diel light/dark cycle on the stress response

There were no differences in resting plasma cortisol, lactate and glucose levels (Figs. 1–3)

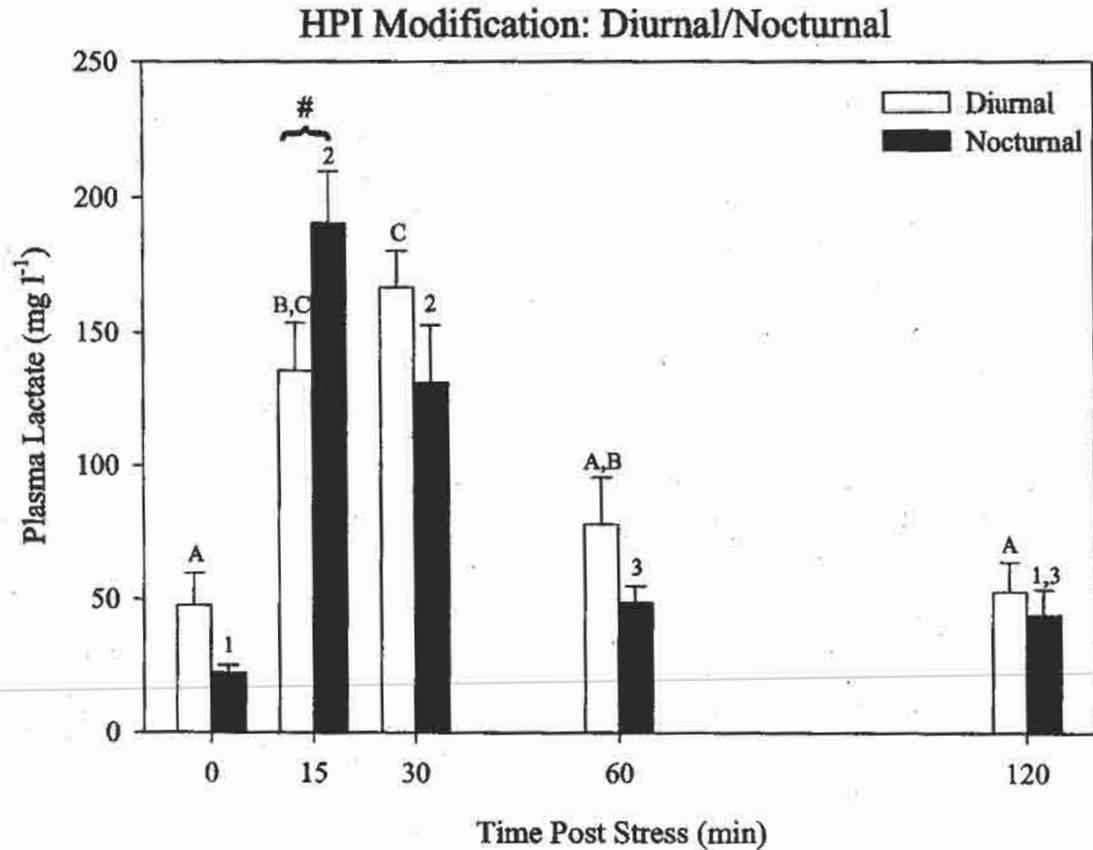


Fig. 2. Plasma lactate responses to a 1-min air emersion in green sturgeon stressed at 08.00 h (open bars) or 20.00 h (black bars). Values are means  $\pm$  S.E. ( $n=7-8$ ) and data were log transformed when necessary. Bars with same symbol, within a single treatment, are not significantly different (one-way ANOVA with Tukey's multiple comparison test;  $P<0.05$ ); # denotes a significant difference between diel groups (two-way ANOVA;  $P<0.05$ ).

between the diel treatment groups. Plasma cortisol and lactate concentrations were significantly increased above control levels in both treatments by 15 min post-stressor, but the peak levels reached were significantly higher when fish were stressed at 20.00 h (19.1 ng ml<sup>-1</sup> and 190.6 mg l<sup>-1</sup>, respectively) compared with those stressed at 08.00 h (4.9 ng ml<sup>-1</sup> and 166.7 mg l<sup>-1</sup>, respectively; Figs. 1 and 2). Cortisol concentrations in both treatments returned to control levels by 120 min post-stressor, but lactate levels appear to recover faster during the day (by 60 min). There was a significant increase in plasma glucose when compared to the control at 60 min post-stressor in fish stressed at 08.00 h (Fig. 3). However, there was no difference between plasma glucose levels between treatments (Fig. 3).

### 3.2. Experiment 2: effect of temperature on the stress response

There was no difference in resting plasma cortisol and glucose levels between treatments (Figs. 4 and 6), however, resting plasma lactate levels were significantly elevated in fish acclimated to 19 °C (Fig. 5). Plasma cortisol levels in both treatments were significantly elevated above resting levels by 10 min post-stressor (Fig. 4). Although both treatments reached similar peak plasma cortisol concentrations (56.7 and 50.3 ng ml<sup>-1</sup> at 11 °C and 19 °C, respectively), the response in fish acclimated to 11 °C was delayed (Fig. 4). Both treatments resulted in stressor-induced increases in plasma lactate, and there was a trend for increased plasma lactate peak means at

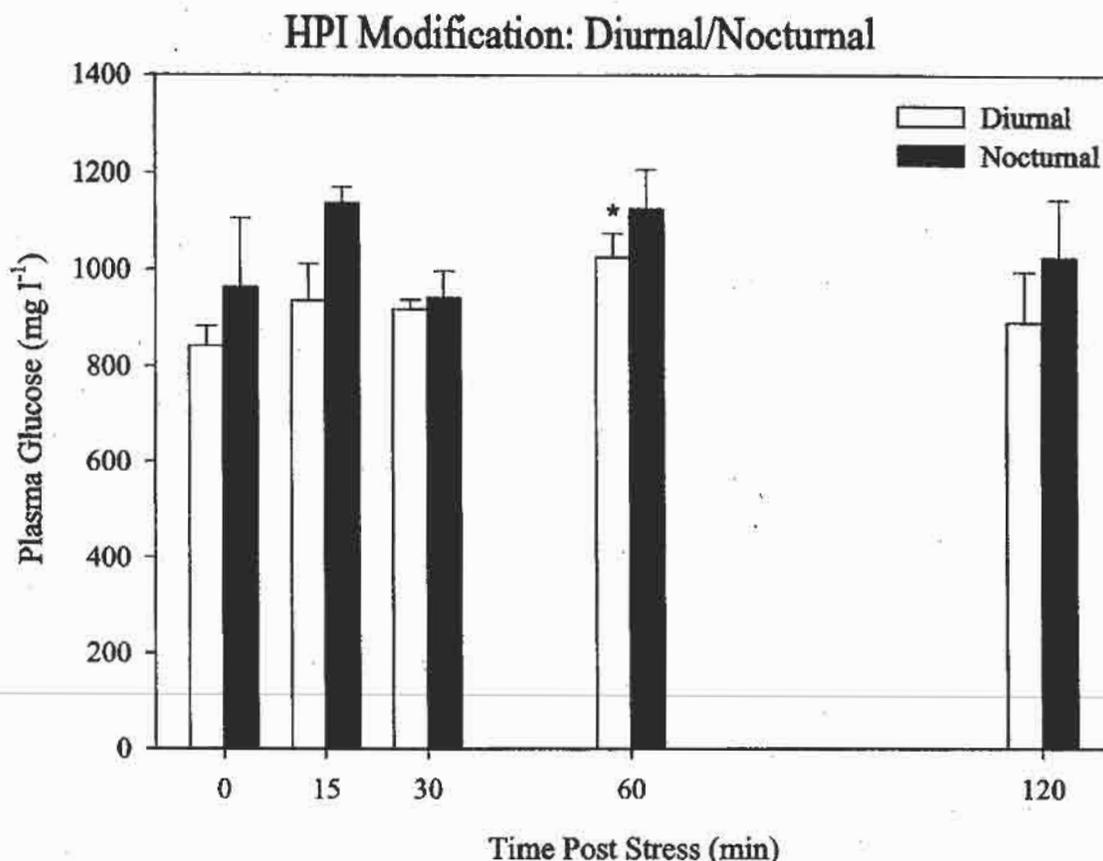


Fig. 3. Plasma glucose responses to a 1-min air-emersion in green sturgeon stressed at 08.00 h (open bars) or 20.00 h (black bars). Values are means  $\pm$  S.E. ( $n=7-8$ ) and data were log transformed when necessary. \* denotes a significant difference from control ( $t$ -test;  $P<0.05$ ).

every time period measured in fish acclimated to 19 °C. Fish acclimated to 11 °C also took longer to return to resting lactate levels when compared with the 19 °C group (Fig. 5). Plasma glucose was only increased at 120 min post-stressor in fish acclimated to 19 °C, but at 11 °C levels were significantly increased by 20 min post-stressor and remained elevated throughout the entire 360-min sampling period (Fig. 6).

#### 4. Discussion

The data presented here are the first to examine the stress response in the green sturgeon. These data describe an acute stress response that is similar to the acute stress response reported for other fishes. In addition we note that the magnitude, duration and temporal course of onset of the physiological stress response in green sturgeon is significantly influenced by time of day and accli-

mation temperature. In both experiments green sturgeon responded to a stressor with significant increases in plasma cortisol (Figs. 1 and 4) and lactate (Figs. 2 and 5) by 10 or 15 min post-stressor, and there is also some evidence of stressor-induced plasma glucose increases (Figs. 3 and 6). Compared with other chondrosteans (the freshwater *Polyodon spathula* and *Scaphirhynchus albus*; Barton et al., 1998, 2000), green sturgeon appear to have a more robust cortisol response, while the response is similar to levels reported in the semi-anadromous white sturgeon (*Acipenser transmontanus*; Belanger et al., 2001), its closest congener (Moyle, 2002). However, resting and stressor-induced concentrations of cortisol and glucose in green sturgeon are still on the low end of the 'typical' ranges reported in other fishes, and resting and stressed levels of plasma lactate are well below 'typical' values (Barton, 2000). It is possible that green sturgeon are less sensitive to

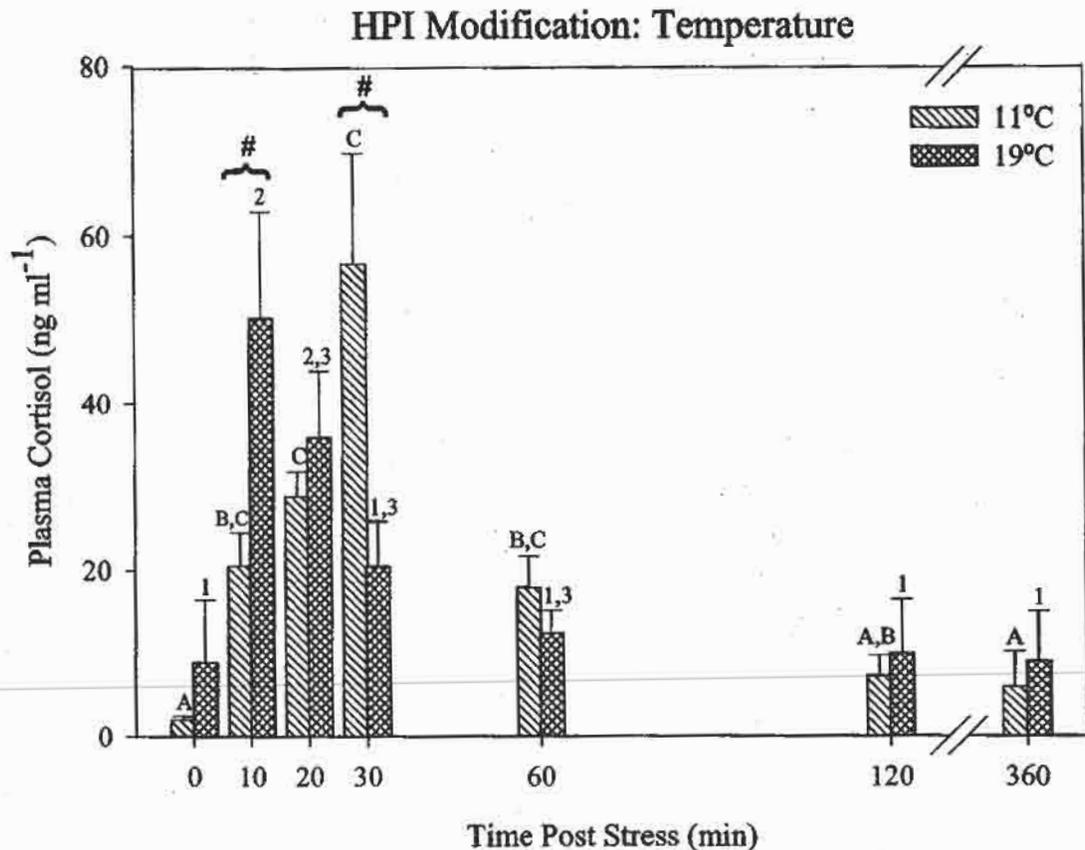


Fig. 4. Plasma cortisol responses to a 1-min air emersion in green sturgeon acclimated to 11 (hatched bars) or 19 (cross-hatched bars) °C. Values are means  $\pm$  S.E. ( $n=5-6$ ) and data were log transformed when necessary. Bars with same symbol, within a single treatment, are not significantly different (one-way repeated measures AVOVA with Tukey's multiple comparison test;  $P<0.05$ ); # denotes a significant difference between temperature groups (two-way ANOVA;  $P<0.05$ ).

stress than are other fishes. However, it is difficult to relate these measured differences in plasma concentrations to differences in biological function among species due to variations in sampling protocols and interspecies differences in the sensitivity and structure of endocrine tissues. Interestingly, the maximal secretory response to the air emersion stressor differed markedly between the two experiments. The maximum stressor-induced level of cortisol in experiment 1 was  $19.1 \text{ ng ml}^{-1}$  (Fig. 1), while fish stressed at the same temperature ( $19^\circ\text{C}$ ) in experiment 2, approximately 3 months later, elevated cortisol to  $50.2 \text{ ng ml}^{-1}$  (Fig. 4) despite the fact that the same stressor was used. Resting cortisol or basal concentrations between experiments 1 and 2 ( $0.4 \text{ ng ml}^{-1}$  and  $8.9 \text{ ng ml}^{-1}$  respectively; Figs. 1 and 4) also appear to be significantly different. We suggest this difference is due to age or mass-related differences in the

sensitivity or synthetic ability of the HPI axis. A similar phenomenon was suggested to exist in full-sib families of Chinook salmon (*Oncorhynchus tshawytscha*; Heath et al., 1993). It is also possible that differences in the protocols used in experiment 1 and 2, namely the timing and method of blood collection, resulted in the noted differences. However, the pattern of lactate production did not differ between experiments 1 and 2 (Figs. 2 and 5).

Experiments examining the modification of the stress response by the diel cycle were initiated after observing a pronounced nocturnal behavior in captive green sturgeon. Fish during the day typically group together at the bottom of the tank, with comparatively low gill ventilatory rates and swimming activity. Conversely, the fish actively swim throughout the entire water column at night. By 15 min post-stressor, a four-fold increase in plasma cortisol (Fig. 1) and a significant increase

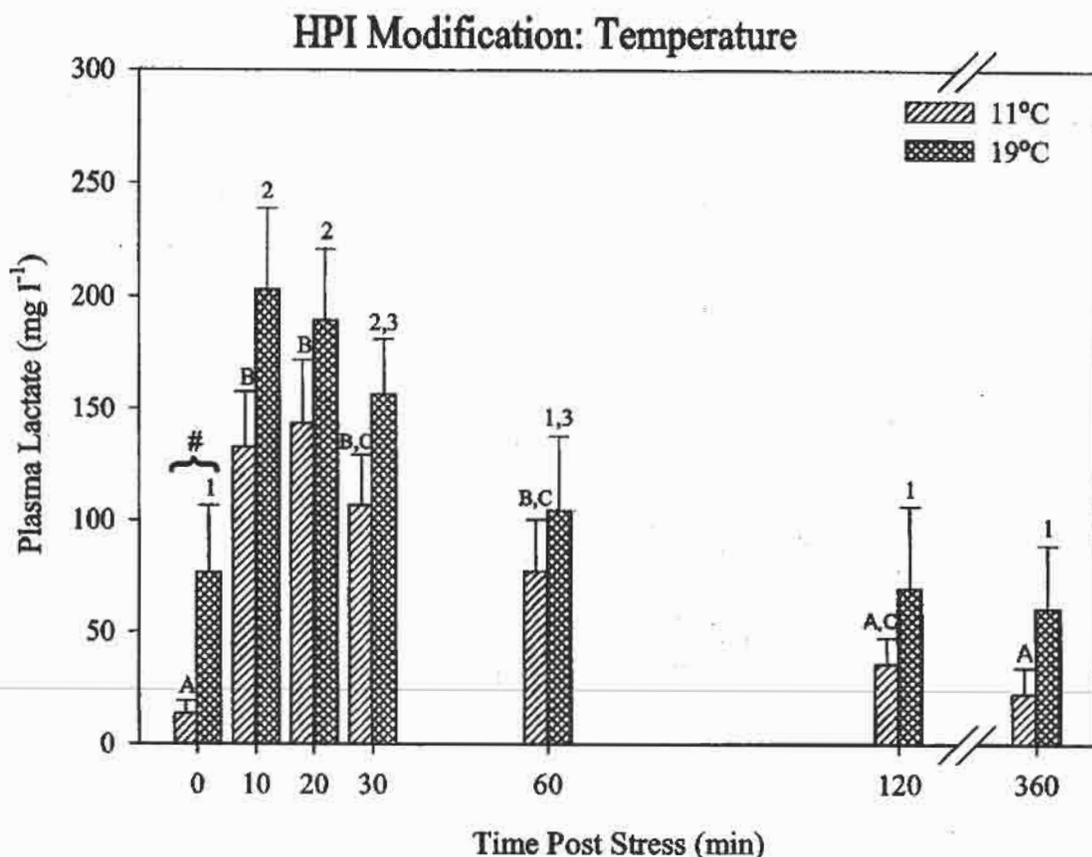


Fig. 5. Plasma lactate responses to a 1-min air-emersion in green sturgeon acclimated to 11 (hatched bars) or 19 (cross-hatched bars) °C. Values are means  $\pm$  S.E. ( $n=5-6$ ). Bars with same symbol, within a single treatment, are not significantly different (one-way repeated measures AVOVA with Tukey's multiple comparison test;  $P<0.05$ ); # denotes a significant difference between temperature groups (two-way ANOVA;  $P<0.05$ ).

in plasma lactate (Fig. 2) were measured at night when compared with those same parameters during daylight hours. This observation supports our hypothesis that the stress response is augmented at night. However, by 30 min post-stressor there was no plasma lactate difference between treatments, suggesting a slower rate of lactate accumulation during the day (Fig. 2). Only minor changes were measured in post-stressor plasma glucose levels (i.e. 60 min; Fig. 3), possibly due to plasma glucose variations among animals. The mechanism for these measured behavioral and physiological differences is unknown, but our data do not support the existence of circadian rhythm control of the resting HPI axis (no pre-stress cortisol differences between the 08.00 h and 20.00 h groups, Fig. 1). However, our sampling protocol was not designed to measure resting HPI axis diel rhythms, which

have been reported in other fishes (Barton et al., 1986; Belanger et al., 2001).

The influence of the diel cycle on fish behavior and physiology has been examined to a limited extent. For example, the timing of migratory movements of Atlantic menhaden (*Brevoortia tyrannus*) and foraging efforts of rainbow trout (*Oncorhynchus mykiss*) are influenced by the diel cycle (Forward et al., 1996; Sanchez-Vazquez and Tabata, 1998). Furthermore, Emata et al. (1991) found daily and seasonal differences in cortisol, estradiol, progesterone, testosterone, thyroxin and thyroid hormone levels in gulf killifish (*Fundulus grandis*) sampled every 4 h for 3 days, demonstrating complex endocrine control. The diel cycle was reported to have little influence on the acute stress response in Chinook salmon (Barton et al., 1986), however, this phenomenon has not been studied in

## HPI Modification: Temperature

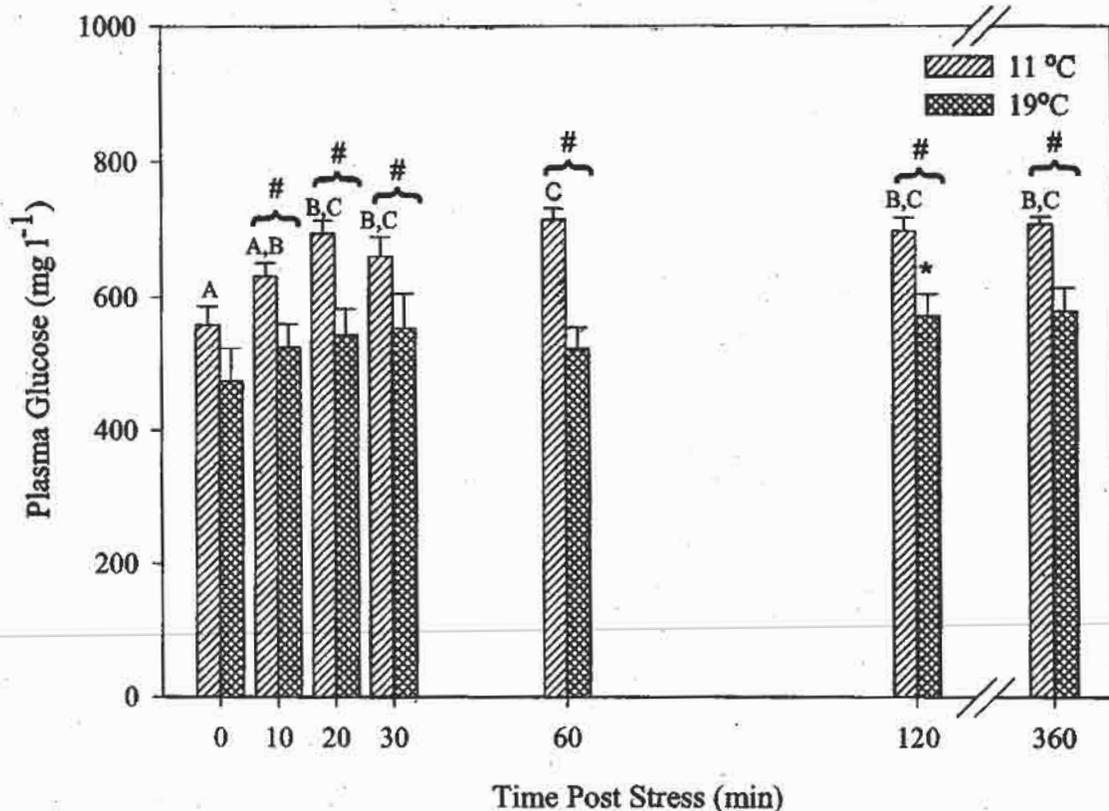


Fig. 6. Plasma glucose responses to a 1-min air emersion in green sturgeon acclimated to 11 (hatched bars) or 19 (cross-hatched bars) °C. Values are means  $\pm$  S.E. ( $n=5-6$ ). Bars with same letter are not significantly different (one-way repeated measures ANOVA with Tukey's multiple comparison test;  $P < 0.05$ ); # denotes a significant difference between temperature groups (two-way ANOVA;  $P < 0.05$ ) and \* denotes a significant difference from control ( $t$ -test;  $P < 0.05$ ).

most fishes. Breuner et al. (1999) reported that the maximal glucocorticoid response to a stressor corresponded to the onset of the active period in Gambel's white-crowned sparrows. Our data are consistent with this finding. Indeed, we demonstrate that increased responsiveness to a stressor occurs at nightfall in green sturgeon, which corresponds to their increased activity period. An increased stress response during the onset of the active period could be associated with increased swimming activity (e.g. foraging) and increased conspicuousness to potential predators and prey. This information is useful to fisheries and (potential) aquaculture managers to decrease stress-related impacts.

The rate of synthesis of cortisol was apparently delayed in green sturgeon acclimated to 11 °C compared with those acclimated to 19 °C (Fig. 4).

Although similar cortisol levels were reached in both groups, maximal concentrations were attained by 10 min post-stressor at 19 °C, compared with 30 min post-stressor in fish maintained at 11 °C (Fig. 4). The data also suggest a slower rate of plasma cortisol clearance at 11 °C, with levels returning to resting values by 90 min after the measured peak compared to the 20-min clearance time in fish maintained at 19 °C. Juvenile Chinook salmon acclimated to 7.5, 12.5 or 21 °C also demonstrate increasing cortisol synthesis and clearance rates with increasing temperatures (Barton and Schreck, 1987). This delay in the cortisol response to the stressor may be due to a temperature dependent reduction in enzymatic activity resulting in decreased synthesis and clearance rates for cortisol. The physiological impact of this temperature dependent modification of the cortisol

response on the animal is uncertain, given the possibility that similar alterations of other metabolic components might be expected. Finally, the basal or resting concentrations of cortisol noted here in green sturgeon are consistent with the observations of Rotlant et al. (2000) for gilthead sea bream (*Sparus aurata*), in that their resting plasma cortisol levels were no different between the two temperature groups after 15 days of acclimation.

Resting plasma lactate levels were significantly higher in green sturgeon acclimated to 19 °C compared with those at 11 °C and there was a trend for increased lactate levels at 19 °C during every time point measured (Fig. 5). The physiological basis for these temperature dependent differences is unknown, perhaps relatable to greater excitability (including swimming bursts) at the warmer temperature. Our data show a sustained stressor-induced elevation of plasma glucose concentrations, presumably via both glycogenolysis and gluconeogenesis, at 11 °C. The rapid (20–120 min) increase in plasma glucose levels is probably due to the glycogenolytic actions of catecholamines acting at the muscle and liver, thereby freeing up glucose from glycogen stores (Larsson, 1973; Randall and Perry, 1992). The prolonged (360 min) increase in plasma glucose is more consistent with a gluconeogenic mechanism, which increases plasma glucose via conversion of amino acids, lactate and glycerol by phosphoenolpyruvate carboxykinase (PEPCK) and is known to be under direct cortisol control (Foster and McGarry, 1996; Vijayan et al., 1996). Why virtually no increase in plasma glucose was measured in fish at 19 °C (except at 120 min; Fig. 6) is unknown. Possible reasons could be slower rates of glucose uptake by tissues at 11 °C or an increased utilization of free glucose at 19 °C, associated with increased metabolic rates at elevated temperatures (Brett and Groves, 1979). However, we never measured a significant and prolonged increase in plasma glucose in any of the fish stressed at 19 °C (Figs. 3 and 6). Taken together with the trend of increased plasma lactate levels, the plasma glucose data suggest that green sturgeon held at 19 °C might be depleted of adequate glycogen stores (e.g. to assure free glucose for CNS function) and relying upon alternative sources of energy to maintain glucose homeostasis. There is also a possibility that the HPI axis, like other physiological mechanisms, has an optimal temperature at which its

response is the most adaptive to the organism. For example, reproductive function (Kelley et al., 1991), cardio-respiratory status (Schreer et al., 2001) and swimming performance (Myrick and Cech, 2000) have optimum temperature ranges in fishes, which if exceeded, result in reduced function and efficiency.

Our data suggest that environmental factors such as time of day and temperature should be considered when designing stress-related experiments and when interpreting their results. Some phenomena described in previous studies on the effects of stress in fishes and other vertebrates might be explainable by differences in sampling time or environmental conditions during the experiment. This information could also be used in the design of management protocols for fisheries and aquaculture applications to minimize stress-related impacts of operational procedures.

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## Cortisol response of green sturgeon to acid-infusion stress

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### Abstract

Cortisol and lactate are classic indicators of stress in fishes and their interactive effects on metabolism during recovery from stress have recently become a subject of more intense study. We examined how stressing green sturgeon through acid infusion affected the cortisol response and lactate metabolism in green sturgeon (*Acipenser medirostris*). Both lactic acid (0.3 M) and HCl (0.3 N) infusion (infusion volumes 1.5 ml kg<sup>-1</sup>) elicited an immediate cortisol response (21.61 ± 4.61 ng ml<sup>-1</sup> and 17.50 ± 3.00 ng ml<sup>-1</sup>, respectively). Lactic acid prolonged the cortisol response compared to HCl (90 min vs. 25 min). Neutralized lactate (0.23 M; with 1 N NaOH; final pH 7.8) and NaCl (0.9%) infusion (infusion volumes 1.5 ml kg<sup>-1</sup>) did not affect plasma cortisol. Sturgeon infused with lactic acid showed a faster rate of lactate disappearance from plasma than those with neutralized lactic acid. We relate these findings to lactate metabolism following exercise, acid-infusion and air immersion stress in fishes.

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**Keywords:** Acid infusion; *Acipenser*; Cortisol; Lactate; Stress; Sturgeon

### 1. Introduction

The physiology of lactate metabolism in fish has long been a subject of intense study, with most investigators focusing on the processes following exercise stress (Black, 1957; Turner and Wood, 1983a; Turner et al., 1983b; Milligan and Wood, 1986; Milligan and Girard, 1993; Wood, 1991; Milligan, 1996). Exercise stress results in a metabolic acidosis caused by the accumulation of lactic acid in muscle and blood and rapid depletion of glycogen stores from exercised muscle. The time required for total recovery, measured by a restoration of blood pH, lactate and muscle glycogen is

dependent on a variety of factors, including species, temperature and activity level during the recovery period.

Exercise stress in fish also elicits the classic stress response, characterized by increases in circulating catecholamines and glucocorticoids (Butler et al., 1986; Milligan, 1996). Like lactate metabolism post-exercise, this hormonal stress response in fishes has been studied extensively (Pickering, 1981; Pickering and Pottinger, 1995; Wendelaar Bonga, 1997; Barton, 2002). The increase in catecholamines, secreted by chromaffin cells of the head kidney in teleosts, is short-lived and stimulates changes in the cardiorespiratory system that enhance oxygen transport during the exercise and initial recovery periods, including increased ventilation rate, branchial perfusion and blood oxygen carrying capacity. Their metabolic effects include stimulation of hepatic glycogeno-

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lysis, mobilization of fatty acids and an elevation of overall oxygen consumption rate, the latter of which is thought to enhance repayment of the oxygen debt incurred during the burst exercise period. The primary glucocorticoid in teleosts is cortisol, which is secreted by the interrenal cells in the head kidney in response to stimulation of the hypothalamo-pituitary axis and subsequent secretion of adrenocorticotrophic hormone (ACTH) (Donaldson, 1981). Once cortisol enters the circulation, it acts on hepatocytes to stimulate gluconeogenesis and glycogen synthesis (Mommsen et al., 1999).

The interactive effects of cortisol and lactate metabolism following exercise stress in fish have recently become a subject of closer study (Pagnotta et al., 1994; Milligan, 1996, 1997; Eros and Milligan, 1996). These studies have found the rate of lactate clearance and glycogen repletion are enhanced when cortisol increases are prevented pharmacologically with metyrapone or dexamethasone. Cortisol prevents net glycogen synthesis from lactate in trout muscle and, therefore, prolongs metabolic recovery (Milligan, 2003). Mommsen et al. (1999) have proposed that elevated cortisol following exercise stress functions to provide a glycogenic/gluconeogenic substrate for liver glycogen repletion at the expense of muscle glycogen stores and prolonged recovery time.

We set out to determine if lactic acid infusion into green sturgeon (*Acipenser medirostris*) sufficient to elevate plasma lactate near concentrations observed after exercise stress, elicited a cortisol response different from that seen in fish infused with hydrochloric acid, sodium chloride, or neutralized lactic acid and whether that cortisol response affected the rate of lactate disappearance from plasma. In addition, we monitored blood  $P_{O_2}$  and pH to gauge any effects on ventilation and acid–base balance caused by the infusions. We measured plasma glucose to determine if any cortisol increase observed was sufficient to induce hyperglycemia. No previous exercise stress data were available for this species; therefore we strenuously exercised green sturgeon to exhaustion and monitored their lactate recovery. We discuss our results in the context of lactate metabolism during recovery from various kinds of stress in fishes.

## 2. Materials and methods

### 2.1. Experimental animals

Green sturgeon (*A. medirostris*) with body mass 1203–5134 g and total body length (TBL) 70.5–113.7 cm were obtained from the University of California, Davis, Center for Aquatic Biology and Aquaculture (CABA), where they were hatched and reared indoors under natural photoperiod for approximately 6 months, then transferred to outdoor tanks. The animals were maintained at temperatures ranging from 15–25 °C, were provided a continuous flow of fresh, unchlorinated well water, and fed commercial fish pellets (Silver Cup, Murray, UT, USA) ad libitum daily prior to experimentation. Fish were transferred from their outdoor tanks at CABA, in coolers salted to 10 parts per thousand (ppt) with rock salt (NaCl), to our laboratory where they were held for 12 h without flow in indoor tanks also salted to 10 ppt. Fresh well-water flows were turned on and maintained for 1–2 days until cannulation. The fish were not fed while held in the laboratory.

### 2.2. Cannulation

Cannulation of the dorsal aorta was performed as described previously (Crocker et al., 2000). Briefly, all fish were anesthetized in buffered MS222 (Sigma, St. Louis, MO, USA) and placed on a surgical board ventral side up. The mouth was protracted and a heat tapered PE50 cannula (Intramedic) threaded over a stainless steel guide wire was inserted into the dorsal aorta and the wire withdrawn. The catheter was flushed with heparinized saline (50 IU sodium heparin  $ml^{-1}$  0.9% NaCl), sutured to the roof of the buccal cavity and led out the side of the mouth. An additional suture secured the catheter to the animal's flank. Animals were allowed one to two days to recover from cannulation before the start of experiments.

### 2.3. Exercise experiments

The goal of the infusion experiments was to elevate plasma lactate levels to physiologically relevant concentrations. Since there were no such published values for green sturgeon at the time of the study, preliminary strenuous exercise experiments were carried out on four green sturgeon

cannulated as described above. Sturgeon (mean  $\pm$  S.D. body mass  $1576 \pm 252$  g; mean  $\pm$  S.D. TBL  $76 \pm 3$  cm;  $n=4$ ) were chased by hand until exhausted (approx. 5 min), at which time the animals were unwilling to swim. Blood samples were taken at rest and immediately, 30 min, 2 h and 4 h post-exercise for analysis of plasma lactate.

Thirty minutes after chasing until exhaustion, plasma lactate peaked at a mean ( $\pm$  S.E.M.)  $4.98 \pm 1.17$  mmol  $l^{-1}$ , which we chose as our target concentration for the lactic acid infusions. We determined from subsequent preliminary lactic acid infusion experiments that 1.5 ml 0.3 M lactic acid per kilogram sturgeon would allow us to approach the plasma concentrations seen during recovery from exercise.

#### 2.4. Infusion experiments

After two days of recovery from surgery, sturgeon were randomly chosen for infusion with 1.5 ml  $kg^{-1}$  of one of the following solutions: 0.3 M lactic acid (mean  $\pm$  S.D. body mass  $2163 \pm 487$  g; mean  $\pm$  S.D. TBL  $78 \pm 4$  cm;  $n=9$ ), 0.3 N HCl (mean  $\pm$  S.D. body mass  $4149 \pm 705$  g; mean  $\pm$  S.D. TBL  $101 \pm 9$  cm;  $n=8$ ), 0.3 M lactic acid titrated to 7.8 with 1 N NaOH, final lactate concentration approximately 0.23 M (mean  $\pm$  S.D. body mass  $4190 \pm 586$  g; mean  $\pm$  S.D. TBL  $102 \pm 7$  cm;  $n=8$ ; subsequently 0.23 M neutralized lactic acid) or 0.9% NaCl (mean  $\pm$  S.D. body mass  $2515 \pm 1295$  g; mean  $\pm$  S.D. TBL  $85 \pm 13$  cm;  $n=10$ ). Infusions were done acutely by hand and infusion rates were standardized by watching a hand timer. Infusion rates and total times ranged from 0.82–1.82 ml  $min^{-1}$  and 1.2–6.9 min, respectively. Cannulae were flushed with 250  $\mu$ l 0.9% NaCl after acid infusion. Blood samples of 700  $\mu$ l were drawn into glass syringes at rest, 5, 25, 45, 90 and 120 min after infusion. Approximately 400  $\mu$ l were used for determination of blood  $Po_2$  and pH. The remainder of the sample was centrifuged at  $2000 \times g$ , and the plasma placed in a cryovial and then frozen for subsequent analysis of lactate, glucose and cortisol.

#### 2.5. Analytical methods

Blood  $Po_2$  and pH were measured using a Cameron Instruments (BGM 200) blood gas analysis system thermostatted to the tank temperature

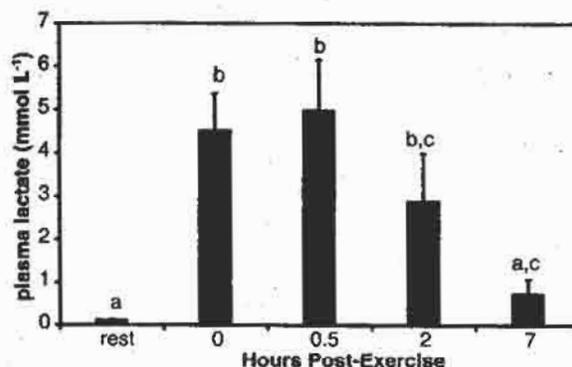


Fig. 1. Plasma lactate concentration in green sturgeon (lactate,  $n=4$ ) after a 5 min bout of exhaustive exercise (mean  $\pm$  S.E.M.). Differing letters indicate a significant difference between time points (one-way repeated measures MANOVA,  $P < 0.05$ , Student's  $t$ -test post-hoc).

(18.5–19.4 °C) of the experimental animal. Lactate and glucose were measured in duplicate using a YSI 2700 Select biochemistry analyzer. Cortisol was determined using an ELISA performed by the Endocrine laboratory, Department of Population Health and Reproduction in the UC Davis School of Veterinary Medicine using a protocol adapted from Barry et al. (1993).

#### 2.6. Statistical analysis

Resting and post-exercise plasma lactate was analyzed with one-way repeated measures MANOVA to determine if there were any differences between time points. Resting and post-infusion parameters were analyzed using two-way repeated measures MANOVA. Student's  $t$ -tests were used to determine if means were different between treatments and time points when MANOVAs detected a significant difference. Differences were considered significant when  $P < 0.05$ . All statistical computations were carried out using JMP 4.0 (SAS Institute).

### 3. Results

#### 3.1. Exercise experiments

Five minutes of chasing to exhaustion increased plasma lactate to  $4.50$  mmol  $l^{-1}$  almost immediately, remained significantly elevated from resting values ( $4.98$  mmol  $l^{-1}$ ) at 30 min into recovery and approached full recovery at 4 h post-exercise (Fig. 1).

Table 1

Blood pH from green sturgeon at rest and after infusion with one of four solutions (mean ± S.E.M.)

Time (min)	0.9% NaCl	0.3 M lactic acid	0.3 N HCl	0.23 M lactate, pH 7.8
Rest	7.89 ± 0.05 <sup>1</sup> (10)	7.88 ± 0.05 <sup>1</sup> (9)	7.95 ± 0.05 <sup>1</sup> (8)	7.98 ± 0.06 <sup>1</sup> (8)
5	7.91 ± 0.05 <sup>1</sup> (10)	7.76 ± 0.05 <sup>2</sup> (9)	7.89 ± 0.05 <sup>1</sup> (8)	7.97 ± 0.04 <sup>1</sup> (8)
25	7.89 ± 0.04 <sup>1</sup> (10)	7.81 ± 0.03 <sup>1</sup> (9)	7.90 ± 0.03 <sup>1</sup> (8)	7.95 ± 0.04 <sup>1</sup> (8)
45	7.91 ± 0.05 <sup>1,2</sup> (10)	7.87 ± 0.04 <sup>2</sup> (9)	7.96 ± 0.04 <sup>1</sup> (8)	7.93 ± 0.06 <sup>1,2</sup> (8)
90	7.89 ± 0.04 <sup>1</sup> (10)	7.88 ± 0.03 <sup>1</sup> (9)	7.98 ± 0.04 <sup>1</sup> (8)	7.97 ± 0.03 <sup>1</sup> (8)
120	7.92 ± 0.03 <sup>1</sup> (10)	7.87 ± 0.03 <sup>1</sup> (9)	7.97 ± 0.04 <sup>1</sup> (8)	7.98 ± 0.05 <sup>1</sup> (8)

Differing numbers indicate significant difference ( $P < 0.05$ ) between treatments at a specific time point. Number in parentheses indicates number of animals per group ( $n$ ).

### 3.2. Infusion experiments

Lactic acid infusion decreased blood pH to levels significantly lower than animals in the other treatments at 5 min post-infusion, but was never statistically different between time points within the treatment (Table 1). HCl, neutralized lactic acid and NaCl had no effect on blood pH. Arterial  $P_{O_2}$  was unaffected by the infusions within each treatment except in animals infused with HCl, which showed slightly elevated blood  $P_{O_2}$ 's at 45 min (Table 2). Overall, the ventilatory status of the animals with respect to blood  $P_{O_2}$  was unaltered by the infusions.

Plasma lactate (Fig. 2) was significantly increased by infusion of 0.3 M lactic acid, but to levels slightly less than half of those seen after exercise: 2.22 mmol  $l^{-1}$  from infusion vs. 4.98 mmol  $l^{-1}$  from exercise. Plasma lactate remained significantly elevated from resting values within the treatment up until 90 min post-infusion. The infusion of 0.23 M neutralized lactic acid also significantly elevated plasma lactate, but was significantly lower at 5 min than animals infused with 0.3 M lactic acid. This was expected because the infusions were of equal volumes not equal molarities. Despite this, neutralized lactic acid plasma lactates were significantly higher than lac-

Table 2

Blood  $P_{O_2}$  from green sturgeon at rest and after infusion with one of four solutions (mean ± S.E.M.)

Time (min)	0.9% NaCl	0.3 M lactic acid	0.3 N HCl	0.23 M lactate, pH 7.8
Rest	98.71 ± 3.79 <sup>a,1</sup> (10)	98.92 ± 6.01 <sup>a,1</sup> (9)	79.42 ± 4.35 <sup>a,b,2</sup> (8)	76.51 ± 8.80 <sup>a,2</sup> (8)
5	105.15 ± 6.05 <sup>a,1</sup> (10)	95.46 ± 3.38 <sup>a,1</sup> (9)	71.80 ± 7.18 <sup>a,2</sup> (8)	75.80 ± 8.26 <sup>a,2</sup> (8)
25	102.96 ± 4.51 <sup>a,1</sup> (10)	94.91 ± 5.76 <sup>a,1,2</sup> (9)	85.99 ± 7.31 <sup>a,b,2</sup> (8)	81.76 ± 5.46 <sup>a,2</sup> (8)
45	98.54 ± 4.48 <sup>a,1,2</sup> (10)	102.69 ± 3.98 <sup>a,2,3</sup> (9)	90.71 ± 5.62 <sup>b,1,3</sup> (8)	83.45 ± 7.34 <sup>a,1</sup> (8)
90	99.75 ± 4.32 <sup>a,1,2</sup> (10)	106.59 ± 4.87 <sup>a,1</sup> (9)	88.29 ± 7.74 <sup>a,b,2,3</sup> (8)	78.30 ± 7.57 <sup>a,3</sup> (8)
120	99.58 ± 6.13 <sup>a,1</sup> (10)	106.53 ± 5.07 <sup>a,1</sup> (9)	74.94 ± 10.44 <sup>a,b,2</sup> (8)	81.70 ± 6.16 <sup>a,2</sup> (8)

Differing letters indicate significant differences ( $P < 0.05$ ) between time points within a treatment. Differing numbers indicate significant difference ( $P < 0.05$ ) between treatments at a specific time point. Number in parentheses indicates number of animals per group ( $n$ ).

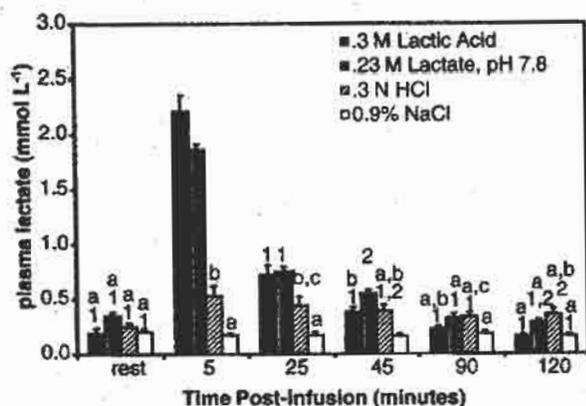


Fig. 2. Plasma lactate concentration in green sturgeon after infusion of 0.3 M lactic acid ( $n=9$ ), 0.3 N HCl ( $n=8$ ), 0.23 M lactic acid solution neutralized to pH 7.8 ( $n=8$ ) or 0.9% NaCl ( $n=9-10$ ; one sample lost during processing) (mean  $\pm$  S.E.M.). Common letters indicate non-significant differences between time points within a treatment. Common numbers indicate non-significant differences between treatments at a specific time point. Two-factor repeated measures MANOVA, Student's  $t$ -test as post-hoc,  $P < 0.05$ .

tic acid-infused animals at 45 min. This suggests plasma lactate is cleared more quickly in animals infused with the acid form. Infusion of HCl resulted in endogenous lactate production as indicated by the slight increase in plasma lactate that peaked 5 min post-infusion, but returned to control values by 45 min post-infusion. Infusion of 0.9% NaCl did not affect plasma lactate.

The 0.3 M lactic acid infusion elicited a cortisol response (Fig. 3) that was nearly immediate and maximal ( $21.6 \text{ ng ml}^{-1}$ ) after 5 min and remained significantly elevated from saline and within treat-

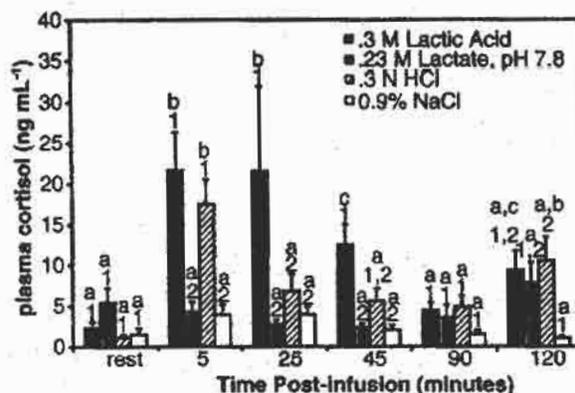


Fig. 3. Plasma cortisol concentration in green sturgeon after infusion of 0.3 M lactic acid ( $n=9$ ), 0.3 N HCl ( $n=7-8$ ; one sample lost during processing), 0.23 M lactic acid solution neutralized to pH 7.8 ( $n=8$ ) or 0.9% NaCl ( $n=10$ ) (mean  $\pm$  S.E.M.). Common letters indicate non-significant differences between time points within a treatment. Common numbers indicate non-significant differences between treatments at a specific time point. Two-factor repeated measures MANOVA, Student's  $t$ -test as post-hoc,  $P < 0.05$ .

ment resting values until 90 min post-infusion. Infusion of 0.3 N HCl also caused a significant and immediate increase in plasma cortisol to  $17.5 \text{ ng ml}^{-1}$ , not different from that in lactic acid infused fish. However, unlike the lactic acid-infused fish, HCl-infused fish had returned to resting values by 25 min, suggesting lactic acid prolongs elevated cortisol. Neither 0.23 M neutralized lactic acid or 0.9% NaCl elicited any significant cortisol response indicating the infusion, per se, did not stress the fish. Plasma glucose concentrations (Table 3) were not affected by any of the

Table 3

Plasma glucose concentrations from green sturgeon at rest and after infusion with one of four solutions (mean  $\pm$  S.E.M.)

Time (min)	0.9% NaCl	0.3 M lactic acid	0.3 N HCl	0.23 M lactate, pH 7.8
Rest	$3.50 \pm 0.26$ (10)	$3.35 \pm 0.14$ (9)	$3.66 \pm 0.36$ (8)	$3.37 \pm 0.14$ (8)
5	$3.44 \pm 0.23$ (10)	$3.57 \pm 0.15$ (9)	$3.81 \pm 0.38$ (8)	$3.33 \pm 0.10$ (8)
25	$3.41 \pm 0.23$ (10)	$3.76 \pm 0.15$ (9)	$3.80 \pm 0.38$ (8)	$3.33 \pm 0.11$ (8)
45	$3.35 \pm 0.25$ (10)	$3.87 \pm 0.17$ (9)	$3.90 \pm 0.41$ (8)	$3.41 \pm 0.09$ (8)
90	$3.39 \pm 0.21$ (10)	$3.83 \pm 0.14$ (9)	$3.96 \pm 0.46$ (8)	$3.32 \pm 0.11$ (8)
120	$3.40 \pm 0.22$ (10)	$3.79 \pm 0.12$ (9)	$3.98 \pm 0.49$ (8)	$3.36 \pm 0.14$ (8)

Number in parentheses indicates number of animals per group ( $n$ ).

infusions, despite the significant and prolonged increases in plasma cortisol seen in lactic acid infused fish.

#### 4. Discussion

These experiments revealed three important findings. First, that the amount of lactate accumulated in the blood as a result of exercise stress in green sturgeon is similar to other sturgeon species (Kieffer et al., 2001) but different from rainbow trout (Milligan, 1996). Second, that infusion of lactic acid prolongs the cortisol response, when compared to the effect of HCl infusion. Lastly, that the rate of lactate removal from plasma post-infusion is dependant on whether the lactate is infused in the acid form. We discuss these findings in the context of lactate and cortisol recovery metabolism after exercise and air-immersion stress in fishes.

##### 4.1. Metabolic and endocrine responses to exercise stress

A small sample size in the preliminary exercise experiments precludes us from drawing any strong conclusions about green sturgeon metabolic recovery from exercise stress. We can say, however, that plasma lactate levels in green sturgeon are similar to but somewhat higher ( $4.98 \text{ mmol l}^{-1}$  vs.  $1.5 \text{ mmol l}^{-1}$ ) than those seen in shortnose sturgeon after strenuous exercise (Kieffer et al., 2001) but much less than rainbow trout ( $10\text{--}15 \text{ mmol l}^{-1}$ ; Milligan, 1996).

##### 4.2. Metabolic and endocrine responses to acid-infusion stress

To our knowledge, this is the first study to examine the stress response of any fish to acid infusion. The infusion of a strong acid, organic or inorganic, clearly elicits a stress response, the dynamics of which are dependent on the type of acid infused. In this study, lactic acid extended the time plasma cortisol concentrations were elevated compared to those infused with HCl. Elevated plasma cortisol did not prolong the rate of lactate clearance from the plasma, as observed in trout recovering from exercise stress (Pagnotta et al., 1994; Eros and Milligan, 1996; Milligan 1997, 2003). Animals infused with lactic acid removed the lactate at a faster rate than those infused with

the sodium lactate solution, even though they were infused with slightly more lactate and had elevated cortisol levels.

The lack of expected correlation between plasma lactate disappearance and plasma cortisol might be explained by fundamental differences in how the two stressors, acid infusion and exercise, affect the animal's physiology independent of the stress response. Exercise leaves muscle glycogen stores depleted and most of the lactate produced is retained within the muscle that produces it (Milligan, 1996). Therefore, most of the metabolic recovery processes occur in the muscle post-exercise. Cortisol inhibits glycogen repletion from lactate in muscle; therefore, the rate of lactate metabolism is slowed (Milligan, 2003).

For animals infused with lactic acid, the situation is different. Muscle glycogen stores are unaffected and muscle lactate concentrations, presumably, start very low. Although perfused trout trunks have been demonstrated to take up lactate via a putative lactate transporter (Wang et al., 1997), recent evidence from trout sarcolemmal preparations suggest that fish muscle is relatively impermeable to lactate. A small amount is still able to diffuse passively in its undissociated form (Sharpe and Milligan, 2003). A previous study examining the distribution of  $^{14}\text{C-L-lactate}$  in resting rainbow trout showed that after 2 h, 78% of the injected label distributed to areas outside of the muscle, the largest fraction (36%) was excreted as  $\text{CO}_2$ . The specific activities of the blood, heart and liver were 32, 12 and six-fold higher, respectively, than muscle. Assuming sturgeon muscle is similar to that of trout, then little of the infused lactate actually enters the muscle, leaving oxidation by other tissues and hepatic gluconeogenesis as principal disposal routes. If one assumes the lactate load was distributed within the animal by the five-minute sample, then 70% of the lactate infused remained in the extracellular fluid in both lactate infused treatments. Therefore, the elevated cortisol seen after acid infusion should not inhibit lactate recovery. Although we did not measure muscle lactate in this study, it is unlikely that these measurements would have been useful in this study because of the high ratio of muscle mass to lactate load infused.

Our observation that the cortisol response is prolonged by lactic acid might be related to cortisol's stimulation of hepatic gluconeogenesis (Mommensen et al., 1999). If fish muscle is imper-

meable to lactate i.e. deficient of lactate transporters; (Sharpe and Milligan, 2003), lactate entering the plasma will be 'trapped' and mainly oxidized by other tissues or converted to glucose or glycogen via hepatic gluconeogenesis (Milligan and Girard, 1993). As cortisol stimulates these processes (Mommensen et al., 1999), the prolonged cortisol elevation following lactic acid infusion might function to enhance lactate clearance from plasma. Without cortisol, we would have expected a slower rate of lactate disappearance for a similar lactate load, which is what we found in our experiments (Figs. 2 and 3).

The results of Lankford et al. (2003) in their study of air immersion stress in green sturgeon show a similar temporal relationship between plasma lactate and cortisol during recovery. In that study, air immersion elevated plasma lactate to levels that were similar to our infusion study and an exercise study in shortnose sturgeon (1–2 mmol l<sup>-1</sup>, Kieffer et al., 2001). Plasma lactate returned to resting within 1 h in the air immersion study. Cortisol also peaked to levels that were similar to our study (approx. 18 ng ml<sup>-1</sup>) and remained elevated until 2 h post-stress. Although it is likely that air-immersed green sturgeon accumulated lactate in muscle, they likely also produced lactate in most other tissues, which would contribute to an elevated plasma lactate. If sturgeon muscle is even less permeable to lactate than trout muscle, the muscle lactate load might augment plasma lactate only to a limited extent, a probable situation because it is known that more benthic fish species, like sturgeon, retain a larger portion of the total lactate load in the muscle post-exercise stress (Wood, 1991). Elevated cortisol post-air immersion would lead to a greater rate of lactate disappearance from plasma and, because little lactate diffuses from the muscle into the plasma, lactate concentrations would return to resting values more rapidly. Muscle lactate measurements in sturgeon after exercise and air-immersion stress are required to confirm this hypothesis.

Our observations beg the question 'Does the presence of the lactate ion per se prolong the elevated cortisol concentrations?' The fact that HCl-infused animals had plasma cortisol levels that returned to resting values sooner suggests this might be the case. Also, we would expect cortisol to lag slightly behind plasma lactate as they both decline towards resting from their peak levels, which occurs in sturgeon after lactic acid infusion

and air immersion stress. Siberian sturgeon, after exposure to severe hypoxia, also shows a similar relationship between cortisol and plasma lactate, although the recovery was not followed to completion (Maxime et al., 1995). Infusing an animal with sodium lactate after infusion of HCl to elicit the initial cortisol response followed by subsequent monitoring of plasma cortisol would help to answer this question.

In conclusion, exercise stress in green sturgeon elicits the typical lactate responses seen in other fishes, albeit less than rainbow trout. Acute infusion of a strong acid is a stressor in this species and elicits a cortisol response. However, the duration of the stress response is longer for lactic acid than HCl. Lactate disappears more rapidly from plasma in green sturgeon infused with the acid form, a result we attribute to the elevated cortisol's stimulatory effect on hepatic gluconeogenesis. The temporal relationship between cortisol and lactate metabolism post-acid infusion is similar to that in other sturgeon studies during recovery from stress. Further studies should determine whether the presence of the lactate ion in plasma after the stress response is the stimulus for prolonged cortisol elevations.

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## Temperature Effects on Green Sturgeon Bioenergetics

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**Abstract.**—The green sturgeon *Acipenser medirostris* is relatively rare, and little is known about the effects of important habitat-related influences on its life history. Temperature directly or indirectly impacts the distribution of fish as well as their physiological and behavioral processes. Because the Klamath and Rogue rivers may reach 24–25°C and egg and embryo incubation temperatures above 22°C are associated with deformities in juvenile green sturgeon, data on the temperature-related bioenergetic responses of these juveniles would be especially useful in designing effective conservation and management strategies. We measured the food consumption, growth, food conversion efficiency, oxygen consumption, volitional activity, ventilatory frequency, thermal preference, and swimming performance of juveniles at several temperatures spanning their warm-season temperature range (11–24°C). Food consumption, growth, and food conversion efficiency generally increased with temperature between 11°C and 15°C but stayed constant between 15°C and 19°C. Growth increased and food conversion efficiency decreased with ration size. Oxygen consumption, volitional activity rate, and ventilatory frequency generally increased with temperature, while preferred temperature increased and swimming performance decreased with temperature from 19°C to 24°C. Relative to other sturgeon species, the green sturgeon has high growth and oxygen consumption rates. Overall, we found that bioenergetic performance was optimal between 15°C and 19°C under either full or reduced rations, thus providing a temperature-related habitat target for conservation of this rare species.

The North American green sturgeon *Acipenser medirostris* is an anadromous chondrosteian with a distribution ranging from the Bering Sea to Ensenada, Mexico (Moyle et al. 1995). Despite this wide range, less is known about the green sturgeon than the other 24 living sturgeon species worldwide (Erickson et al. 2002). Green sturgeon are known to spawn in three Pacific river systems: the Rogue River in Oregon and the Sacramento and Klamath rivers in California (Moyle 2002). The temperature in the Klamath River varies from 4°C to 25°C (C. Chamberlain, Yurok Tribal Fisheries Program, personal communication) due to natural (e.g., seasonal and precipitation-related cycles) as well as human-induced (e.g., impoundments) effects. The Rogue River is the most northern of the three, with temperature ranging from 4°C to 24°C during 2000–2001 (Erickson et al. 2002). Because egg and embryo incubation temperatures above 22°C are associated with deformities in juvenile green sturgeon (Van Eenennaam et al. 2003) and temperature directly or indirectly affects the distribution of fish as well as their physiological and

behavioral processes (Cech et al. 1990; Schmidt-Nielsen 1999), it is important to determine the temperature-related effects on green sturgeon. The use of environmental temperatures in defining optimal fish habitat or restricting the distribution of species is well documented (McCauley and Fry 1986; Armour 1991; Hurst and Conover 2002).

Green sturgeon are considered an “at risk” species by the California Bay-Delta Authority and a species of special concern by the California Department of Fish and Game. Habitat changes (e.g., due to dams, loss of riparian cover, and thermal pollution) leading to temperature increases are known to have serious consequences for resident fish populations (Chart and Bergesen 1992). Data on this species’ temperature-related responses, particularly regarding their patterns of resource use and survival (Wainwright 1994; Cech and Crocker 2002) would be particularly useful in designing effective conservation and management strategies. Temperatures in regulated rivers such as the Rogue, Sacramento, and Klamath can be altered with releases of reservoir water from various depths. Warm temperatures associated with low, drought-induced flows in a major Sacramento tributary, the Feather River, produced major changes in its fish community (Moyle et al. 1983).

The energetic (metabolic) demands of fish typically increase with temperature (reviewed by Fry

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1971). As a result, food consumption (energy ingested) often increases with increasing water temperature to satisfy increasing body maintenance demands and, often, increasing growth and reproduction demands. Our objectives were to assess the effects of temperature on important bioenergetic variables in juvenile green sturgeon and to compare these effects with those documented for other sturgeons. These variables can be modeled as

$$I = G + A + M + R + E + SDA, \quad (1)$$

where the energy ingested ( $I$ ) is balanced by energy allocated to growth ( $G$ ), activity ( $A$ ), maintenance ( $M$ ), reproduction ( $R$ ), excretion ( $E$ ), and specific dynamic action (SDA, i.e., the energy associated with digestive and anabolic processes following feeding; Jobling 1994).

#### Methods

The age-0 and age-1 green sturgeon (mean body weight range, 150–851 g) used in this 2-year study were progeny of wild-caught Klamath River sturgeon that had been artificially spawned during late May 1999 (Van Eenennaam et al. 2001). Eggs were incubated at the University of California-Davis and juveniles reared in aerated water at temperatures similar to those in the Klamath River (11–15°C) during late spring. The age-0 fish were fed commercial Silvercup trout pellets at 3–5% of body weight per day based on a feeding table for white sturgeon *A. transmontanus*. Fish (age, 31-d posthatch) were acclimated (1°C/d) to 19°C (ambient well water temperature) and held in round 284-L fiberglass holding tanks. Because tanks received continuous flows of the air-equilibrated, 19°C well water, high water quality conditions were ensured until fish were needed for experiments. Within the temperature control limitations of the growth laboratory facility, the experimental temperatures that were chosen spanned the warm-season temperature ranges of the Klamath and Rogue rivers. Dissolved ammonia concentrations were measured twice weekly and were always below detection limits (<0.01/L).

#### Experiment 1: Food Consumption, Growth, and Food Efficiency

At 144 d posthatch, fish (body weight, 149.8 ± 41.0 g [mean ± SD];  $n = 144$ ) from a holding tank were randomly distributed into 24 round 110-L tanks (six fish/tank) and either held at 19°C or acclimated to 11°C or 15°C (at 1°C/d). There were four replicate tanks for each of the six treatments

(three temperatures × two ration levels) for the 33-d experiment, which was sufficient to measure substantial growth. Tanks were indoors and were maintained on a natural photoperiod for 38°55'N in October–December using both natural (translucent roof panels) and artificial (fluorescent lights) sources. Incoming water flow (4 L/min) was adjusted by means of angled spray bars to provide a current of 10 cm/s, and current direction was reversed every 5 d to uniformly exercise the fish.

**Food consumption.**—Half of the age-0 fish (tanks selected randomly) at each temperature were fed to satiation (range, 8.0–13.1 g Silvercup pellets per tank of 6 fish per feeding), and the rest were fed at 50% of satiation (as measured from the observed feeding levels of sated fish at each temperature). Fish were fed twice daily, and the amount of food consumed was quantified by collecting (after 20 min) and counting the uneaten pellets and subtracting their predetermined weight from that of the total fed at each feeding. Due to observable erosion of the pellets in the water, feeding was limited to 20 min so that uneaten pellets could still be counted. This erosion may have resulted in an overestimate of food consumption rates and an underestimate of food conversion efficiency. The mean daily food consumption rate (CR; grams of feed per gram of fish per day) for each tank of fish was calculated from the equation

$$CR = \frac{\sum C \log_e(W_2/W_1)}{t(W_2 + W_1)} \quad (2)$$

where  $C$  = total food consumed (dry weight [g]) for the duration of the experiment;  $W_1$  and  $W_2$  = individual fish wet weights (g) at the beginning and end of the experiment, respectively; and  $t$  = experiment duration (i.e., 33 d).

**Growth.**—The age-0 fish were individually weighed and measured at 11-d intervals and were fasted 24 h prior to being weighed and measured to minimize food weight contributions. Just prior to weighing, the fish were blot-dried. Fork and total lengths were measured (mm), and weight (nearest 0.1 g) was determined with an electronic balance. Mean live weights were used to calculate the instantaneous specific growth rate (SGR; percent change in weight per day) with the equation

$$SGR = \frac{\log_e(W_2/W_1)}{t} (100) \quad (3)$$

**Food conversion efficiency.**—After the experiment, six age-0 fish from each treatment were dried

to a constant weight at 60°C in a drying oven to derive the following relationship between dry weight (DW) and wet weight:

$$DW = 0.203W - 3.435 \quad (R^2 = 0.92),$$

where  $W$  = wet weight. We calculated gross food conversion efficiencies (CE; weight gained per gram of feed) for each tank from the equation

$$CE = \frac{DW_2 - DW_1}{\sum C} \quad (4)$$

where  $DW_1$  = the mean dry weight (g) of fish sampled from the holding tank at the start of the experiments and  $DW_2$  = the mean dry weight of the experimental fish in each tank at the end of the experiment.

#### Experiment 2: Metabolic Rate and Activity

Metabolic (oxygen consumption) rates were measured for both age-0 (routine rates) and age-1 fish (resting routine rates). Routine metabolic rates were determined by measuring oxygen consumption rates via closed respirometry (Cech 1990). Fish (age-0; body weight,  $65.6 \pm 362.2$  g,  $n = 39, 33, \text{ and } 33$  for 11, 19, and 24°C, respectively) were randomly taken from their holding tanks and transferred to three indoor experimental tanks also receiving air-equilibrated well water at 19°C. Two randomly chosen tanks were cooled or warmed at 1°C/d to reach the acclimation temperatures of 11°C and 24°C, respectively. This temperature range spanned the warm-season range of the Klamath and Rogue rivers (Chamberlain, personal communication; Erickson et al. 2002). Respirometers were of clear polyvinyl chloride (PVC) pipe construction with an opaque PVC end cap and neoprene stopper and were equipped with water flushing and sampling tubes on the ends. Individual postabsorptive fish were acclimated for at least 6 h in the respirometers before the start of the experiments. A blank respirometer (i.e., one without fish) was used to account for microbial respiration. As a pilot study showed that age-0 fish continued to exhibit activity (tail beats) even after 8 h of acclimation, we quantified the tail beats using videotapes from an overhead video camera. Metabolic rates ( $MO_2$ ; mg  $O_2$ /h) were calculated from the equation

$$MO_2 = \frac{(CO_{2i} - CO_{2f})}{t} (V_R) \quad (5)$$

where  $CO_{2i}$  and  $CO_{2f}$  = the respirometer  $O_2$  con-

centrations (mg/L) at the beginning and end of the experiment;  $V_R$  = the respirometer volume (L); and  $t$  = the experiment time (h). The  $O_2$  partial pressures ( $PO_2$ ) were measured with a Radiometer PHM71/E5046/D616  $O_2$  analyzer system and converted to  $O_2$  concentrations using an  $O_2$  solubility nomogram (Green and Carrit 1967).

The resting routine metabolic rates of juvenile green sturgeon (age-1;  $n = 19, 21, \text{ and } 20$  for 11, 19, and 24°C, respectively) were determined by measuring oxygen consumption rates via open respirometry (Cech 1990). Age-1 fish were transferred and acclimated as described above. Fish (body weight,  $851.2 \pm 240.2$  g) were completely quiescent in their triangular (cross-sectional) 11-L respirometers after 8 h of respirometer acclimation. Respirometer water flow rates were measured by means of the timed collection of water in a calibrated, graduated cylinder, and oxygen contents were calculated from inflow and outflow  $PO_2$ s, as above. Measurements from a blank respirometer accounted for microbial respiration. Age-1 fish  $MO_2$ s were calculated from the equation

$$MO_2 = (CO_{2in} - CO_{2out}) (V_w), \quad (6)$$

where  $CO_{2in}$  and  $CO_{2out}$  = the  $O_2$  contents (mg  $O_2$ /L) of water flowing into and out of the respirometer and  $V_w$  = the water flow rate through the respirometer (L/h). The effects of temperature on metabolic rates were determined from  $Q_{10}$  values (Schmidt-Nielsen 1999), which express the rate of change in a process with temperature as follows:

$$Q_{10} = (R_2/R_1)^{10/(T_2-T_1)} \quad (7)$$

where  $R_2$  is the rate at  $T_2$  (the higher temperature) and  $R_1$  is the rate at  $T_1$  (the lower temperature).

#### Experiment 3: Thermal Preference

Thermal preference experiments on age-0 green sturgeon (weight,  $38.4 \pm 17.2$  g;  $n = 20, 20, \text{ and } 9$  for 11, 19, and 24°C, respectively) were conducted in an annular, 1-m-diameter, flow-through thermal gradient tank constructed of clear acrylic plastic and designed to avoid vertical stratification (Myrick et al. 2004). Age-1 fish were too large for this apparatus. A light-colored shade cloth cover shielded the apparatus and fish from investigators, minimizing tank position effects. Observations of the individual fish positions were made via a CRT monitor wired to an overhead video camera. Water flows throughout the thermal gradient tank were isothermal at the acclimation temperature for the first hour after each fish was placed in the appa-

ratus. Then the thermal gradient (11.5–31.0°C) was established. During each 1-h experiment, the location of the fish and the corresponding water temperature data (YSI 44TD telethermometer with 10 calibrated YSI 401 thermistor probes placed at regular intervals around the gradient tank) were recorded at 10-min intervals. Fish could easily swim around the entire annulus in 30 s, minimizing any possible space and time autocorrelation.

#### Experiment 4: Swimming Performance

The critical swimming velocities ( $U_{crit}$ ) of age-1 sturgeon (weight,  $1,132.5 \pm 424.2$  g;  $n = 19$ , 11, and 8 for 11, 19, and 24°C, respectively) were determined during year 2 of the study with a 200-L recirculating-water flume (Brett 1964) incorporating a variable-speed motor. The flume was partly immersed in a temperature-controlled water bath, and the velocities were calibrated with a digital Marsh-McBirney (Model 201D) water current meter. Individual fish were placed in the swimming chamber, and after 1 h of acclimation at 10 cm/s, the  $U_{crit}$  was measured by 10-cm/s increases in water velocity every 20 min until the fish became fatigued (Beamish 1978). A fish was considered fatigued when it impinged three times at the downstream end of the chamber. Absolute  $U_{crit}$  ( $U_{crit_a}$ ) was calculated (Brett 1964) from the equation

$$U_{crit_a} = U_i + (10 \text{ cm/s}) \cdot (T_i \cdot 20/\text{min}), \quad (8)$$

where  $U_i$  = the highest velocity maintained for 20 min and  $T_i$  = the time elapsed at fatigue velocity. Tail beat frequencies were measured for each fish by counting the number of tail beats over a 1-min period at each swimming velocity.

#### Statistical Analyses

A one-way analysis of variance (ANOVA; parametric data) was conducted (SIGMASTAT software) to test for significant effects of experimental factors (e.g., temperature;  $n > 2$ ), Tukey's tests being used for post hoc pairwise comparisons. A two-way ANOVA was also conducted to test for significant interactions between experimental factors (temperature and ration), Tukey's tests being used for post hoc pairwise comparisons. A Kruskal-Wallis test was conducted to test for significant effects and Dunn's test for post hoc pairwise comparisons. Student's *t*-tests were used to compare the two food ration levels. Analysis of covariance (ANCOVA) was used to determine significant interacting effects of body weight and activity on metabolic rate (SAS software). Differences were considered significant at  $P < 0.05$ .

## Results

### Food Consumption

Mean food consumption rates increased significantly with temperature (Table 1) for both ration levels between 11°C and 15°C ( $Q_{10} = 3.33$ ), but no significant difference was found for either ration level between 15°C and 19°C ( $Q_{10} = 1.16$ ).

### Growth

Both temperature and ration level influenced green sturgeon growth rates. Specific growth rates were higher among fish held at 15°C than among those held at 11°C ( $Q_{10} = 4.65$ ) for both rations, whereas no significant differences were detected between the 15°C and 19°C ( $Q_{10} = 1.47$ ) groups at either ration level (Table 1). Specific growth rates were also higher at 100% rations than at 50% rations at all temperatures (Table 1). No significant ( $P = 0.25$ ) interaction occurred between temperature and ration level.

### Food Conversion Efficiency

Temperature did not affect green sturgeon food conversion efficiency at 100% rations ( $P = 0.38-1.0$ ), whereas efficiency was significantly higher at 15°C than at 11°C ( $Q_{10} = 1.42$ ) at 50% rations (Table 1). Increases in ration level significantly decreased food conversion efficiencies only in the 15°C and 19°C treatments. There was a significant ( $P = 0.02$ ) interaction between temperature and ration level.

### Metabolism and Activity

The temperature increase from 11°C to 19°C did not affect the  $MO_2$ s of age-0 green sturgeon ( $Q_{10} = 1.50$ ), but the increase from 19°C to 24°C resulted in a significant increase in  $MO_2$  ( $Q_{10} = 4.12$ ; Table 1). Significant increases in volitional activity (tail beat frequencies) occurred with all temperature increases (Table 1).

In age-1 fish, significant increases in mean resting routine metabolic rate were associated with increases in temperature in all treatments (Table 1); there was greater similarity in the  $Q_{10}$  values of the 11–19°C (1.36) and 19–24°C rates (1.72) than there was for the age-0 fish. Ventilatory frequency increased with temperature (Table 1).

As would be expected, the larger, age-1 fish consumed more oxygen than the smaller, age-0 fish (Figure 1). The slopes of these relationships at the three temperatures were similar (weighted mean = 1.07).

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TABLE 1.—Summary (mean  $\pm$  SD) of temperature and ration (where applicable) effects on juvenile green sturgeon. Within rows, different letters indicate significant differences (ANOVA;  $P < 0.05$ ).

Response variable	Ration	Acclimation temperature (°C)				P
		11	15	19	24	
Food consumption (g feed·g fish <sup>-1</sup> ·d <sup>-1</sup> )	50%	0.0057 $\pm$ 0.0007 z	0.009 $\pm$ 0.0008 y	0.01 $\pm$ 0.0008 y		<0.008
	100%	0.01 $\pm$ 0.01 z	0.02 $\pm$ 0.002 y	0.02 $\pm$ 0.001 y		<0.001
Specific growth rate (% weight gain/d)	50%	0.65 $\pm$ 0.06 z	1.39 $\pm$ 0.13 y	1.59 $\pm$ 0.16 y		<0.001
	100%	1.06 $\pm$ 0.15 z	1.96 $\pm$ 0.10 y	2.29 $\pm$ 0.31 y		<0.001
Food conversion efficiency (g weight gained/g feed)	50%	0.23 $\pm$ 0.02 z	0.32 $\pm$ 0.01 y	0.32 $\pm$ 0.02 y		<0.001
	100%	0.2 $\pm$ 0.02 z	0.23 $\pm$ 0.01 z	0.23 $\pm$ 0.02 z		1.000
Age-0 metabolic rate (mg O <sub>2</sub> ·h <sup>-1</sup> ·g fish <sup>-1</sup> )		0.1 $\pm$ 0.05 z		0.13 $\pm$ 0.07 z	0.27 $\pm$ 0.08 y	<0.001
Activity (Tail beats)		537 $\pm$ 567 z		1,108 $\pm$ 1,074 y	1,802 $\pm$ 1,013 x	<0.001
Age-1 metabolic rate (mg O <sub>2</sub> ·h <sup>-1</sup> ·g fish <sup>-1</sup> )		0.16 $\pm$ 0.08 z		0.20 $\pm$ 0.08 y	0.27 $\pm$ 0.06 x	<0.02
Ventilatory frequency (Strokes/min)		67.7 $\pm$ 9.0 z		104.8 $\pm$ 13.2 y	117.6 $\pm$ 17.2 x	<0.01
Preferred temperature (°C)		15.9 $\pm$ 1.7 z		15.7 $\pm$ 2.9 z	20.4 $\pm$ 3.1 y	
Swimming performance (Ucrit [cm/s])		68.3 $\pm$ 11.9 z		80.4 $\pm$ 16.1 z	56.4 $\pm$ 20.8 y	<0.005

Thermal Preference

Fish acclimated to 11°C and 19°C did not differ significantly ( $P = 0.95$ ) in their thermal preferences (15.9  $\pm$  1.7°C and 15.7  $\pm$  2.9°C, respectively); however, fish acclimated to 24°C exhibited a significantly higher ( $P < 0.001$ ) preferred temperature (20.4  $\pm$  3.1°C; Table 1).

Swimming Performance

There was no difference ( $P = 0.10$ ) between the Ucrit values for the 11°C and 19°C treatments (Table 1). However, the Ucrit for the 24°C group was significantly lower than that of the 19°C group. Approximately 60% of the 24°C fish died after transport but before being tested for swimming performance.

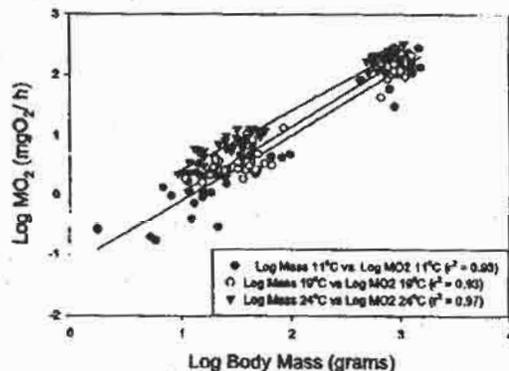


FIGURE 1.—Relationships between log metabolic rate ( $MO_2$ ) and log body mass of age-0 and age-1 green sturgeon acclimated to water temperatures of 11, 19, and 24°C. The intercepts and slopes of the equations for the three temperature groups are as follows: -1.20, 1.10 (11°C); -1.06, 1.11(19°C); and -0.56, 0.99 (24°C).

Discussion

Temperature affected several bioenergetic variables of juvenile green sturgeon. Given the large temperature ranges measured for the Klamath and Rogue rivers (Chamberlain, personal communication; Erickson 2002) when juvenile green sturgeon are likely to be present, effective conservation and management strategies should include water temperature criteria.

Food Consumption

Temperature changes are a major factor influencing oxygen and food demand in fish (Lovell 1989; Nikinmaa 2002). The average food consumption rate increase for green sturgeon associated with the increase from 11°C to 15°C can be at least partly attributed to the increased energetic demands of the fish at the higher temperature. In-

TABLE 2.—Summary of studies on sturgeon growth rates.

Species	Weight (g)	Temperature (°C)	Growth rate (% Body weight/d)	Feed rate (% Body weight/d)	Reference
White sturgeon	0.5–5	15, 20, 25	1.6, 2.6, 2.9	Ad libitum	Cech et al. (1984)
Chinese sturgeon <i>Acipenser sinensis</i>	3.8	16	4.5	6.0	Xiao et al. (1999)
Sterlet <i>A. ruthenus</i>	20	23	1.1	3.5	Jahnichen and Rennert (1999)
White sturgeon	26.2	20	2.1	2.0	Hung and Lutes (1987)
White sturgeon	30.3	23, 26	2.2, 1.9	2.0	Hung et al. (1993)
Atlantic sturgeon <i>A. oxyrinchus</i>	10.0–70.0	19, 26	5.0, 3.6	2.5	Secor and Gunderson (1998)
Siberian sturgeon	181	11 to 19	1.4	1.5–2.1	Prokes et al. (1997)
Oreca sturgeon	184	11, 15, 19	1.1, 2.0, 2.3	1.3, 1.8, 2.1	This study
White sturgeon	250	18	1.5	2.0	Hung et al. (1989)
Lake sturgeon	10–1,322	18	–1.5, 0.0, 1.8, 2.6	0.0, 2.2, 9.4, 13.2	Diana et al. (2000)

creased food consumption fueled the increased growth, activity, and maintenance demands. Increased food consumption at higher water temperatures is not unique to sturgeon species. Given adequate access to food, fish increase their consumption as temperature rises; peak consumption occurs before the temperature aches the upper thermal tolerance limit for the species (Jobling 1994). Larsson and Berglund (1998) found this pattern for age-0 Arctic char *Salvelinus alpinus*, for which food consumption peaked at 16°C over a 5–20°C range. In contrast, the food consumption of the green sturgeon in this study never declined at temperatures up to 19°C, although this result suggests the need for more data at higher temperatures and at temperatures between 15°C and 19°C to determine a peak temperature for food consumption.

#### Growth

When food is available, fish growth increases with temperature, followed by a decline when lethal temperatures are approached (Jobling 1994). In this study, the diminishing growth effect at temperatures above 15°C was linked to increased energy demands for maintenance and activity associated with higher temperatures. The maximum growth rates of our fish may have been limited by the twice-daily (rather than continuous) feeding schedule.

Despite the potentially suboptimal feeding regime in our study, the fish had high growth rates (given their acclimation temperature and ration levels) compared with other sturgeon species of similar body size. For example, Siberian sturgeon *A. baeri*, lake sturgeon *A. fulvescens* of a large size range, and sympatric white sturgeon all grew more slowly at temperatures similar to those in our study (Table 2) despite having equal or higher ration

levels (Hung and Lutes 1987; Hung et al. 1989; Prokes et al. 1997; Diana et al. 2000). Furthermore, despite a higher temperature and ration level, sterlet (20 g) grew more slowly than our green sturgeon (Jahnichen et al. 1999). At comparable or higher temperatures, Chinese sturgeon (Xiao et al. 1999) and Atlantic sturgeon (Secor et al. 1998) did grow faster than our green sturgeon, but they were fed higher ration levels (Table 2). These latter comparisons can be deceiving, however, because the smaller body size of sterlet and Chinese and Atlantic sturgeon contributes to higher specific growth rates (Ricker 1979).

#### Food Conversion Efficiency

Food conversion efficiency in fish typically increases with temperature to some maximum, followed by a decline (Jobling 1981). Common carp *Cyprinus carpio* increased their food conversion efficiency from 0.28 to 0.55 with temperature increases over the 12–30°C range (Goolish and Adelman 1984). Furthermore, peak food conversion efficiency may typify intermediate rations rather than maintenance (no growth) and maximal rations (Ricker 1971). At all temperatures, Goolish and Adelman's (1984) carp showed peak food conversion efficiency at intermediate rations. Because our green sturgeon grew moderately well at the 50% ration level, this level could be considered a moderate one, with a food conversion efficiency pattern comparable to that of the carp.

Despite a possible underestimate of food conversion efficiency owing to the partial erosion of the pellets, green sturgeon appear to have food conversion efficiencies similar to those of other *Acipenser* species. Using feeding efficiency (wet weight gained per amount of feed consumed during the experiment) for comparative purposes (Ta-

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TABLE 3.—Summary of studies on sturgeon feeding efficiencies. Feeding efficiency = wet weight gained by fish/total amount of feed fed to the fish.

Species	Weight (g)	Temperature (°C)	Feeding efficiency (g weight gain/g feed)	Feed rate (% Body weight/d)	Reference
Sterlet	20.0	23	2.0	3.5	Jahnichen and Rennert (1999)
White sturgeon	30.0	23, 26	1.3, 1.1	2.0	Hung et al. (1993)
Green sturgeon	184.0	11, 15, 19	1.0, 1.1, 1.1	1.3, 1.8, 2.1	This study
Siberian sturgeon	190.7	11.0–18.0	1.4	1.5–2.1	Prokes et al. (1997)
White sturgeon	250.0	18.0	0.9	2.0	Hung et al. (1989)

ble 3), the feeding efficiencies of our juvenile green sturgeon approximate those of juvenile white sturgeon at similar temperatures (Hung et al. 1989, 1993). Hung et al. (1993) and Hung and Lutes (1987) found that the highest feeding efficiency for juvenile white sturgeon occurred between 20 and 23°C, which was beyond the range used in our studies.

Metabolism and Activity

The metabolic and activity patterns of the age-0 and age-1 green sturgeon reflect different temperature sensitivities. While the high  $Q_{10}$  value (4.12) for age-0 green sturgeon between the 19°C and 24°C treatments shows a high metabolic temperature sensitivity, the lower  $Q_{10}$  value (1.50) and the lack of a significant difference between the  $MO_2$ s for the 11°C and 19°C treatments demonstrates a relative insensitivity. Assuming that this relative insensitivity does not stem from increased relative error at lower  $MO_2$ s, it would be advantageous to allow age-0 green sturgeon to forage in thermally diverse habitats. The larger  $Q_{10}$  value and the significant difference in  $MO_2$ s between the two higher temperature treatments suggests that age-0 green sturgeon inhabiting water warmer than 19°C would be at an energetic disadvantage in having to expend significantly more energy on maintenance and activity and comparatively less on growth.

Increases in volitional swimming and activity with temperature typify poikilothermic animal behavior (e.g., Wurtsbaugh and Cech 1993), including that of our age-0 sturgeon ( $r^2 = 0.98$ ). White sturgeon also significantly increase their activity with temperature over a 10–25°C range (Cech et al. 1984; Crocker and Cech 1997). Such increases presumably facilitate more widespread foraging to meet temperature-associated increases in energy requirements for growth (Cech et al. 1984) and metabolism (Crocker and Cech 1997). Concurrent measurements showed that volitional activity (tail beats) increased with temperature over the 11–

19°C range, whereas routine metabolism remained unchanged over the same range. Without being penalized energetically for increased activity (e.g., for foraging) over the above temperature range, a fish would be able to devote more energy to growth. Faster growth can decrease predation risk by minimizing the time spent as small, relatively vulnerable prey (Werner and Hall 1988). Although the age-1 green sturgeon significantly increased their  $MO_2$ s with each temperature increase, they were quiescent in flow-through respirometers and their  $Q_{10}$ s were more consistent across the temperatures than those of age-0 fish. The quiescence of the age-1 fish may reflect either an age-related difference in volitional activity or the more confining dimensions of the flow-through respirometers. All metabolic measurements were conducted during daylight hours. Lankford et al. (2003) noted that green sturgeon in the size range of our age-1 fish were more nocturnally active.

Green sturgeon exhibited high metabolic rates relative to other sturgeon species (Table 4). For example, McKenzie et al. (1997) reported an  $MO_2$  in Adriatic sturgeon (mean weight, 198 g) at 23°C that was less than one-half the value for our green sturgeon at 24°C. The higher  $MO_2$ s of our fish may be due to proportionally larger red muscle masses. Moyle (2002) describes the extensive red muscle in green sturgeon. Red muscle, with its higher mitochondrial density than white muscle, exhibits higher tissue  $MO_2$ s (Gordon 1968).

In terms of the bioenergetic model (equation 1), temperature-related increases in  $I$  were allocated proportionately more to  $G$  at temperatures up to 15°C and proportionately more to  $M$  at temperatures above 15°C. Volitional activity ( $A$ ) increased throughout the temperature range. While  $E$  and  $SDA$  were not measured, they have been shown to increase with  $I$  (Kitchell et al. 1977), and  $R$  is irrelevant in these juvenile fish.

Thermal Preference

Conservation efforts will also require knowledge of sturgeon behavior (thermal preference)

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TABLE 4.—Summary of routine metabolism studies for sturgeons.

Species	Weight (g)	Temperature (°C)	Routine metabolism (mg O <sub>2</sub> ·h <sup>-1</sup> ·g fish <sup>-1</sup> )	Reference
White sturgeon	02–63	10, 16, 20	0.26, 0.20, 0.18	Crocker and Cech (1997)
Green sturgeon	22.3	11, 19, 24	0.10, 0.13, 0.27	This study
Atlantic sturgeon	12.0–69	19, 26	0.20, 0.30	Secor and Gunderson (1998)
Adriatic sturgeon	198	23	0.11	McKenzie et al. (1997)
Green sturgeon	851	11, 19, 24	0.16, 0.20, 0.27	This study
White sturgeon	950	15	0.08	Burggren and Randall (1978)
Siberian sturgeon	1,800	15	0.06	Nonnette et al. (1993)
White sturgeon	2,000	18	0.10	Ruez et al. (1987)

and locomotory (swimming) performance to develop the best management plans. Although both the 11°C and 19°C acclimation temperatures fall within the thermal regimes of the Rogue and Klamath rivers (Erickson et al. 2002; Chamberlain, personal communication), the similar thermal preference values (15.9°C and 15.7°C, respectively) suggest that 15–16°C is the temperature range preferred by age-0 green sturgeon in their natural habitats. Interestingly, the 11–15°C range was the one in which the fish showed the sharpest increase in growth rates and in food conversion efficiency (followed by slight increases beyond 15°C). Fish commonly select temperatures that promote optimal growth (Jobling 1981) and those at which physiological functions operate at maximum efficiency (Crawshaw 1977). The less pronounced temperature effect beyond 15°C suggests a lesser benefit with respect to growth for age-0 green sturgeon at higher temperatures. Conversely, regimes cooler than 15°C would decrease the growth rate regardless of food availability and can decrease muscle twitch time (Rome 1990), increasing vulnerability to predators via slower escape initiation or swimming performance. Marbled rockfish *Sebastes marmoratus* (weight, 10.7 ± 1.4 g) preferred 21°C within the 15–25°C range (Kita et al. 1996). Further, they found that the observed preferred temperature coincided with the temperature range at which  $\dot{M}O_2$  increases were less pronounced. This pattern was similar to that seen in our study, in which the preferred temperatures (although based on short laboratory observations) were between 11°C and 19°C and the corresponding  $\dot{M}O_2$ s were not significantly different.

Thermal tolerance was originally planned to be part of this study; however, the anatomy (large pectoral fins) and demersal nature of green sturgeon made it difficult to define a loss of equilibrium for some of the fish, which was our endpoint for measuring this variable. Jobling (1981) re-

ported the following linear relationship between preferred ( $X$ ) and lethal ( $Y$ ) temperatures in fish:

$$Y = 0.66X + 16.45 \quad (r = 0.880). \quad (8)$$

Applying this equation to the juvenile green sturgeon's preferred temperature (15.8°C) results in a 27°C lethal temperature. Whereas many of the age-1 fish acclimated to 25°C died after being transported to a holding tank for high-temperature (24°C) swimming performance measurements, identical transportation of age-1 fish acclimated and swum at cooler temperatures never resulted in mortality, suggesting that 25°C approximates the maximum temperature for transporting age-1 green sturgeon. The lethal temperature would thus be near the value calculated from the regression equation. The continuous flow of water (with non-detectable ammonia levels) in these experiments ensured that the calculated lethal temperature was not influenced by dissolved substances.

#### Conclusions

The presence of significant differences in swimming performance associated with temperature may have been confounded by the thermal sensitivity of the fish to handling (see above). Peake et al. (1997) recommended 14°C for adult lake sturgeon migrating up their spawning rivers. Swimming ability could be relevant to the successful instream rearing (foraging and predator avoidance) and downstream migration of juvenile sturgeon. Reduced swimming performance at temperatures above 19°C could jeopardize those essential life history activities.

Overall, management plans for watersheds containing juvenile green sturgeon should include water temperature criteria that prevent prolonged exposure to regimes beyond 19°C, which represent the species' growth and locomotory optima. Using temperature as a management tool to provide optimal environmental conditions for protecting fish

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is well documented. Magnuson et al. (1979) described the temperature-related habitat partitioning of several freshwater fishes using laboratory preference and field distribution data sets. Amour (1991) related individual temperature tolerance data to predicted population-level responses to protect fish in natural habitats. Future studies on green sturgeon should examine the effects of other relevant factors (e.g., dissolved oxygen) to foster better understanding of this species' environmental requirements and better protection of its populations.

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**The Cost of Chronic Stress: Metabolic Scope for Activity, Critical Swimming Velocity  
and Liver Glycogen Levels in Sturgeon**

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## Summary

Metabolic scope for activity (MSA), critical swimming velocity ( $U_{crit}$ ), and liver glycogen levels were measured in green sturgeon (*Acipenser medirostris*) exposed to a 28-d chronic stress regime. We hypothesized that green sturgeon would not habituate to chronic stress, resulting in 1) an increased maintenance metabolic rate (MMR), 2) decreased MSA, 3) decreased  $U_{crit}$ , and 4) decreased liver glycogen content. Green sturgeon were placed into indoor, flow-through tanks and exposed to two of three randomized acute stressors (5-min chasing stressor, 10-min water depth reduction stressor, or 5-min confinement stressor) twice daily for 28 days. Acutely, all stressors significantly increased plasma cortisol and there was no evidence of habituation in sturgeon subjected to the chronic stress regime. Oxygen consumption and  $U_{crit}$  were measured using a Brett-type swimming-tunnel respirometer. The chronic stress regime decreased MSA, increased MMR, and decreased liver glycogen content, compared with non-stressed controls. Conversely, the  $U_{crit}$  was unaffected. These are the first measurements of chronic stress in chondrosteian fishes, and clearly indicate that chronic stress represents a significant metabolic cost, as measured by decreased MSA and liver glycogen content. A non-habituating stress response, unlike what is reported in teleost, is suggested as a potential cause for the chronic stress-induced metabolic costs.

## Introduction

The physiological and behavioral mechanisms that have evolved in vertebrates to cope with stressors, both perceived and real, have been the focus of many biologists since the introduction of the general adaptation syndrome (GAS) by Selye (1950).

Examination of the stress response in fish has revealed a complex set of coordinated responses (Barton et al., 2002; Greenberg et al., 2002; Idler and Truscott, 1972; Wendelaar Bonga, 1997), which have been highly conserved among fishes and other vertebrates (Greenberg et al., 2002). Catecholamines (epinephrine/norepinephrine) and the products of the hypothalamo-pituitary-interrenal (HPI) axis (corticotropin-releasing hormone [CRH], adrenocorticotropin [ACTH], and cortisol) are widely accepted as primary components and, therefore, indicators of an active stress response in fishes (Donaldson, 1981; Idler and Truscott, 1972; Mazeaud et al., 1977; Sumpter, 1997; Wendelaar Bonga, 1997). Additional regulatory compounds, such as heat-shock proteins (HSPs), serotonin, arginine vasotocin (AVT), mineralocorticoids, and central brain monoamines also appear to be involved in the stress response of fishes (see Barton et al., 2002 for a review). These diverse regulatory elements appear to enhance the effectiveness of the acute stress response, but their effects during times of prolonged stress are not as well studied.

Most studies that examine stress describe the acute response, which is known to involve immediate release of stored catecholamines followed by the synthesis and release of cortisol (Gamperl et al., 1994; Mazeaud et al., 1977; Wendelaar Bonga, 1997). The initial perception of a stressor stimulates the release of catecholamines, which rapidly increase blood glucose concentrations via glycogenolysis (Larsson, 1973; Randall and

Perry, 1992; Reid et al., 1998). Cortisol then stimulates catabolic processes to liberate amino acids (AA) and free fatty acids (FFA) for the prolonged production of glucose via gluconeogenesis (Milligan, 1997; Vijayan et al., 1997; Vijayan et al., 1994b). Cortisol has also been shown to directly regulate the transcription and activity of phosphoenolpyruvate carboxykinase (PEPCK) in fishes, which is the rate limiting enzyme in gluconeogenesis (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). The free glucose synthesized by hepatic gluconeogenesis primarily is believed to be converted back into glycogen to repay the debt incurred during the initial stress response, assuming the stressor is removed (Mommsen et al., 1999; Vijayan et al., 1994a). During periods of persistent, prolonged, or repetitive stress, cortisol is known increase hepatic glycogenolysis *in vitro*. However, the liberated glucose is believed to be primarily used by the liver to enhanced hepatic gluconeogenesis (Vijayan et al., 1994b) and not as a systemic glucose source. This would provide glucose for systemic systems via gluconeogenesis, while conserving hepatic glycogen stores. However, the effects of cortisol on liver glycogen content are still not clear (Barton and Schreck, 1987; Mommsen et al., 1999; Vijayan et al., 1997; Vijayan et al., 1994b). Other known secondary/tertiary components of the stress response include reduced or altered immune function (Dhabhar and McEwan, 1997; Maule et al., 1988), reduced growth rate (Carr, 2002; Heath et al., 1993), and reduced reproductive function (Campbell et al., 1994; Carragher and Sumpter, 1990; Haddy and Pankhurst, 1999). The attenuation of these processes during acute stress is believed to allow reallocation of the liberated metabolic resources toward vital functions necessary for survival (Moberg, 1985). While the responses described above provide the animal with increased energetic resources to cope

with a stressor, there is a short-term biological cost associated with the shifting of these resources. Barton and Schreck (1987) examined the metabolic cost associated with acute stress in juvenile steelhead (*Oncorhynchus mykiss irideus*) and concluded that the mechanisms involved in the acute stress response reduced metabolic scope for activity (MSA) by approximately 25%. Despite the association of the acute stress response with negative impacts on the animal, there is little doubt regarding the adaptive significance of this neuroendocrine-systemic response on short-term survivability (Greenberg et al., 2002).

On the other hand, situations where the stressors are persistent or chronic are commonly regarded as maladaptive (Barton et al., 2002; Moberg, 1985; Reid and Perry, 1991; Selye, 1950). We know little about the organismal effects of chronic stress in fish, but the available literature suggests an extension of the effects mentioned above for acute stressors, which over time would reduce the fitness of the individual. Campbell and coworkers (1994) reported that two episodes of chronic confinement stress (2 weeks episode<sup>-1</sup>) reduced egg size and progeny survival in brown (*Salmo trutta*) and rainbow trout (*O. mykiss*). Similarly, food consumption, growth rate, and food conversion efficiency are reduced in rainbow trout receiving stress-like levels of exogenous cortisol (Gregory and Wood, 1999). Clearly, chronic stress impacts fitness on multiple levels, but if one considers the glucose metabolism paradigm presented above, chronic stress would present a bioenergetic conundrum for the animal. During a period of chronic stress the animal must either compromise glycogen reserves, increase the catabolism of tissue protein (free AA), or potentially both to repetitively up regulate the energetic resources required to maintain the components of the acute stress response mentioned above. This

increased utilization of energetic reserves is theorized to be a contributing factor in the development of the pre-pathological state (Moberg, 1985), which leaves the animal vulnerable to the development of pathology. Thus, if the animal became habituated during chronic exposure to stress the energetic problem would be alleviated, but the adaptive stress response would be compromised.

There is growing evidence suggesting that chronic stress alters the components of the acute stress response. Barton and coworkers (1987) detected an inhibition of the HPI axis in juvenile rainbow trout fed cortisol-supplemented feed or physically stressed once daily for 10 weeks. Furthermore, Shrimpton and Randall (1994) reported that the concentration of glucocorticoid receptors (GR) in the gill tissue of coho salmon (O. kisutch) was reduced during 18 days of exogenous cortisol treatment or endogenous cortisol increases via exposure to a single stressor day<sup>-1</sup>. The reduction of gill GR resulted in reduced sensitivity of the gills to cortisol. Similarly, repeated physical stress in rainbow trout decreased the number of  $\beta$ -adrenergic receptors in red blood cells, which are positively correlated with cortisol (Reid and Perry, 1991). However the sensitivity of the RBC's adrenergic response ( $\text{Na}^+/\text{H}^+$  exchange) to epinephrine was enhanced due to increased receptor affinity (Perry et al., 1996). These results suggest that chronic stress significantly alters the components of the acute stress response in fish, although due to different experimental protocols and the limited number of species that have been examined the implications are not fully understood.

In the following experiments, the effects of chronic stress were investigated in juvenile green sturgeon (*Acipenser medirostris*), a "species of special concern" in California, by exposing fish to 2 stressors day<sup>-1</sup> for 28 consecutive days. Our purpose

was to measure, for the first time, the responses of this phylogenetically ancient order of fishes to chronic stress and to compare the results to those of more derived teleosts in hopes of elucidating factors contributing to the decline of this species. We measured acute plasma cortisol responses to the individual stressors selected for the chronic stress regime, changes in plasma glucose, lactate, and cortisol in response to a single acute stressor at 7-day intervals during the chronic stress regime, and changes in maintenance metabolic rate (MMR), MSA, critical swimming velocity ( $U_{crit}$ ) and liver glycogen concentrations after the completion of the 28-day chronic stress regime. We hypothesized that green sturgeon would not habituate to chronic stress, which would give rise to increased maintenance requirements resulting in 1) increased MMR, 2) decreased MSA, 3) decreased  $U_{crit}$ , and 4) decreased liver glycogen.

## **Materials and methods**

### *Fish Source and Care*

Juvenile green sturgeon were obtained from artificially spawned, wild-caught brood stock from the Klamath river (Van Eenennaam et al., 2001) and maintained in outdoor flow-through tanks supplied with 19 °C well-water under a natural photoperiod (38.6 N, 121.7 W) at the Center for Aquatic Biology and Aquaculture (CABA), University of California, Davis. Fish were fed Silver Cup™ extruded, nonfloating trout chow diet at the rate of 1.0% body weight  $d^{-1}$  via a 24-h belt feeder. All fish were held in similar, round (3.66-m-diameter) tanks until being transferred into experimental tanks where they were allowed to acclimate for 2 weeks before initiating experiments. The experiments described below utilized fish from two different year classes (2000 & 2001); however, year classes were not mixed within a single experiment. In all experiments

feeders were emptied 24 h prior to sampling to ensure all fish were in a post-absorptive metabolic state.

### *Experimental procedures*

#### *Chronic stress regime*

Chronic stress was simulated by subjecting fish ( $n = 3 \text{ tank}^{-1}$ ) to a randomized sequence of stressors (1 of 3), twice daily (1000 and 1600 h) for 28 consecutive days. This chronic stress regime was adapted from the regime developed by Shrimpton and Randall (1994). The stressors included: a 5-min chasing stressor, which involved a small aluminum dip net with only incidental direct contact with the fish, a 10-min water depth reduction stressor that consisted of a rapid reduction in the water depth until the dorsal fins of the fish were slightly exposed to air followed by a gradual return to the original water level, or a 5-min confinement stressor where the fish were held underwater in a large, fine mesh, tube net measuring at least 1.5 times the length of the fish. The weights of the fish were recorded at the beginning and end of the 28-day period and fish received feed the entire treatment period.

#### *Acute responses to chronic stressors*

The acute cortisol response to each of the three stressors used in the chronic stress regime ( $n = 6 - 7 \text{ fish stressor}^{-1}$ ) were measured in age 0+ fish (2000 year class; 1.0 - 1.4 kg live weight) acclimated to individual 1-m diameter, indoor, flow-through tanks. Laboratory lights were adjusted to simulate a natural photoperiod and 24-h disk feeders provided food at a rate of 1.0% body weight  $\text{day}^{-1}$ . All other aspects of fish care were similar to the care of fish in the holding tanks. In-dwelling catheters were surgically implanted into the caudal vasculature 48 h prior to sampling, using the procedure

described by Crocker and Cech (1998). Briefly, fish were rapidly anaesthetized in a buffered MS 222 ( $350 \text{ mg l}^{-1}$ ,  $9 \text{ g l}^{-1} \text{ NaCl}$ , and  $0.42 \text{ g l}^{-1} \text{ NaHCO}_3$ ) bath and transferred to a surgery table where the gills were continuously irrigated with a similar bath containing  $150 \text{ mg l}^{-1}$  MS 222. Using a modified 17-Ga Touhy needle as a guide, polyethylene tubing (Intramedic Clay-Adams PE-50, Becton Dickson and Company, Sparks, Maryland) was inserted into the caudal vasculature and sutured in place. Cannulae were flushed with heparinized saline ( $50 \text{ units heparin ml}^{-1}$ ) and tied off after sampling so that the fish could swim freely after return to the original tank. The entire cannulation procedure was completed within 15 min. On the sampling day, blood was collected via the cannulae before exposure to the stressor (time 0) and 15, 30, 60, 120, and 180 min post-stressor. The blood samples were stored on ice until centrifugation and plasma was collected and stored at  $-80 \text{ }^\circ\text{C}$  until analysis for cortisol.

#### *Chronic stress habituation*

Habituation of the acute stress response by exposure to the chronic stress regime (see description above) for 21 days was investigated by acclimating groups ( $n = 3$ ) of age 1+ fish (2000 year class;  $1.6 - 2.9 \text{ kg}$  live weight) in round (2.1-m-diameter), outdoor, flow-through tanks ( $n = 5$ ) maintained as described for the holding tanks. Fish were exposed to the chronic stress regime daily except for the days (0, 7, 14, and 21) that their acute response to a water depth reduction stressor was determined. To determine the acute response, tanks were randomly assigned a "post stressor" sampling time of 0, 15, 30, 60, or 120 min post-stressor, the morning of each sampling day. Fish in the time-0 tank were always sampled first to avoid indirectly disturbing the tank, thereby assuring

the measurement of accurate resting cortisol levels. After collection of the time 0 tank, the 4 remaining tanks were rapidly drained until the water level approached the fish's dorsal surface, but did not expose the fish to air. The water level remained depressed for 10 min and then was allowed to return to the initial level. Individual timers were set for each tank and blood was sampled at the selected time post-stressor, according to the following protocol. All fish ( $n = 3$ ) in the assigned tank were quickly netted and transferred into a buffered MS 222 anesthetic bath ( $350 \text{ mg l}^{-1}$ ,  $6 \text{ g l}^{-1}$  NaCl and  $0.42 \text{ g l}^{-1}$   $\text{NaHCO}_3$ ) until undulations weakened and ventilatory movements ceased (ca. 1 min). Fish were then transferred to one of three V-shaped tables for blood sampling via cardiac puncture with heparinized syringes and 20 Ga needles. All blood samples were collected within 5 min of initial tank disturbance, before plasma cortisol increases due to the sampling techniques were detectable (Gamperl et al., 1994; Wedemeyer et al., 1990). Blood was processed as described above.

#### *Determination of MSA, MMR, and $U_{crit}$*

Groups ( $n = 3$ ) of age 0+ green sturgeon (2001 year class; 0.7 – 1.6 kg) were chronically stressed in 1-m, indoor, flow-through tanks ( $n = 5$ ) maintained as described in the “acute responses to chronic stressors” section. Two of the three fish in each tank were used for the measurement of MSA and  $U_{crit}$  and one for liver glycogen measurements (see description below). The initiation of each tank of fish into the chronic stress regime was staggered by 3 days to facilitate a continuous availability of fish for the post-chronic stress regime experiments (detailed below) and to assure that all fish would be tested within 2 d of completing the chronic stress regime. Control fish were held at

similar densities in a 2-m, outdoor, flow-through tank maintained as described for the holding tanks.

After the completion of the stress regime fish were placed into the Brett-type swim-tunnel respirometer (ca. 655 l, of plastic and stainless steel construction with a variable speed motor and propeller) no later than 1600 h and allowed to acclimate at a velocity of  $10 \text{ cm sec}^{-1}$  until 0800 - 0900 h the next morning to provide adequate time for the fish to recover from the transfer stress and adjust to the swimming chamber. The acclimation speed ( $10 \text{ cm sec}^{-1}$ ), at which green sturgeon would remain calm in the apparatus and not need to actively swim to maintain position, was determined by pilot experiments. To assure that the water inside of the respirometer remained equilibrated with the aerated water bath, 2 large capacity, submersible water pumps forced water into the respirometer through an opening equipped with a ball valve. The opposite side of the loop had an identical opening to allow water to return to the bath.

The morning of the experiment, the respirometer oxygen partial pressure ( $P_{O_2}$ ) was adjusted, if needed, to 140 - 160 mm Hg by bubbling oxygen into the water bath. Closing the ball valves and switching off the flushing pumps sealed the respirometer and a water sample was collected for initial  $P_{O_2}$  (Cameron Instruments BGM 200) measurement via micro-bore Tygon™ tubing, stopcock, and glass syringe. Water samples (in duplicate) were removed every 30 min until the  $P_{O_2}$  decreased by at least 10 mm Hg, and ventilatory rates and tail beat frequencies were monitored (remote video camera and monitor). If resting ventilatory rates and/or tail beat frequencies were above 80 or 10 beats  $\text{min}^{-1}$ , respectively, fish were not used in the experiment. The MMR (oxygen consumption) was calculated by converting  $P_{O_2}$  into oxygen content ( $C_{O_2}$ ) with a

nomogram (Green and Carritt, 1967) and using the equation in Cech (1990) for closed respirometers.

After the MMR  $P_{O_2}$  data were collected, oxygen was again bubbled into the water bath, the flushing pumps switched on, and the ball valves opened to re-equilibrate respirometer water to near-air-saturation conditions. Respirometer water velocity was rapidly increased to  $25 \text{ cm sec}^{-1}$  for 20 min and then slowly increased to  $35 \text{ cm sec}^{-1}$  over 10 min to induce steady swimming. The respirometer was then resealed and an initial  $P_{O_2}$  sample removed. Tail beat and ventilatory frequencies were recorded 15 min into the interval, a final  $P_{O_2}$  sample was collected at 45 min for calculation of a swimming metabolic rate ( $M_{O_2}$ ), and the respirometer was unsealed and flushed for 15 min, resulting in a 60-min velocity interval. The velocity was rapidly increased by  $5 \text{ cm sec}^{-1}$  increments and the protocol was repeated until the fish became exhausted. Exhaustion was defined as three consecutive impingements (60 s of caudal fin contact with the posterior screen) at the same velocity. The  $M_{O_2}$  for each interval that resulted in at least a 10-mm Hg  $P_{O_2}$  decrease were calculated as above with the highest  $M_{O_2}$  used as the active metabolic rate (AMR). The MSA was then calculated ( $AMR - MMR$ ; Fry, 1947). The above protocol was also used to calculate the  $U_{crit}$  (Brett, 1964) of green sturgeon using the 60 min velocity intervals.

#### *Liver Collection and Glycogen assay*

Individual fish were rapidly netted and immediately over-anesthetized in a buffered MS 222 anesthetic bath ( $350 \text{ mg l}^{-1}$  MS 222,  $6 \text{ g l}^{-1}$  NaCl, and  $0.42 \text{ g l}^{-1}$   $\text{NaHCO}_3$ ) two days after the completion of the chronic stress regime ( $n = 8$ ) or after being

held in the same tank system for 30 d, but not stressed (n = 8). After body undulations ceased (ca. 1.5 min) liver tissue was quickly excised and small pieces were frozen in liquid nitrogen. Samples were then stored at  $-80^{\circ}\text{C}$  for measurement of glycogen content.

Liver glycogen levels were analyzed in duplicate by an amyloglucosidase method (Hung et al., 1989; Murat and Serfaty, 1974). Approximately 400 – 500 mg (wet weight) of liver tissue was homogenized in 2 ml of 0.1 M citrate buffer ( $4^{\circ}\text{C}$ ) containing NaF and centrifuged at 3000 rpm for 5 min. One set of duplicate aliquots of the clear supernatant was used to determine the total glucose (enzyme digested) in the homogenate, while a second set was used to determine free glucose. The net amount of glucose liberated from liver glycogen was determined by subtracting the total glucose from the free glucose. All samples were measured with a calibrated, YSI 2700 biochemical analyzer in triplicate. Standards and a pooled tissue control were used to monitor analyzer and assay accuracy.

All procedures described above were approved by the UC Davis Animal Care and Use Committee (Protocol # 9368).

#### *Statistical analysis*

Statistical analyses were performed using Sigmastat 2.03 (Jandel Scientific, San Rafael, California) software. Data from the acute responses to chronic stressors experiment were analyzed either by a one-way analyses of variance (ANOVA) model followed by Tukey's tests, or by a Kruskal-Wallis one way analysis of variance on ranks to determine differences in cortisol levels for a single stressor, as well as between the 15

min post-stressor responses for all 3 stressors. Cortisol, glucose, and lactate data from the chronic stress habituation experiment were analyzed by a one-way ANOVA followed by Tukey's tests or t-tests to determine differences between pre-stress and 15-min post-stressor plasma concentrations. Differences between the 15-min post-stressor plasma concentrations between days (0, 7, 14, and 21) were compared by a one-way ANOVA. Differences between the MMR, MSA, and liver glycogen levels of control and chronically stressed fish were compared using studentized t-tests, and regression analysis was used to compare the initial  $P_{O_2}$  to  $M_{O_2}$ .

## Results

### *Acute responses to chronic stressors*

Exposure to the chasing, water depth reduction, and confinement stressors resulted in a graded plasma cortisol response, reaching significantly elevated concentrations of 18.8, 60.8, and 113.4 ng ml<sup>-1</sup> respectively (Fig. 1) by 15 min post-stressor. The time needed to return to resting levels appears to be related to the stressor severity, with those in response to the chasing, water depth reduction, and confinement stressors requiring 30, 60, and 120 min respectively (Fig. 1).

### *Chronic stress habituation*

Exposure to the chronic stress regime for 21 days did not result in habituation of the green sturgeon's acute stress response supporting our general hypothesis. Fish remained responsive to a water depth reduction stressor at 0, 7, 14, and 21 days into the chronic stress regime, as determined by significantly elevated ( $p < 0.05$ , t-test) plasma cortisol concentrations (means  $\pm$  SEM of  $9.4 \pm 1.3$ ,  $24.3 \pm 4.1$ ,  $20.8 \pm 3.6$ , and  $19.5 \pm 2.9$  ng ml<sup>-1</sup>, respectively) compared to baseline levels. In addition, there were no differences

in the plasma cortisol responses at any time post-stressor on days 7, 14, and 21 ( $p < 0.05$ , Tukey's test). The cortisol response measured on day 0 was significantly lower ( $9.4 \text{ ng ml}^{-1}$ ) than the responses on all other days, and the responses measured to the water depth reduction stressor during the "acute response" experiment ( $60.8 \text{ ng ml}^{-1}$ ; Fig. 1) were higher than those measured during the "chronic stress" experiment (c.a.  $20 \text{ ng ml}^{-1}$ ). We also report significantly higher resting glucose levels on days 14 and 21 when compared to day 0 of the chronic stress regime (Fig. 2), as well as a trend suggesting an acute reduction in lactate at 15 min post-stressor throughout the chronic stress regime, but only significantly different from the control on day 21 (Fig. 3).

### *Effects of the chronic stress regime*

#### *Growth*

There was no significant difference between initial ( $1026.1 \pm 57.8 \text{ g}$ ) and final ( $1117.5 \pm 40.8 \text{ g}$ ) weights of fish exposed to the 28-day chronic stress regimes, but there was a trend suggesting positive growth during this short period of time. Fish were observed to be actively feeding throughout the chronic stress regime and showed no signs of "poor" health. The weights of control fish were not monitored.

#### *MMR and AMR*

The 28-day chronic stress regime significantly increased the MMRs of juvenile green sturgeon (mean  $\pm$  SEM:  $0.19 \pm .02 \text{ mg O}_2 \text{ hr}^{-1} \text{ g}^{-1}$ ) when compared to control fish ( $0.27 \pm 0.01$ ), supporting hypothesis 1 (Fig. 4). The AMRs were unaffected by the chronic stress regime with  $0.60 \pm 0.04 \text{ mg O}_2 \text{ hr}^{-1} \text{ g}^{-1}$  consumed by control fish and  $0.57 \pm 0.02 \text{ mg O}_2 \text{ hr}^{-1} \text{ g}^{-1}$  by chronically stressed fish (Fig. 4). There was no correlation between initial  $P_{O_2}$  and  $M_{O_2}$ , and solid blocking effects were ignored because the total cross-sectional area of

the fish was <10% of the swimming chamber (Bell and Terhune, 1970). Bacterial respiration was not accounted for, as weekly 60 min “blank” runs resulted in no  $P_{O_2}$  decreases and the entire respirometer was bleached weekly.

#### *MSA*

The MSA was significantly decreased in chronically stressed fish ( $0.29 \pm 0.04 \text{ mg O}_2 \text{ hr}^{-1} \text{ g}^{-1}$ ) compared to control fish ( $0.4 \pm 0.04 \text{ mg O}_2 \text{ hr}^{-1} \text{ g}^{-1}$ ), supporting hypothesis 2 (Fig. 5).

#### *U<sub>crit</sub>*

Swimming performance, as measured by  $U_{crit}$ , was not affected by the chronic stress regime (Table 1). Chronically stressed and control fish (of similar length, Table 1) achieved similar relative sustained swimming speeds of  $0.83 \pm 0.02$  and  $0.78 \pm 0.03 \text{ BL sec}^{-1}$ , rejecting hypothesis 3.

#### *Liver glycogen*

Exposure to the chronic stress regime significantly reduced the green sturgeons' liver glycogen content when compared to control fish (Fig. 6). Mean liver glycogen content decreased by 53% ( $48.9 \pm 6.06 \text{ mg g}^{-1}$  in control fish vs.  $23.2 \pm 5.77 \text{ mg g}^{-1}$  in chronically stressed fish), supporting hypothesis 4, while the free glucose present in the liver tissue before enzymatic breakdown of glycogen was significantly higher in chronically stressed fish (Fig. 6).

## Discussion

We investigated the impacts of chronic stress on green sturgeon, a chondrosteian fish. The studies described here noted that the hormonal and metabolic effects of chronic stress in green sturgeon differ from the pattern of responses reported in teleosts. Our intent was to quantify the development of the pre-pathological state (Moberg, 1985), which is theorized to be induced by chronic stress and involve increased metabolic demand and vulnerability to additional threats.

To study the impacts of chronic stress, we first needed to simulate this phenomenon in the laboratory. Past studies have employed cortisol injections, slow-release cortisol implants, mini-osmotic pumps, and cortisol-enriched feed to study chronic stress in fish (Barton et al., 1987; Gamperl et al., 1994; Gregory and Wood, 1999; Vijayan et al., 1994b). All of these methods resulted in cortisol increases; however, the majority resulted in a persistent increase in cortisol, often to hyper-physiological levels. The impacts of these manipulations are not fully understood, but because cortisol participates in a self-regulatory, negative feedback loop (Donaldson, 1981; Wendelaar Bonga, 1997), they should be considered. For example, juvenile rainbow trout that were fed cortisol-enriched feed for 10 weeks displayed decreases in liver glycogen, circulating lymphocytes, growth rate, and condition factor; while fish stressed once daily showed only a decrease in lymphocytes (Barton et al., 1987). These results suggest a significant difference between the impacts of a feed-based, cortisol delivery resulting in extended exposure to hyper-physiological levels, and the endogenous cortisol response with its a relatively quick peak (15 – 60 min) followed by a brief clearance period (Barton, 2002; Gamperl et al., 1994). Because a constant elevation of cortisol in non-mammalian

vertebrates most likely occurs in only extreme situations, we utilized the modified Shrimpton and Randall (1994) method to simulate chronic stress.

We initially quantified the acute stress response to each of the selected stressors. These stressors were selected to activate the non-specific stress response with different intensity levels, resulting in a graded cortisol response; the chasing, water depth reduction, and confinement stressors stimulating mild, moderate, and severe response, respectively (Fig. 1). None of these stressors involved air emersion to avoid the development of a respiratory acidosis, which would likely extend the recovery time. After establishing the acute response to the stressors, we determined that green sturgeon did not habituate to our chronic stress protocol. These results differ from the response noted in teleosts, which appear to habituate to chronic stress. For example, rainbow trout exposed to a single daily stressor or to cortisol-enriched feed for 10 weeks show habituation of the HPI axis by becoming less responsive to a 30-s air-emersion stressor, despite normal resting cortisol concentrations (Barton et al., 1987). Rainbow trout exposed to a single daily stressor for 6 days have decreased mRNA levels of AVT, which is a hypothalamic neuropeptide involved in ACTH stimulation, suggesting habituation at the hypothalamus (Gilchrist et al., 2000). In addition, Sloman et al. (2002) reported a reduced sensitivity of the interrenal cells (in situ) to ACTH and a reduced cortisol synthetic capability in subordinate rainbow trout, which are known to have elevated resting cortisol levels when compared to dominate fish. These results suggest habituation of the HPI axis to chronic stress at the hypothalamus, pituitary, and interrenal tissue levels in teleosts. In contrast, our studies suggest that green sturgeon remained responsive. Interestingly, the cortisol response to the water depth reduction stressor in

this experiment was reduced when compared to the response to the identical stressor in the “acute response to chronic stressors” experiment. The use of larger tanks, grouping 3 fish tank<sup>-1</sup>, and the fact the animals were not cannulated are key differences in the “chronic stress habituation” experiment that could have accounted for the reduced responses. All of these factors would probably reduce the perceived severity of this stressor, yet the fish still remained responsive.

The chronic stress regime also affected secondary stress responses in green sturgeon. Resting levels of plasma glucose on days 0 and 7 of the regime were similar (Fig. 2) and consistent with resting concentrations reported for unstressed green sturgeon (Lankford et al., 2003). However, on days 14 and 21 of the chronic stress regime, resting glucose levels were significantly elevated (Fig. 2), suggesting an increased glucose homeostatic set point in fish exposed to chronic stress. A potential explanation for this phenomenon could be a prolonged up-regulation of gluconeogenesis, due to the repetitive increases of cortisol. However, reduced glucose uptake by peripheral tissues may also account for this change in blood glucose. Additionally, there was a trend for an acute decrease in plasma lactate in response to a water depth reduction stressor (Fig. 3), which is quite different than the response to air emersion or other physically challenging stressors in teleosts and chondrosteans (Barton et al., 2002; Mazeaud et al., 1977). Most reports indicate an increase in lactate levels after exposure to a stressor. Indeed, this response is often accepted as a common secondary measure of stress (Barton et al., 2002; Wendelaar Bonga, 1997). Our data suggest that this response is dependent on the nature of the stressor (e.g., requiring a switch from aerobic to anaerobic metabolic processes, consistent with an intense physical challenge or hypoxia exposure due to air emersion) to

elicit the lactate increase. Our results could be explained by increased hepatic clearance rates of lactate and/or utilization of lactate by gluconeogenesis (Suarez and Mommsen, 1987). Despite this novel finding, our chronic stress regime appears to potentiate the post-stressor reduction in lactate at 21 days (Fig. 3) when compared to 0, 7, and 14 days. We suggest that this lactate decrease might be due to a post-stressor gluconeogenesis enhancement consistent with the elevated glucose set point noted above. Additional investigations are needed to elucidate these mechanisms more completely, but the limited information available for a teleost does not report similar changes due to chronic stress (Barton et al., 1987).

Green sturgeon exposed to the chronic stress regime for 28 consecutive days increased their MMR approximately 30%, compared with control fish (Fig. 4), indicating a significant increase in maintenance costs associated with chronic stress. This finding supports our hypothesis, as well as the development of the "pre-pathological state" due to chronic stress. The MMR of control fish were similar to that of unstressed Adriatic sturgeon (McKenzie et al., 2001) and to unstressed green sturgeon acclimated to flow-through respirometers (Mayfield and Cech, 2003) corroborating the  $M_{O_2}$  data from the Brett-type swim-tunnel respirometer used in these experiments. There were no differences between the AMR of chronically stressed or control fish (Fig. 4).

To estimate the fish's ability to perform work and the impact that chronic stress has on this ability, we calculated the MSA. Exposure to chronic stress significantly decreased the MSA by approximately 25% (Fig. 5), indicating that the increased maintenance cost associated with coping with stress is not compensated for by an increased AMR. Barton and Schreck (1987) investigated the metabolic costs of exposure

to an acute stressor immediately prior to the measurement of MSA in juvenile steelhead. They reported a 25% reduction in MSA associated with the cost of stress, which is remarkably similar to our results. It is worth clarifying that our protocol did not involve an acute stress, as all fish were acclimated to the apparatus for at least 16 h and the control and chronically stressed fish were treated identically. These results suggest that fish exposed to the chronic stress regime would have an attenuated ability to perform work when compared to control fish and provides direct support for the development of the "pre-pathological state," from a bioenergetic point of view. This reduction in MSA might manifest itself by decreasing reproductive or somatic growth, immunocompetence, or performance-based measures, which have all been investigated as potential impacts of chronic stress in fish (Dhabhar and McEwan, 1997; Gregory and Wood, 1999; Haddy and Pankhurst, 1999; VanWeerd and Komen, 1998).

To assess changes in a performance variable, we measured the  $U_{crit}$ , or maximal sustained aerobic swimming velocity of control and chronically stressed green sturgeon and found no difference between the two treatments (Table. 1), rejecting our hypothesis that chronic stress would reduce  $U_{crit}$ . Interestingly, juvenile rainbow trout implanted with cortisol pellets have reduced food consumption, growth rates, and food conversion efficiency, but have no change in  $U_{crit}$  (Gregory and Wood, 1999), corroborating our results, and suggesting that  $U_{crit}$  might not be a good measure of chronic stress in fishes. Green sturgeon have a lower  $U_{crit}$ , compared to teleosts, although it is in accordance with  $U_{crit}$ s measured in other sturgeons (Adams and Adams, 2003; Adams et al., 1999; McKenzie et al., 2001). Sturgeons are believed to be less-efficient swimmers due to their heterocercal tail (Lauder, 2000; McKenzie et al., 2001). Although our results reveal a

lower  $U_{crit}$  in green sturgeon compared to these other sturgeons, the size of fish investigated most likely accounts for the difference, as our fish were at least 3 times the length of fish used in the studies cited above.

Finally, we investigated changes in free glucose and glycogen in the livers of chronically stressed and control fish. Chronically stressed fish had approximately 50% more free glucose in the hepatic tissue and vascular space than control fish, while the glycogen content of the liver was reduced by approximately 50 % in chronically stressed fish (Fig. 6). These results suggest an increased conversion of glycogen to glucose either for use as a hepatic substrate for gluconeogenesis, as suggested by Vijayan et al. (1994b), or to be released into the systemic circulation for use by other tissues, both accounting for the increased plasma glucose levels found at days 14 and 21 of our chronic stress regime (Fig. 2). Regardless, it is evident that the source for the increased glucose was at least in part liver glycogen, which was significantly decreased in the stressed fish (Fig. 6). This is somewhat contrary to results in teleosts, which appear to conserve liver glycogen during times of stress by altering hepatocyte responsiveness to glucoregulatory hormones after activation of the stress response (Vijayan et al., 1994a). Barton et al. (1987) also report no change in trout liver glycogen after 10 weeks of exposure to a single stressor day<sup>-1</sup>; however, fish fed cortisol-enriched feed for 10 weeks did significantly reduce liver glycogen levels in the same experiment and fish implanted with cortisol pellets had reduced liver glycogen (Vijayan et al., 1994b). Experiments on glycogen changes in response to acute stressors typically result in significant reductions in liver glycogen (Barton et al., 1986; Vijayan et al., 1997; Vijayan et al., 1994a). However, these experiments either involved multiple acute stressors applied within 3 h of each other or

appear to be short-term changes consistent with glycogenolytic processes due to catecholamines (Larsson, 1973; Randall and Perry, 1992). Our results suggest a long-term depletion of liver glycogen in feeding fish, which were not stressed for 2 days prior to liver collection. It is important to point out that our results were likely not due to the fish going off feed, as the fish were observed to be eating on several occasions. The energetic consequences of this glycogen depletion are unknown, but presumably the fish would have 50% less glycogen resources to mobilize during exposure to an acute stressor, reducing the fish's ability to cope with that stressor. It is probable that this situation would be exacerbated if food were not continuously supplied as it was in our experiments.

Taken together, these results suggest that the green sturgeon, a "species of special concern" in California, was metabolically compromised by exposure to our chronic stress regime, which we believe is the most accurate method to simulate chronic stress in the laboratory. We argue that the metabolic changes incurred are due to the inability of the green sturgeon to habituate to the stress regime, as evidenced by significant increases in plasma cortisol throughout the entire stress regime. Because green sturgeon remain responsive to stress, MMR is increased, resulting in a reduction in the MSA. These results suggest an increase in the maintenance requirements due to the additional resources needed to cope with stress, leaving fewer resources for alternative processes such as reproduction, the immune system, growth, and replenishment of energy stores. The decrease in MSA and liver glycogen levels suggests the development of the "pre-pathological state" that is theorized to decrease fitness and survivability of the animal.

We feel that these findings, which are not reported in teleosts, might be a factor associated with population declines in this species.

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## Acute Responses to Chronic Stressors

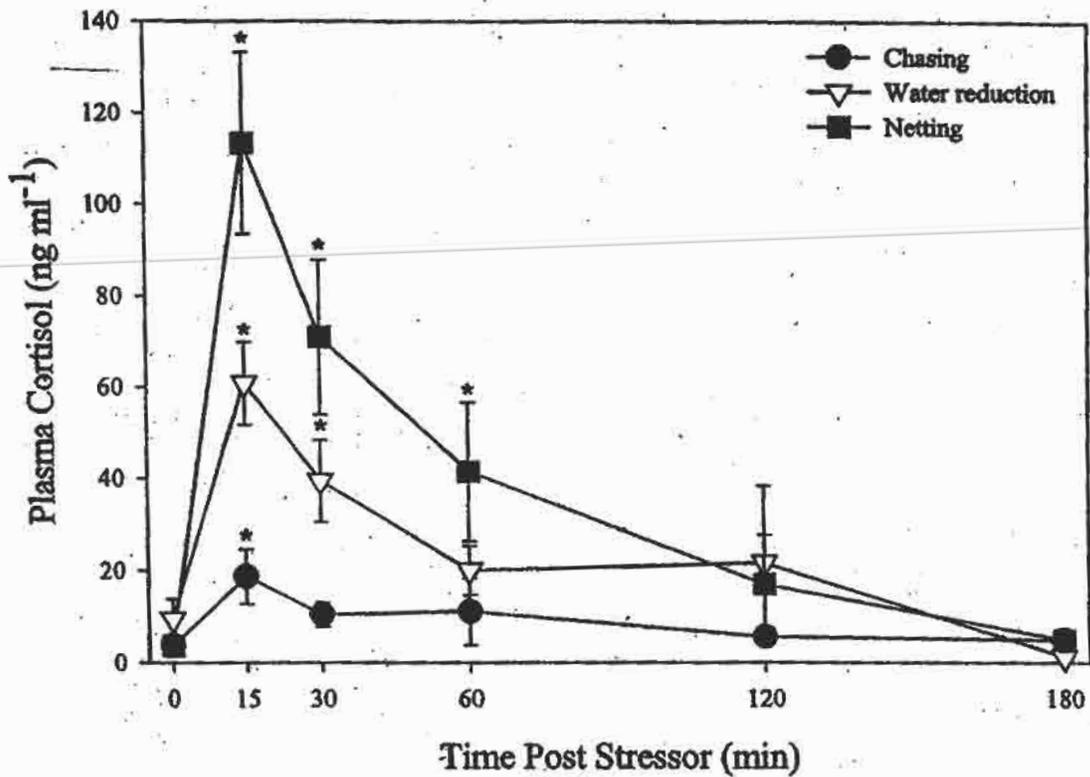


Fig. 1. Acute plasma cortisol responses (means  $\pm$  SEM) to three chronic stressors in cannulated age 0+ green sturgeon ( $n = 6 - 7$ ). The \* denote a significant difference ( $p \leq 0.05$ ; t-test or Mann-Whitney Rank Sum test) from control.

### Chronic Stress Habituation: Resting Glucose

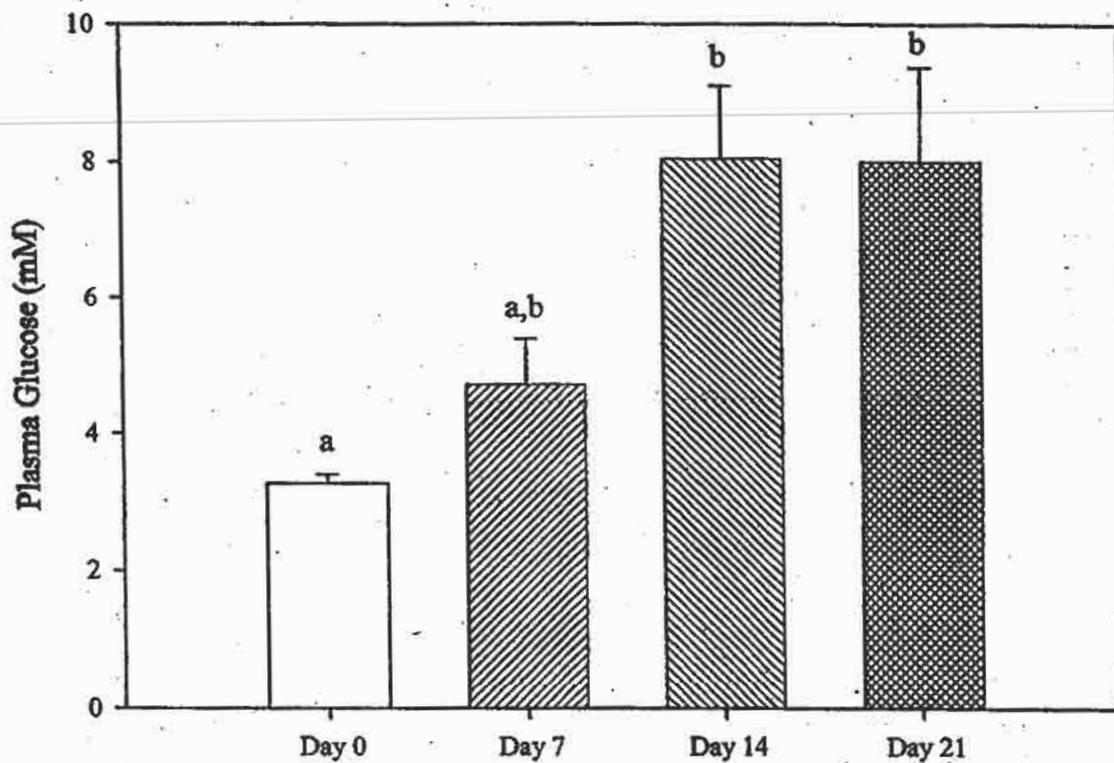


Fig. 2. Resting plasma glucose levels in age 1+ green sturgeon on days 0, 7, 14, and 21 of a chronic stress regime, which involved exposure to 2 of 3 stressors day<sup>-1</sup> for 21 consecutive days. The bars represent means ( $\pm$  SEM;  $n = 3$ ) and bars with the same symbol are not significantly different.

### Chronic Stress Habituation: Lactate

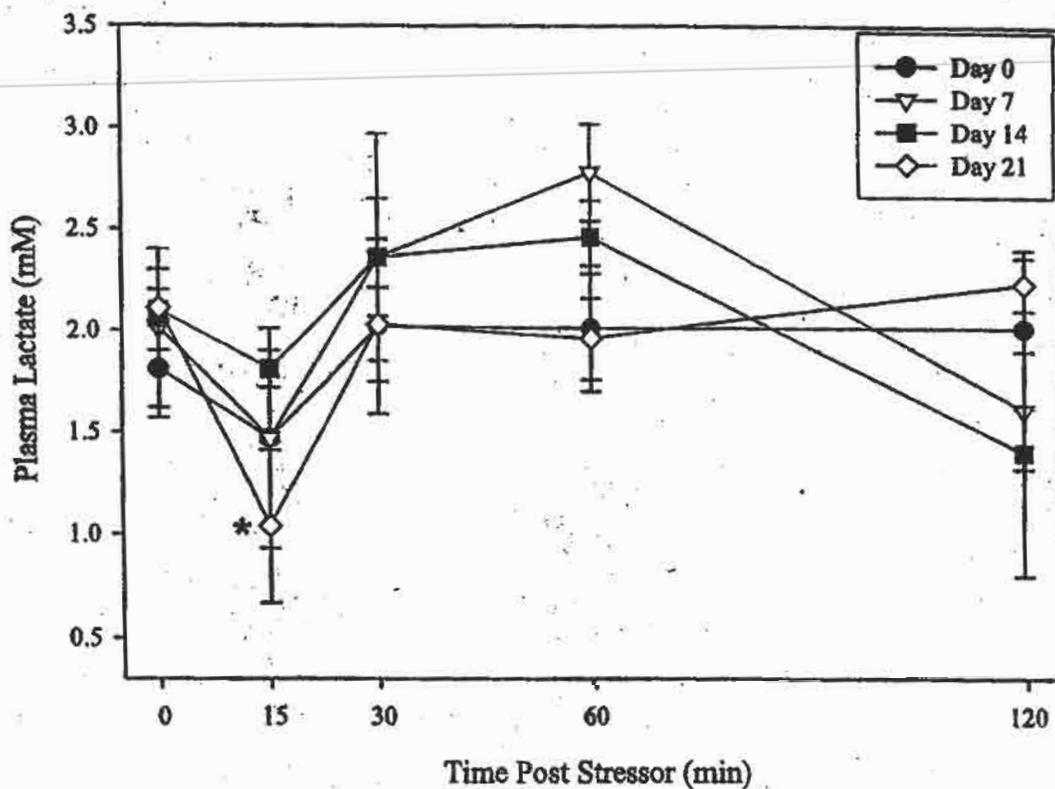


Fig. 3. Plasma lactate response to a water reduction stressor in age 1+ green sturgeon on days 0, 7, 14, and 21 of a chronic stress regime, which involved exposure to 2 of 3 stressors day<sup>-1</sup> for 21 consecutive days. The bars represent means ( $\pm$  SEM; n = 3) and the \* denotes a significant difference from time 0, at day 21.

Control vs. Chronic Stress:  
Maintenance and Active Metabolic Rates

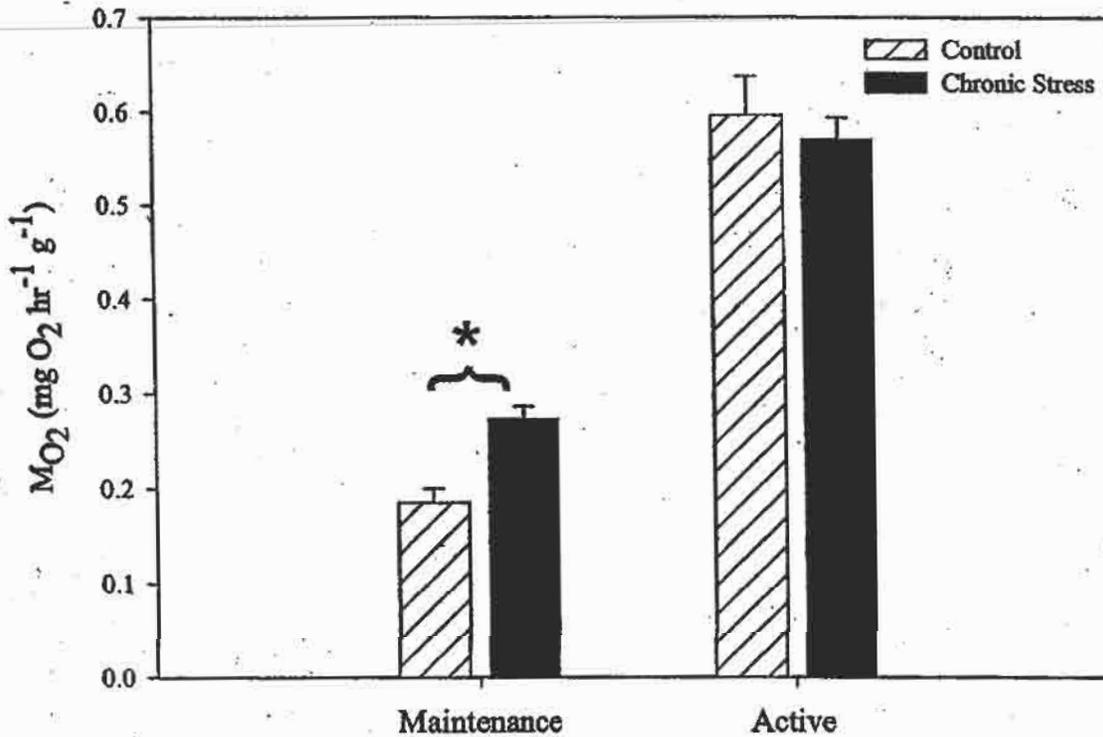


Fig. 4. Mean (+SEM) maintenance and active metabolic rates for age 0+ green sturgeon exposed to a control (unstressed, n = 11) or chronic (n = 10) stress regime. Means were compared using t-tests ( $p < 0.05$ ; \* denotes significant difference between groups).

Control vs. Chronic Stress:  
Scope for Activity

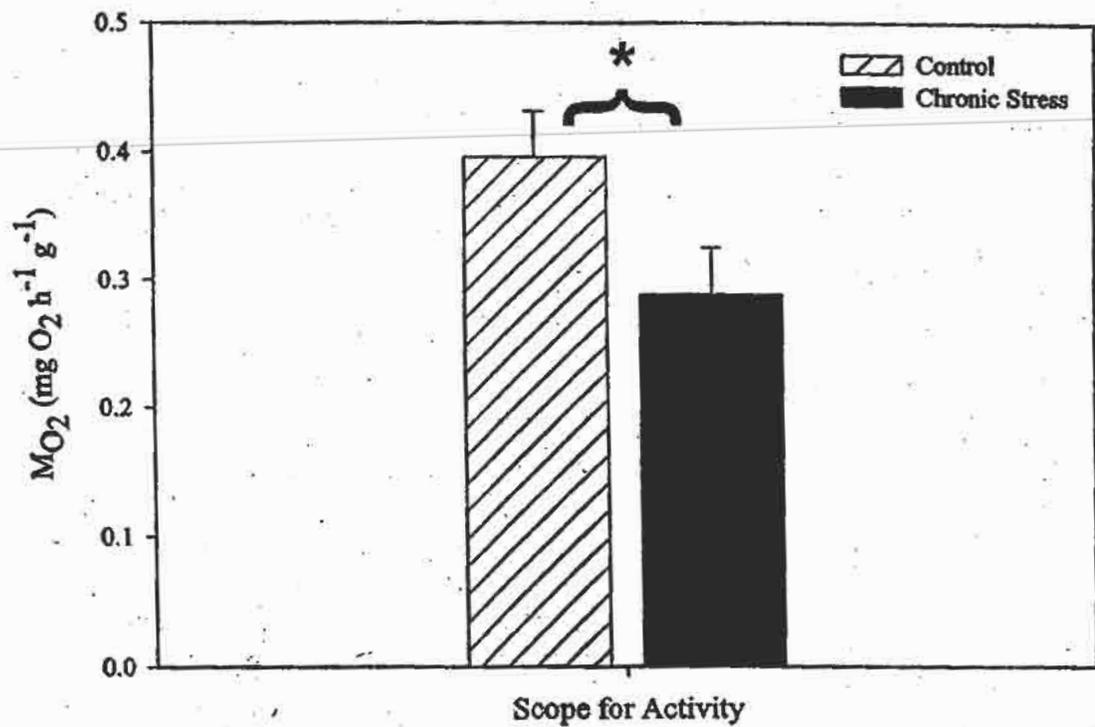


Fig. 5. Mean (+SEM) metabolic scope for activity in age 0+ green sturgeon exposed to a control (unstressed) or chronic stress regime. Means were compared using t-tests ( $p < 0.05$ ; \* denotes significant difference between groups).

Control vs. Chronic Stress:  
Liver Glucose and Glycogen

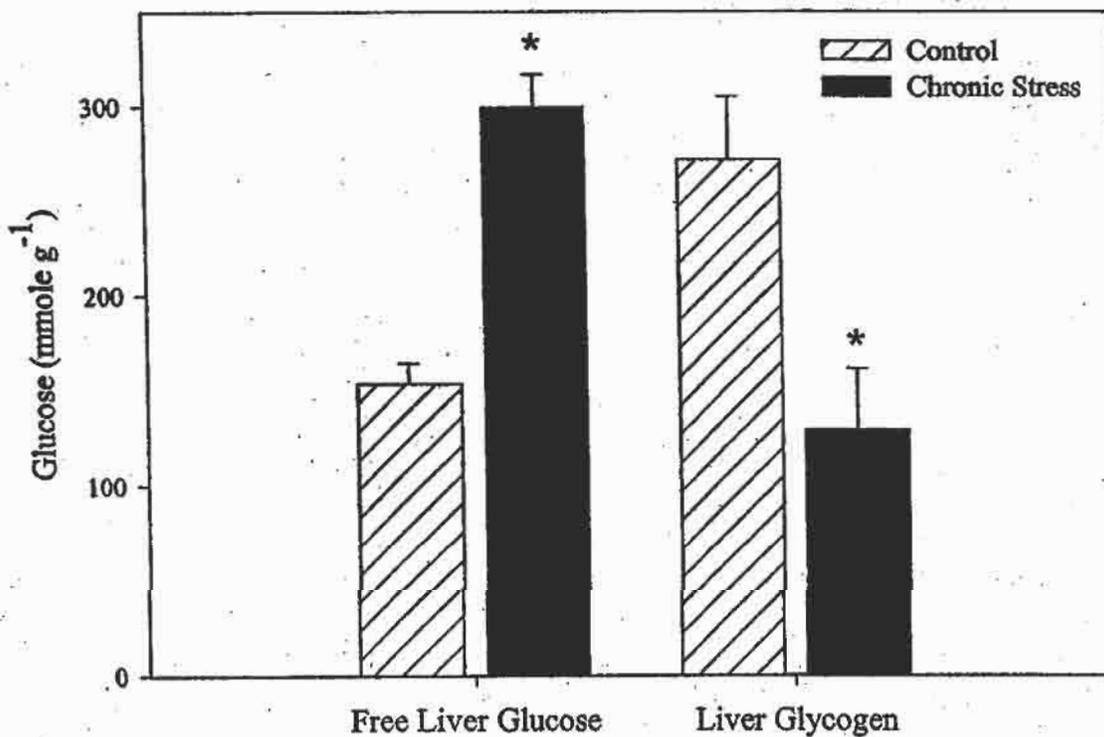


Fig. 6. Mean (+ SEM) free glucose and glucose liberated from liver glycogen in age 0+ green sturgeon liver homogenates sampled from either undisturbed fish (n = 8) or fish subjected to the chronic stress regime (n = 8) for 28 days. The \* denotes a significant difference ( $p \leq 0.05$ , t-test) from the control (unstressed).

**Table 1: The means ( $\pm$  SEM) of the critical swimming velocity ( $U_{crit}$ ) and the total lengths of age 0+ green sturgeon exposed to control (no stress; n=11) or chronic stress (n = 10) regimes. Means were compared using an unpaired Student's t-test. There were no significant differences between the means.**

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	<u>Control</u>	<u>Chronic Stress</u>	<u>P-Value</u>
$U_{crit}$ (BL sec <sup>-1</sup> )	0.78 $\pm$ 0.03	0.833 $\pm$ 0.02	0.137
Total Length (cm)	67.6 $\pm$ 1.0	64.8 $\pm$ 1.3	0.117

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*INVESTIGATIONS INTO THE EFFECTS OF CHRONIC STRESS ON SWIMMING PERFORMANCE, STANDARD METABOLIC RATE AND METABOLIC SCOPE FOR ACTIVITY IN GREEN STURGEON, ACIPENSER MEDIROSTRIS*

We investigated the impacts of chronic stress on the "standard" metabolic rate (SMR), "active" metabolic rate (AMR), metabolic scope for activity (MSA), and critical swimming velocity ( $U_{crit}$ ) in green sturgeon, Acipenser medirostris. Our approach was based upon a bioenergetic relationship, which hypothesizes that chronic stress will increase maintenance requirements for the fish resulting in a significantly increased SMR, decreased MSA, and reduced  $U_{crit}$ . To simulate chronic stress, groups of three young-of-the-year green sturgeon were placed into one of five identical, flow-through, indoor tanks and maintained on a natural photoperiod. Fish were stressed twice a day (1000 and 1600 h) for 28 consecutive days by exposure to two of three randomized acute stressors: a 5-min confinement stressor, a 5-min chasing stressor, or a 10-min water depth reduction stressor. These stressors were previously shown to elicit a significant plasma cortisol response. Measurements of SMR, AMR, MSA, and  $U_{crit}$  in both chronically stressed and control fish were conducted using a closed, variable speed, Brett-type respirometer (Brett, 1964). The MSA was calculated by subtracting the SMR from the highest AMR measured, and the  $U_{crit}$  was calculated following Brett (1964). Exposure to the chronic stress regime resulted in a significantly increased SMR and a 25% reduction in MSA, consistent with the hypotheses that acute stressors sum to simulate chronic stress and that chronic stress affects the SMR and MSA. Interestingly, there was no difference in  $U_{crit}$  between the stressed and control fish, and the stressed fish displayed positive growth throughout the 28-day regime. We conclude that our chronic stress regime resulted in a significant maintenance metabolic cost to green sturgeon, as indicated by the SMR and MSA measurements, but may not have jeopardized the overall health of these fish, as indicated from the  $U_{crit}$  and growth measurements.

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**The Effects of Size on Juvenile Green Sturgeon (*Acipenser medirostris*)  
Swimming Performance**

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Co-authors: Brian Hodge and Joseph J. Cech, Jr., Department of Wildlife, Fish, and Conservation Biology, and the Center for Aquatic Biology and Aquaculture, University of California, Davis

Studies of smolting salmonids have generally found a marked decrease in maximum aerobic swimming performance (Ucrit) compared to freshwater parr as a result of morphological and physiological changes

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**Metabolic and Hormonal Responses  
to Changing Flow Velocities in Green Sturgeon, *Acipenser medirostris***  
**Ryan A. Miller**

Sponsor: Joseph J. Cech, Ph.D., Wildlife, Fish and Conservation Biology  
Contributor: Scott Lankford, Ph.D. candidate

The steroid hormone, cortisol, is a highly conserved component of the physiological stress response in all vertebrates, including fish. Transient blood cortisol elevations are associated with many physiological disturbances in most fishes. Cortisol's identified functions include regulation of energy metabolism and water and salt balance. Increases in plasma lactate and glucose levels may accompany a cortisol stress response. Lactate accumulation results from either hypoxia (low oxygen) or severe exercise (anaerobic) stress. Although fish swimming performance is routinely measured by stepped velocity increases in a water tunnel, no one has measured the stress associated with this protocol. We measured the green sturgeons' swimming performance via 10 cm/s step increases in water velocity at 45-minute intervals until they fatigued. Water samples were taken at the beginning and end of each interval to measure energy metabolism and oxygen consumption during swimming. Blood samples from implanted catheters were taken once during every interval until exhaustion. I hypothesize a cortisol and lactate increase with increasing water velocities, followed by dissipation during the same interval. These increases will not exactly match metabolic activity suggesting that a change in water velocity itself is enough to elevate levels of blood cortisol. These increases will also display a general trend of increasing baseline cortisol and lactate levels while exhibiting an initial spike of cortisol accompanied by each velocity change.

313.13

Swimming performance, "standard" metabolic rate, and metabolic scope for activity as measures of chronic stress in green sturgeon, *Acipenser medirostris*

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The impacts of chronic stress on "standard" and "active" metabolic rates (SMR & AMR), metabolic scope for activity (MSA), and critical swimming velocity (Ucrit) were investigated in green sturgeon, *Acipenser medirostris*. We hypothesized that chronic stress would increase maintenance requirements with 1) increased SMR, 2) decreased MSA, and 3) decreased Ucrit. To simulate chronic stress, groups of three 0+ green sturgeon were placed into flow-through, indoor tanks and stressed twice d-1 (1000 and 1600 h) for 28 consecutive d. Fish were exposed to two of three randomized acute stressors: a 5-min confinement stressor, a 5-min chasing stressor, or a 10-min water depth reduction stressor. These stressors resulted in a graded acute plasma cortisol response, and fish were responsive to a stressor over the entire 28-d regime. Measurements of SMR, AMR, MSA, and Ucrit were conducted using a closed, variable speed, Brett-type respirometer. Exposure to the chronic stress regime resulted in a 25% reduction in MSA due to a significantly increased SMR (supporting hypotheses 1 & 2). There was no difference in Ucrit between the stressed and control fish (rejecting hypothesis 3). We conclude that our chronic stress regime resulted in a significant maintenance cost to green sturgeon, without decreasing their swimming performance. Interestingly, stressed fish showed positive growth. (US Anadromous Fish Restoration Program funding)

**[P026] Ontogeny Of Salinity Tolerance In Juvenile Green Sturgeon (*Acipenser medirostris*)**

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**Abstract:** Green sturgeon (*Acipenser medirostris*) generally occur in rivers with small estuaries, indicating that their ability to osmoregulate in seawater may occur at an early age. We measured salinity tolerance through gradual and abrupt salinity exposures using seawater. Experiments using freshwater or brackish water-acclimated (>14 days at 15 ppt) fish were repeated every two weeks until 100% survival at 34 ppt was achieved. Treatment groups (0, 15, 20, 25, 30, 34 ppt; and direct exposure: 30, 34 ppt) were adjusted as fish developed and exhibited greater tolerance. Fish were held in individual containers (4-L, 9-L, 25-L, or 50-L depending on size/age), randomized by treatment groups (6 fish/treatment), and subjected to a step increase in salinity (5 ppt/12 h) that culminated in a 72-h exposure to a target salinity. Surviving fish were sampled for plasma ionic concentrations ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ), osmolality, hematocrit, hormone concentrations (cortisol, T3 and T4), and gill tissue (chloride cells,  $\text{Na}^+/\text{K}^+$ -ATPase). A long-term (28-day) seawater (33 ppt) exposure conducted after the last 72-h experiment found 100% survival (38 fish). Juvenile green sturgeon increased in salinity tolerance with size and age, achieving seawater tolerance at 7 months or younger. Research funded by CALFED and Anadromous Fish Restoration Program (USFWS, USBR).

From: "Productive Pacific Ecosystems," Proceedings of the 2003 Annual Meeting of the Western Division of the American Fisheries Society, April 14-17, 2003, San Diego, California.

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**Abstract:** Metabolic scope for activity (MSA) and critical swimming velocity ( $U_{crit}$ ) were utilized as measures of chronic stress in green sturgeon, *Acipenser medirostris*. We hypothesized that chronic stress would increase maintenance requirements with 1) increased maintenance metabolic rates (MMR), 2) decreased MSA, and 3) decreased  $U_{crit}$ . To simulate chronic stress, groups of three 0+ green sturgeon were placed into flow-through, indoor tanks and stressed twice daily (1000 and 1600 h) for 28 consecutive days. Fish were exposed to two of three randomized acute stressors: a 5-min confinement stressor, a 5-min chasing stressor, or a 10-min water depth reduction stressor. These stressors resulted in a graded acute plasma cortisol response, and fish were responsive to a stressor over the entire 28-d regime. Metabolic measurements and  $U_{crit}$  were conducted using a closed, variable speed, Brett-type respirometer. Exposure to the chronic stress regime resulted in a 25% reduction in MSA due to a significantly increased MMR (supporting hypotheses 1 & 2). There was no difference in  $U_{crit}$  between the stressed and control fish (rejecting hypothesis 3). We conclude that our chronic stress regime resulted in a significant maintenance cost to green sturgeon, without decreasing their swimming performance.

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**Abstract:** Juvenile green sturgeon (*Acipenser medirostris*) are periodically subjected to high water temperatures in their natal rivers depending on the timing and duration of regulated water releases. Growth was measured at three temperature regimes (19°C, 24°C, 19-24°C -oscillating gradually every 12 hours) toward the warmer end of the expected range. Post yolk-sac fish (19°C) were randomized into 24 tanks (50-L circular, 8 tanks/treatment, 40 fish/tank), and temperature was increased 1°C /day to 24°C in elevated and fluctuating temperature tanks, after which 19-24°C tanks were started on a daily cycle (between 24°C at 1700 and 19°C at 0500). Fish were kept on a natural photoperiod, receiving flow-through well water (~3 L/min) and constant aeration. *Ad libitum* rations (Silver Cup Semi-Moist) were supplied by 24-hr feeders, and wastes and uneaten food were siphoned daily. Dissolved oxygen (always > 80 % of air saturation) and ammonia (always < 300 ppb) were measured daily and weekly respectively, from all tanks. Significant differences (ANOVA,  $\alpha=0.05$ ) were found between final weights (24°C > 19°C) and total lengths (24°C & 19-24°C > 19°C), indicating that temperatures within this range may not adversely affect juvenile fish. Research funded by CALFED and Anadromous Fish Restoration Program (USFWS, USBR).

From: Proceedings of the 133<sup>rd</sup> Annual Meeting of the American Fisheries Society,  
August 10-14, 2003, Quebec City, Quebec, Canada.

SP-38-04 Salinity Tolerance in Developing Juvenile Green Sturgeon (*Acipenser medirostris*)

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Ecosystem Restoration Program (CALFED) and the Anadromous Fish Restoration Program (USFWS, USBR).

From: Proceedings of the 133<sup>rd</sup> Annual Meeting of the American Fisheries Society,  
August 10-14, 2003, Quebec City, Quebec, Canada.

SO-38-16 Effects of Elevated and Fluctuating Temperatures on the Growth of Juvenile Green Sturgeon (*Acipenser medirostris*)

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Water impoundments and diversions alter temperature regimes of Pacific Coast rivers inhabited by anadromous green sturgeon (*Acipenser medirostris*). We measured the growth rates of juvenile green sturgeon at three, elevated, temperature regimes: 19°C, 24°C, and (oscillating) 19-24°C. Post yolk-sac fish (19°C) were randomized into 24, 50-l tanks and temperature was increased 1°C day<sup>-1</sup> to 24°C in appropriate tanks, and oscillating regime (24°C at 1700 and 19°C at 0500) started. Fish were kept on a natural photoperiod, receiving continuous well water (~3 l min<sup>-1</sup>) and aeration. *Ad libitum*, commercial rations were supplied by 24-h feeders, and wastes and uneaten food were siphoned daily. Dissolved oxygen (always > 80 % of air saturation) and ammonia (always < 30 ppb) were measured daily and weekly respectively, from all tanks. Significant differences (ANOVA,  $\alpha=0.05$ ) were found between final weights (24°C > 19°C) and total lengths (24°C & 19-24°C > 19°C), indicating that temperatures within this range do not adversely affect juveniles (body size range: ~1-10 g), with abundant food and oxygen. We thank Dave Hillemeier and the Yurok Tribe for broodstock and Prof. Serge Doroshov for juvenile fish. Research supported by the

Reprinted from the Proceedings of the Annual Meeting of the California-Nevada and Humboldt Chapters of the American Fisheries Society Symposium, March 29-31, 2001, Santa Rosa, CA.

### **MODIFICATION OF THE PHYSIOLOGICAL STRESS RESPONSE OF GREEN STURGEON, ACIPENSER MEDIROSTRIS, BY TIME OF DAY AND TEMPERATURE**

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(530) 752-8659, selankford@ucdavis.edu

Co-author: Joseph J. Cech, Jr., University of California, Davis

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There is limited physiological and life history information available for the green sturgeon, Acipenser medirostris, a species of special concern in California, and no information is available on its stress response. We quantified how the green sturgeon's response to an air emersion (1-minute) stressor is modified by time of day and temperature, by measuring the relative changes in post-stress concentrations of plasma cortisol (via radioimmunoassay), lactate and glucose (via YSI biochemical analyzer). We measured the stress response in young-of-the-year (6 months old) green sturgeon during the dark ( $n = 8$ ) and light hours ( $n = 8$ ) of the day. The stress response was increased at night to peak means of 19.09 ng/ml (cortisol) and 190.57 mg/L (lactate) compared to peak daytime means of 4.9 ng/ml and 166.69 mg/L, respectively, with no change in plasma glucose concentrations. The modification of the stress response by temperature was also investigated by acclimating two groups ( $n = 8$  per group) of green sturgeon (10 months old) to 11 or 19 °C over a 2-week period. Two days before the experiment all fish were cannulated (via dorsal vasculature) to allow for collection of multiple blood samples with minimal disturbance to the fish. While the peak post-stress cortisol concentrations were similar for 11 and 19° C groups (56.66 and 50.27 ng/ml, respectively), the synthesis and/or clearance of the hormone was prolonged at 11° C. No differences were found in post-stress lactate levels between temperature groups, but there was a significant increase, lasting 6 h, in post-stress plasma glucose levels in 11° C fish, providing evidence for glycogenolysis and gluconeogenesis. Our results provide insights into this fish's vulnerabilities to different environmental variables and management techniques that would assist natural resource and aquaculture managers in improving the condition, recruitment, and survivability of green sturgeon. This work supported by the CALFED Bay-Delta program.



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### **Warm Water-induced Growth Depression in Juvenile Green Sturgeon**

Peter Allen<sup>1</sup>, Joseph J. Cech, Jr.<sup>1</sup>, Amy Vlazny<sup>1</sup>, Serge Doroshov<sup>2</sup>, Joel Van Eenennaam<sup>2</sup> and David Hillemeier<sup>3</sup>

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Green sturgeon (*Acipenser medirostris*) spawn in the Klamath and Sacramento river systems, where the young-of-the-year sturgeon rear prior to their outmigration to the Pacific Ocean. Because of this native species' high value in these ecosystems and to their resident Native American cultures and because water temperature can vary dramatically during this rearing period in these regulated river systems, we investigated warm temperature (24°C) effects on juvenile green sturgeon growth. Fish (age: 31 d) were acclimated to 19 or 24°C water for 14 d in individual plastic containers. Containers (1.8-L: first 7 d, 4-L: last 9 d) featured continuous aeration, a well water inflow, and a screened top, and they were situated in temperature-controlled water baths. Water baths (natural photoperiod) were covered with shade cloth covers to lower indwelling light levels. Fish (initial mean wet weight: 1.27 g, mean TL: 58.0 mm; no significant difference between temperature groups,  $p > 0.05$ ) were hand-fed (Silver Cup trout feed) at 08:00, 14:00, and 20:00, and wastes and uneaten food were siphoned from tanks at 09:00 and 16:00, daily, over the 16-d growth experiment. Dissolved ammonia (always  $< 0.38$  mg/L) and oxygen (always  $> 7.34$  mg/L) levels were measured in 5, randomly selected containers at each temperature daily, with all containers being measured every 6 days. Fish reared at 19°C grew significantly (ANOVA,  $p < 0.05$ ) faster in wet weight (final mean  $\pm$  SE wet weights, 19°C:  $2.95 \pm 0.18$  g, 24°C:  $2.27 \pm 0.22$  g), but not ( $p > 0.05$ ) in TL (19°C:  $79 \pm 2$  mm; 24°C:  $74 \pm 2$  mm). These results, along with previous green sturgeon growth data at cooler temperatures from our laboratory, indicate that river temperatures should not increase beyond the 15-19°C range for optimal green sturgeon growth rates. Research funded by CALFED and Anadromous Fish Restoration Program (U.S. Fish and Wildlife Service, Bureau of Reclamation).

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### 36.6

#### Modification of the Physiological Stress Response in Green Sturgeon, *Acipenser medirostris*: The Influence of Time of Day and Temperature.

Scott Edward Lankford, Thomas E Adams, Joseph J Cech, Jr.: University of California, Davis, One Shields Blv., Davis, CA 95616

Physiological processes in poikilothermic animals are largely regulated by their surrounding environmental conditions. The light/dark cycle and temperature are examples of environmental variables that change predictably and unpredictably respectively, with temperature becoming even more unpredictable due to the impact of man. We investigated the modification of the physiological stress response by both time of day and temperature in young-of-the-year (YOY) green sturgeon (*Acipenser medirostris*), which were exposed to a 1-minute air emersion stressor in a net. The stress response (plasma cortisol, lactate, and glucose) was measured at two different times of day (0800 or 2000 h), as well as after a 2-week acclimation to 11 or 19 °C. The stress response was augmented at night, reaching a peak mean of 19.09 ng/ml cortisol and 190.57 mg/L lactate compared to 4.9 ng/ml cortisol and 166.69 mg/L lactate during the day. There was no significant stress-induced change in plasma glucose levels. Temperature did not affect the peak cortisol concentrations (56.66 and 50.27 ng/ml at 11 and 19 °C respectively), however the synthesis and/or clearance rate of cortisol was prolonged at 11 °C. The post-stress rise in lactate was similar between temperature groups, however there was a significant increase in post-stress glucose levels at 11 °C that was maintained for at least 6 h, suggesting both glycogenolysis and gluconeogenesis. This work is supported by the CALFED Bay-Delta program.

GB35

**Time of Day and Temperature Modify the Stress Response of the Green Sturgeon, *Acipenser medirostris*.**

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There is limited physiological and life history information available for the green sturgeon, *Acipenser medirostris*, a species of "special concern" in California, and no information is available on its stress responses. We are investigating how green sturgeon respond to stress (e.g., via activation of the hypothalamic-pituitary-interrenal axis, which results in the synthesis and release of cortisol) and possible factors that modify the stress response. We quantified how the green sturgeon's response to an air emersion (1-minute) stressor is modified by time of day and temperature, by measuring the relative post-stress rise in plasma cortisol (via radioimmunoassay), lactate and glucose (via YSI biochemical analyzer). We measured the stress response in young-of-the-year (6 months old) green sturgeon during the light (n=8) and dark hours (n=8) of the day. The stress response was increased at night to peak means of 19.09 ng/ml (cortisol) and 190.57 mg/L (lactate) compared to peak daytime means of 4.9 ng/ml and 166.69 mg/L, respectively, with no significant change in plasma glucose levels. The modification of the stress response by temperature was also investigated by acclimating two groups (n=8 per group) of green sturgeon (10 months old) to 11 or 19 °C over a 2-week period. Two days before the experiment all fish were cannulated (via dorsal vasculature) to allow for multiple blood samples to be taken with minimal disturbance to the fish. While the peak cortisol concentration post-stress was similar for 11 and 19 °C groups (56.66 and 50.27 ng/ml, respectively), the synthesis and clearance of the hormone was prolonged at 11 °C. No differences were found in post-stress lactate levels between groups, but there was a significant increase, lasting 6 h, in post-stress plasma glucose levels in 11 °C fish, providing evidence for both glycogenolysis and gluconeogenesis. Additional work is planned to determine the stress response to environmental hypoxia and to sudden changes in salinity and temperature for further insights into this fish's vulnerabilities to different environmental variables and management techniques. This information would assist natural resource and aquaculture managers in improving the condition, recruitment, and survivability of green sturgeon. Work supported by the CALFED Bay-Delta program.

**Keywords:** Stress, cortisol, sturgeon, lactate, glucose

**Preference:** oral presentation

**AV Equipment Requirement:** PC and projector for Power Point presentation

### Green Sturgeon Bioenergetic Responses to Temperature

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The green sturgeon (*Acipenser medirostris*) is comparatively rare in the Sacramento-San Joaquin Estuary of northern California, USA. Little is known about this species, which is considered a "species of special concern" in this ecosystem, and a new research program has been started to at the University of California, Davis, to learn the basic life history information needed for its protection. We measured temperature and ration size effects on green sturgeon juveniles' food consumption, growth, and food conversion rates at 11, 15, and 19EC. We also measured temperature effects (11, 19, and 24EC) on their resting routine metabolic rates and thermal preferences. Increases in temperature and ration size generally increased juvenile green sturgeon food consumption rates and growth rates. Conversion efficiency was higher at the reduced (50% of satiation) ration and at the warmer (19 and 24EC) temperatures compared with the satiation rations and cooler (11EC) temperature, respectively. Resting routine metabolic rate increased with increasing temperature and increasing body weight. Juvenile green sturgeon acclimated to 24EC preferred a significantly higher temperature than those acclimated to either 19 or 11EC. These temperature and ration-related responses should assist managers in preserving suitable habitat for green sturgeon in the Sacramento-San Joaquin Estuary. Research supported by CALFED Bay-Delta Program.

Key words: growth, food consumption, conversion efficiency, metabolism, temperature preferences

Poster presentation

## **Biological Assessment of Green Sturgeon in the Sacramento-San Joaquin Watershed Phase 3-4**

Anadromous Fish Recovery Program, Agreement # 11332-1-G005  
Task 2: Reproduction of Green Sturgeon

S.I. Doroshov, J.P. Van Eenennaam, and J. Linares-Casenave

The objectives of Task 2 were to elucidate the reproductive characteristics of green sturgeon and to determine the temperature influences on green sturgeon embryos and larvae. This report includes these two objectives and information on captive breeding. The Yurok Tribe's Fishery Program assisted in collection of the tissue samples from adults and broodstock capture for captive breeding. Work was conducted in collaboration with four other laboratories at UC Davis (J.Cech, P. Klimley, B. May, I.Werner).

### **Reproductive characteristics of wild green sturgeon**

Samples were collected from 198 adults (82 females and 116 males) during the spawning migration in April-June, 1999-2003. Fish were caught by gill nets (mesh 17-19 cm) in the lower Klamath River between the river mouth and RK 70. Tribal biologists measured length (TL and FL), live weight (W) and the gonad weight (GW) of brood fish, and collected samples of gonadal tissue and fin rays. In the laboratory, we estimated individual and relative fecundity (F and RF), egg diameter (ED) and the oocyte polarization index (PI, Dettlaff et al. 1993), using digital image-analysis. The condition factor (CF), gonadosomatic index (GSI), and fish age were determined as previously reported (Van Eenennaam et al., 1996).

The spawning run of green sturgeon in the lower Klamath River was observed from April through June, with the majority of fish caught in May. The stock was composed of multiple age groups and different sizes of fish (Table 1). Females and males differ significantly in mean length, age, condition factor, and gonadosomatic index. All fish were fully mature, indicated by the high GSI, presence of milting males, and large ovarian eggs (ED 4.33 mm) in an advanced stage of germinal vesicle migration (PI < 0.1, Table 1). Mean fecundity of green sturgeon was low (142,000), compared to white sturgeon (218,000; DeVore et al. 1995). Observations on fecundity, egg size, and larval development of the two species indicate that low fecundity of green sturgeon is a trade-off for the larger eggs and higher survival of juveniles (Deng et al. 2002). We plan to prepare manuscript on reproductive characteristics of green sturgeon broodstock for potential publication in the *Transactions of American Fisheries Society*.

### **Temperature influences on green sturgeon embryos and larvae**

Changes in the thermal regime of impounded rivers, such as the Sacramento and Klamath, may negatively affect reproduction and recruitment of sturgeons (Dettlaff et al. 1993). The rise of river temperature during the spring spawning season is of particular concern for the reproduction and early development of green sturgeon. We investigated the temperature effects on the survival and development of green sturgeon, using eggs and larvae obtained by artificial spawning of the Klamath River broodstock.

In a pilot experiment with a temperature range 11-26°C (2000), we determined that temperature 20°C affected embryo development and temperatures 23°C were lethal for green sturgeon embryos. The experiment with eggs of two females and temperatures 16, 17.5, 19, 20.5, 22, 23.5°C (2002) revealed significant effects of temperature and the egg source. Egg incubation at 23.5°C resulted in 100% mortality in both progenies. While the survival of two progenies varied in 20.5°C and 22°C treatments, both temperatures had high rates of embryonic deformities at hatch. The treatments within 16-19°C had high survival rates, but the proportions of abnormal larvae significantly increased in the 19°C treatment. The experimental results indicate that the upper thermal limit in developing green sturgeon embryos is approximately 18°C and Klamath River temperatures may exceed this limit during the springs of dry years (Van Eenennaam et al. in press, attached).

In the spring of 2003, we conducted a similar experiment with two progenies of yolk sac larvae hatched at normal incubation temperature (15.5°C) and held at 18, 20, 22, 24, 26, 28 °C to stage 44 (yolk absorption). There was 100% mortality at 28°C and lower survival at 26°C, compared to temperatures 18-22°C. Larvae held at 22-26 °C exhibited lordosis (notochord deformity) and the incidence and severity of lordosis increased with higher temperature. These results indicate that the temperature range 22-26 °C is suboptimal for green sturgeon larvae and temperatures above 26 °C are close to lethal (manuscript in preparation).

### **Improvement of captive breeding technique**

Captive breeding of green sturgeon provided live embryos, larvae, and juveniles for laboratory research in this project. Four females and 4 males from the Klamath River were used for spawning in the years of 2002 and 2003. We transported broodstock to campus in an insulated tank with oxygenated water and modified the spawning and culture techniques, compared to our initial protocol (Van Eenennaam et al., 2001). To induce gamete maturation, males received a single injection of 10 µg/kg GnRH<sub>a</sub> [D-Ala<sup>6</sup>, des-Gly<sup>10</sup>], while females received priming (0.6-1.0 µg/kg) and resolving (19 µg/kg) injections of GnRH<sub>a</sub>, with the addition of 0-3 mg kg<sup>-1</sup> domperidone (Table 2). Gamete collection followed Conte et al. (1988), and the sperm quality was evaluated by motility (5 µL of sperm : 200 µL of water). The other modifications were higher sperm density at fertilization (dilution 1:100), rinsing ova with water before fertilization (two rinses, 10-15 s each), and a longer fertilization time (4-5 min). Fertilized eggs were incubated in upwelling trout incubators (Eagar, Inc. Utah), with less turbulent flow compared to McDonald jars.

All brood fish responded with spermiation and ovulation to hormonal treatment. Fertilization rates (Table 2) improved, compared to low and more variable rates during the previous years. We identified several factors negatively affecting artificial spawning, including condition of broodfish, condition of river (rising temperature), low sperm: egg ratio in previous fertilization trials, and the sensitivity of fertilized green sturgeon eggs to turbulent flow in the MacDonald jars.

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Werner, I., Linares-Casenave, J., Lee, G., Van Eenennaam, J.P., and S.I. Doroshov. 2003. Temperature tolerance and the heat-shock protein response in larval green sturgeon (*Acipenser medirostris*). 6<sup>th</sup> Biennial State of the Estuary Conference. October 21-23, 2003. Oakland, California.

Table 1. Characteristics of green sturgeon broodstock in the lower Klamath River (mean  $\pm$  sd, range)

OBSERVATION	FEMALE (N=55-82)	MALE (N=74-113)
TL (cm)	196.7 $\pm$ 14.9 (162-242)	178.1 $\pm$ 14.7 (152-216)
FL (cm)	180.6 $\pm$ 13.7 (151-223)	162.9 $\pm$ 12.9 (139-199)
W (kg)	46.1 $\pm$ 8.6 (29.0-72.6)	32.0 $\pm$ 8.0 (19.1-55.8)
Age (yr)	27 $\pm$ 5 (16-40)	20 $\pm$ 4 (14-32)
CF (%)	0.78 $\pm$ 0.07 (0.65-0.94)	0.74 $\pm$ 0.07 (0.60-0.92)
GW (kg)	5.92 $\pm$ 1.97 (2.07-10.25)	1.70 $\pm$ 0.70 (0.40-3.74)
GSI (%)	12.62 $\pm$ 2.52 (7.13-17.19)	5.16 $\pm$ 1.35 (1.67-8.24)
F (x 1,000)	142 $\pm$ 41 (59-242)	-
RF (x 1,000 kg <sup>-1</sup> )	3.0 $\pm$ 0.5 (1.9-4.2)	-
ED (mm)	4.33 $\pm$ 0.14 (4.04-4.66)	-
PI (ratio)	0.04 $\pm$ 0.01 (0.02-0.08)	-

Table 2. Results of green sturgeon spawning at UC Davis in 2002-2003. Latency is an interval between the 2<sup>nd</sup> injection and ovulation.

DATE OF SPAWN	APR 28, 2002	APR 29, 2002	APR 25, 2003	MAY 16, 2003
River temperature	13°C	13°C	8-9°C	12-14°C
Female weight (kg)	50	56	51	48
1 <sup>st</sup> Injection: GnRH <sub>a</sub> Domperidone	0.6 µg/kg -	1.0 µg/kg 1 mg/kg	0.6 µg/kg -	0.6 µg/kg -
2 <sup>nd</sup> Injection: GnRH <sub>a</sub> Domperidone	19 µg/kg 1 mg/kg	19 µg/kg 3 mg/kg	19 µg/kg 1 mg/kg	19 µg/kg 1 mg/kg
Latency (hr)	14	14	18	14.5
Ova collected (x 1,000)	113	108	76	86
Fertilization (%)	82	62	31-44	60-75
Fry produced (x 1,000)	39	26	26	26

## Comparison of Early Life Stages and Growth of Green and White Sturgeon

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**Abstract.**—Gametes of green sturgeon *Acipenser medirostris* (caught in the Klamath River, California) and farm-reared white sturgeon *A. transmontanus* were obtained using hormonal induction of ovulation and spermiation. The offspring of one female in each species were reared in the laboratory, to compare their development and growth. Green and white sturgeon embryos had similar rates of development and hatched after 169 h and 176 h, respectively, at incubation temperature  $15.7 \pm 0.2^\circ\text{C}$ . Embryos of both species exhibited similar holoblastic development and passed through 36 stages characteristic of acipenserids. Green sturgeon fertilization and hatching rates were 41.2% and 28.0%, compared with 95.4% and 82.1% for the white sturgeon. Larval survival to 45 d (metamorphosis) was 93.3% in green and 92.1% in white sturgeon. Newly hatched green sturgeon (length  $13.7 \pm 0.4$  mm, mean  $\pm$  SD) were larger and less pigmented, compared with white sturgeon. They had large ovoid yolk sacs and did not exhibit pelagic behavior that was observed in white sturgeon. The onset of exogenous feeding in green sturgeon occurred at age 10–15 d and length  $24.0 \pm 0.5$  mm, and metamorphosis was completed at age 45 d and length  $74.4 \pm 5.9$  mm (rearing temperature  $18.5 \pm 0.2^\circ\text{C}$ ). Weight and length of green sturgeon larvae and juveniles were considerably greater than in white sturgeon at each sampling time, but the relative growth rate and weight-length relationship were similar in both species. This suggests an effect of larger egg size and maternal yolk supply on the growth of green sturgeon. We conclude that green sturgeon differs from the white sturgeon in their reproductive strategy and, potentially, reproductive habitat.

Green sturgeon *Acipenser medirostris* are widely distributed in the coastal waters along the north Pacific Ocean, having been recorded from at least six countries: the United States, Canada, Mexico, Russia, Japan, and Korea. However, because of low abundance, the green sturgeon are considered a threatened or vulnerable species in Canada and the United States and an endangered species in Russia (Houston 1988; Artyukhin and Andronov 1990; Moyle et al. 1994). Spawning populations of the anadromous green sturgeon have been identified in only a few rivers. In Asia, spawning has been found in the Tumnin River, and a successful artificial spawning of two females was reported (Artyukhin and Andronov 1990). In North America, green sturgeon spawning occurs in the Sacramento and Klamath Rivers, California, and in the Rogue River, Oregon (Moyle et al. 1994). Despite the green sturgeon's wide geographic distribution, there is no information on spawning migrations, spawning and nursery habitats, or the early life stages of this species.

We recently initiated studies on hatchery spawning and reproduction of green sturgeon and conducted the first artificial spawning in 1999 on the Klamath River (Van Eenennaam et al. 2001). As part of a larger project to study the biology of green sturgeon, the objective of this study was to examine the development and growth of green sturgeon in comparison with the sympatric white sturgeon *A. transmontanus*. Although the fertilized eggs of only one green and white sturgeon female were used in this study, we describe the important characteristics of the embryonic development and early life stages, as well as the larval growth in two species.

### Methods

The artificial spawning of green sturgeon was conducted on the lower Klamath River at Weitchpec, during 18–20 May 1999. One female (weight 48 kg) and five males, captured by gill net, were induced to ovulate and spermiate by administration of GnRH $\alpha$  and domperidone (Van Eenennaam et al. 2001). Fertilized with pooled milt, eggs

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were transported (6 h) to facilities of the Center for Aquatic Biology and Aquaculture (CABA) at the University of California, Davis. Fertilized eggs from one female white sturgeon (weight 63 kg) were obtained from Stolt Sea Farm California, LLC (Wilton, California), in July 1999, and transported (1 h) to CABA. Gametes were obtained using the same spawning induction protocol as for the green sturgeon but without domperidone. All husbandry procedures and sampling methods were identical for both species, as described below.

Upon arrival to CABA, fertilized eggs were acclimated to the incubation temperature for 1 h and placed in McDonald jars at a density of 1–1.5 L of eggs per jar (Conte et al. 1988). A 14 L:10 D photoperiod was maintained by artificial illumination throughout the incubation period. The water flow in the incubators was carefully adjusted, to allow the eggs to roll gently during the early stages (cleavage and gastrulation), and was increased at the later stages of development, to reduce the incidence of fungal infection. Dead eggs were periodically removed by siphon and counted as mortality. Incubation was conducted in a semirecirculation system, where the water was filtered and chilled and temperature was maintained at  $15.7 \pm 0.2^\circ\text{C}$  (range  $15.5$ – $15.8^\circ\text{C}$ ).

Hatched larvae were transferred into the circular larval receiving tank (1.2 m-diameter) connected to the semirecirculation system, and during the next 5 d the water temperature, was increased to  $18.5^\circ\text{C}$ , at a rate of  $1^\circ\text{C}$  per day. One-thousand five-hundred 5 d old larvae were stocked into a circular flow-through tank of 1.2 m-diameter and 355 L-volume, in a separate building with skylight windows. Water was supplied by a spray bar, at a rate of 9–10 L/min, creating a circular current in the tank. Fish were fed a commercial semimoist fry feed (Silver Cup, Nelson & Sons, Inc., Murray, Utah), provided continuously over 24 h by 3 automatic feeders placed at equal distances around the wall of the tank. The fish were also hand fed 5 g of chopped *Tubifex* worms twice a day. The tank was exposed to natural photoperiod, but the light intensity was partially reduced by black plastic shade cloth extending over 2/3 of the tank. The tank was cleaned daily, and mortalities were removed and recorded. Larval behavior was observed during the day and at night, with a white dim flashlight with which no obvious disturbance occurred. Water temperature was recorded daily and averaged  $18.5 \pm 0.2^\circ\text{C}$  (range  $18.2$ – $18.7^\circ\text{C}$ ). The dissolved oxygen exceeded 8.6 mg/L during the entire 45 d rearing period.

Staging of embryonic development followed the classification of Dettlaff et al. (1993) that includes 36 stages to hatching. More than 300 eggs were randomly sampled at cleavage (stages 5–6) to determine the rates of fertilization. The embryonic development was closely monitored in McDonald jars, and the published developmental rates of white (Wang et al. 1985) and Russian (Dettlaff et al. 1993) sturgeon *A. gueldenstaedti*, at similar temperatures, were used to estimate sampling time for specific stages of the green sturgeon. To estimate the rate of development, the post fertilization time was recorded when more than 50% of the eggs reached a defined stage. All embryos were fixed in 10% phosphate-buffered formaldehyde solution for further observations and measurements. Thirty preserved ova and stage 5 (second cleavage, fully formed perivitelline space) eggs were weighed ( $\pm 0.1$  mg) and measured ( $\pm 0.01$  mm) for maximum diameters under a dissecting microscope with a digital image-analyzing tablet. The chorions of the additional five eggs at each stage were removed under a dissecting microscope before fixation, to facilitate photomicrography.

Samples of 30 larvae and juveniles were collected at days 0 (hatching), 1, 3, 6, 10, 15, 21, 28, 36, and 45 post hatch. Animals were euthanatized by overdose of MS-222 and preserved in buffered formalin for further observations and measurements. All larvae were individually weighed and measured for length under a dissecting microscope or by using a micrometer caliper. Measurements reflect minor shrinkage due to fixation in formalin.

Mean weight and length of green and white sturgeon were compared by Student's *t*-test. A few larvae (with bent bodies or other defects) were deleted from the samples before calculation of means and standard deviations. The specific growth rates were calculated as  $100 \times (\ln W_t - \ln W_0) / t$ , where  $W_t$  and  $W_0$  are mean body weight at each of the two samplings, and  $t$  is the number of days between the two samplings. Weight-length relationships were evaluated by linear regression analysis using  $\log_{10}$ -transformed data. The regressions were tested for the lack of fit and their slopes compared using a *t*-test. The JMP Statistical Software (Version 3, SAS Institute, Cary, NC) was used for data analysis. The accepted significance level was  $P < 0.05$ .

Photomicrographs of embryos and larvae were scanned by SprintScan 35 plus (Polaroid Cooperation, Cambridge, Massachusetts) and

edited using Adobe Photoshop Software (Version 5.0) to remove the dark background and to adjust contrast for clearer pictures.

## Results

### Survival Rate

Fertilization (Stage 5) and hatching (Stage 36) rates for eggs from the wild green sturgeon were 41.2% and 28.0%, respectively (Van Eenennaam et al. 2001), whereas the domestically reared white sturgeon exhibited higher fertilization (95.4%) and hatching (82.1%) rates. Larval survival from hatching to metamorphosis (age 45 d) was high in both species, 93.3% in green and 92.1% in white sturgeon. No mortalities occurred after 32 d posthatch in green sturgeon, while cumulative mortality of white sturgeon exhibited small but steady increases throughout the 45 d experiment.

### Embryonic Development

Early development of green sturgeon followed the holoblastic style of *Acipenseriformes* described in detail by Dettlaff et al. (1993). We refer to their descriptions and briefly characterize selected stages with the distinguishing characteristics of green sturgeon (Figure 1 and 2). For comparative purposes, similar stages of white sturgeon are given (for more detailed illustrations see Beer, 1981). Enumeration of stages corresponds to the classification of Dettlaff et al. (1993).

**Stage 5 (second cleavage, Figure 1).**—The eggs of green sturgeon, although varying slightly in pigmentation, had a flattened white animal region with a small dark pigmented spot in the center, which was unevenly divided by the two cleavage furrows. Their vegetal hemisphere was brown-olive green. A narrow pigmented ring (with lighter pigmentation on the other side), which was not apparent at this stage in white sturgeon eggs, appeared along the boundary between animal and vegetal regions. The weight of green sturgeon eggs that completed hydration and hardening increased from  $35.6 \pm 0.9$  mg (ova) to  $38.6 \pm 1.2$  mg (stage 5) and their maximum diameter from  $4.17 \pm 0.12$  to  $4.44 \pm 0.15$  mm, respectively ( $N = 30$ ). Smaller white sturgeon eggs were more darkly pigmented and exhibited similar relative increases in weight (from  $17.9 \pm 1.1$  to  $21.6 \pm 0.8$  mg) and diameter (from  $3.40 \pm 0.09$  to  $3.57 \pm 0.11$  mm) at stage 5 ( $N = 30$ ).

**Stage 14 (early gastrula, Figure 1).**—The horizontal blastopore in green sturgeon embryos ap-

peared as a short shallow groove and was darkly pigmented. Blastomeres positioned along the border between animal and vegetal regions (marginal zone) were intermediate in size and darker than those at either pole, forming a wide speckled zone that appeared at the late cleavage stage. A ratio of the distance from blastopore to animal pole to that from blastopore to vegetal pole varied within a range of 0.60–0.95, indicating the variable location of the blastopore above the egg equator. The horizontal blastopore of white sturgeon embryos appeared on the equator (Beer 1981; Bolker 1993b).

**Stage 22 (late neurula, Figure 1).**—The neural plate (in a process of folding) slightly protruded above the surface of the green sturgeon egg with a diamond-shaped opening in the anterior part and incomplete closure of neural fold in the trunk region. The region of the neural plate was grayish, and the rest of the embryo was yellowish. White sturgeon eggs had a similar neurulation pattern with a darker pigmented neural plate. Epiboly was completed in both species before the onset of neurulation.

**Stage 35 (embryo before hatch, Figure 1).**—The egg chorion was softened and became fragile after secretion of a hatching enzyme (Dettlaff et al. 1993). The hatching gland was evident in front of the mouth cleft. With an actively twisting tail and trunk, the tail of an embryo was able to break through the chorion and stretch out. The chorion surrounding the head and ovoid shaped "yolk sac" (endoderm in sturgeon) was discarded by further movement of the trunk.

**Rates of embryo development.**—The chronology of embryonic development of the green and white sturgeon was similar (Table 1). Despite the great difference in egg size, the green and white sturgeon embryos exhibited an overall similar rate of development at 15.7°C. Cleavage and morphogenetic movement appeared to proceed at slower rates in green sturgeon. However, the developmental pace was accelerated before hatching, resulting in an 8 h earlier mass hatching than in the white sturgeon. Hatching occurred over two days in both species, from 144 to 192 h after fertilization in green sturgeon and from 152 to 200 h in white sturgeon. The earlier hatching of the green sturgeon could also be associated with its thin and fragile chorion.

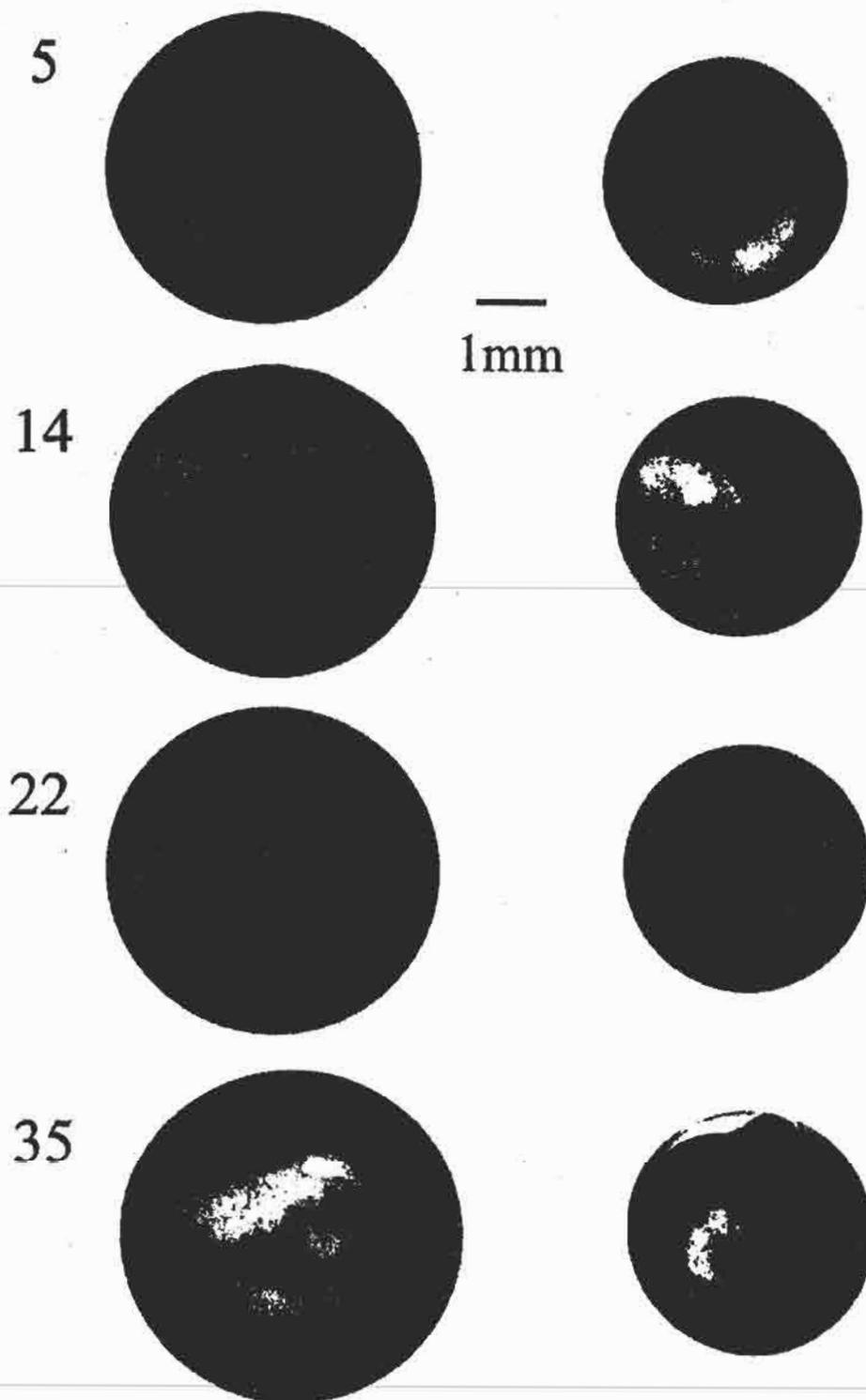


Figure 1. Stages of embryonic development of green sturgeon (left) and white sturgeon (right). Stage 5—second cleavage; Stage 14—early gastrula; Stage 22—late neurula; Stage 35—prehatch embryo (stages by Dettlaff et al. 1993).

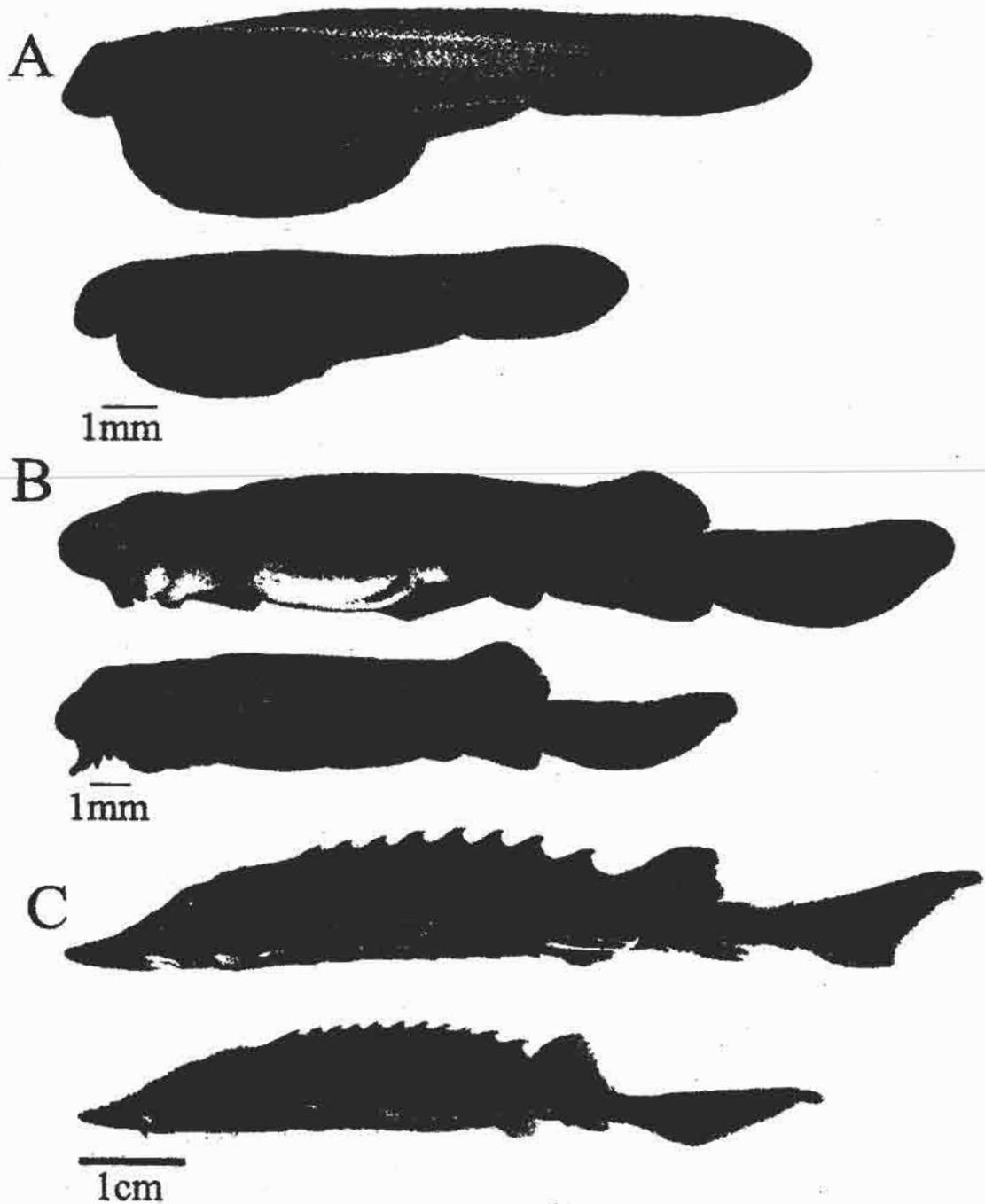


Figure 2. Larvae and juveniles of green and white sturgeon at the same age. Green sturgeon on the top and white sturgeon at the bottom in A, B, and C, respectively. A—posthatch larvae (stage 36); B—larvae at the onset of feeding (10 d posthatch); C—juveniles (45 d posthatch).

Table 1. Chronology of embryonic development of green and white sturgeon at 15.7°C. Time of respective stages (Dettlaff et al. 1993) is in h:min after fertilization.

Stages	Green sturgeon	White sturgeon
1	0	0
4	6:10	4:10
5	7:40	4:55
6	9:40	5:50
7	10:30	7:00
8	10:57	7:30
9	-	9:10
10	-	10:10
11	17:55	13:00
12	23:58	-
13	27:15	23:40
14	31:35	-
15	-	31:20
16	41:45	35:00
17	45:25	39:50
18	47:45	-
19	48:30	-
20	49:25	46:10
21	52:50	49:00
22	55:00	52:50
23	57:30	-
24	65:40	-
25	68:52	70:00
26	-	-
27	80:40	77:20
28	89:50	83:00
29	97:35	-
30	-	95:00
31	-	104:00
32	145:35	120:00
33	154:05	128:30
34	161:50	144:00
35	-	159:40
36	169:00	176:40

#### Larval Development

*Larvae at hatch* (Figure 2A). —Newly hatched green sturgeon (L = 12.6 - 14.5 mm, range) were grayish in the trunk and had a large ovoid yolk sac with yellowish coloration. They were considerably less pigmented than the white sturgeon larvae. The larval body had 63-71 myotomes, of which 36-41 are anterior to the cloaca and 27-31 were posterior to the cloaca. The eyes were well developed, with differentiated lenses and a dark pigmented spots. Olfactory and auditory vesicles were present. Mouth and gill cover differentiation began as a shallow cleft. The posterior intestine contained a dark pigment in the spiral valve. The paired Cuvier's ducts and yolk veins with red

blood were highly developed. The continuous fin fold was interrupted at the cloaca and had a slightly wrinkled area in the preanal region posterior to the yolk sac. The rudiments of pectoral fins appeared as small buds on the dorsal part of the yolk sac behind the pronephros. White sturgeon hatchlings (L = 10.0 - 11.0 mm) were darker, with dense melanin pigmentation of the trunk and yolk sac. White sturgeon larvae appeared to be less developed at hatching, lacking eye lenses and pectoral fin buds. Their fin folds were smooth and not wrinkled at the preanal regions as it was in green sturgeon.

*Larvae at the onset of feeding* (Figure 2B). —At 10 d of age, pigmentation of green sturgeon (L = 23.0 - 25.2 mm) greatly increased on the head and along the trunk, except the ventral region. The larvae became dark gray. Myotomes extended to the ventral side of the yolk sac, which was greatly diminished in size, resulting in a streamlined body shape. The barbels had been elongated. Rays started to form in all fin rudiments, including the lower lobe of the caudal fin. The pectoral fins moved down to the ventral region and acquired a horizontal position. The fin fold discontinued posterior to both the dorsal and anal fins. The lateral lines extended over the mid-body. The third pair of branchial arches had formed. Larval teeth were visible on the upper and lower jaws. The spleen was present as a bright red spot. In white sturgeon of the same age (L = 17.3 - 19 mm), larvae were darkly pigmented in the tail region. Their yolk was practically absorbed, and the lateral lines were completely developed. Some of the larvae started releasing their melanin plugs. White sturgeon larvae started exogenous feeding at this stage.

*Juveniles at metamorphosis* (Figure 2C). —At 45 d of age, the green sturgeon (L = 62.5 - 94.4 mm) had completed metamorphosis, which was characterized by development of dorsal, lateral, and ventral scutes, elongation of barbels, rostrum, and caudal peduncle, full resorption of caudal and ventral fin folds, and development of fin rays. Juveniles were similar to adults in body shape and olive-green coloration, with a dark midventral stripe. Development of lateral scutes started at the anterior portion of the trunk immediately posterior to the gill cover, progressing toward the caudal region, while dorsal and ventral scutes started differentiation in the mid-body region anterior to

the dorsal and ventral fins. The ventral scutes appeared at 28 d after the dorsal and lateral scutes had been differentiated. At age 45 d, green sturgeon juveniles had 25.7 (24–28) lateral scutes, compared with 37.8 (34–40) in the white sturgeon (mean and range,  $N = 5$ ). A few small bony grains and platelets started to develop at age 28 d between the dorsal and lateral scutes, but they were not abundant at metamorphosis and gave the green sturgeon skin a smooth appearance, compared with the white sturgeon. In white sturgeon ( $L = 31.0 - 78.2$  mm at 45 d), the bony grains and platelets began to appear on the dorsal portion of the head as early as 15 d post hatch. They continued to develop and became abundant on the head, operculum and the whole trunk (except the ventral portion), giving the skin of white sturgeon juveniles a rugged appearance. White sturgeon juveniles had the uniform gray coloration of their bodies.

#### *Growth and Weight-Length Relationship*

Changes in length and weight of green and white sturgeon larvae and juveniles are shown in Figure 3. Mean ( $\pm$ SD) weight and length of newly hatched larvae were  $36.3 \pm 2.4$  mg and  $13.7 \pm 0.4$  mm ( $N = 29$ ), for green sturgeon, and  $15.8 \pm 0.9$  mg and  $10.6 \pm 0.3$  mm ( $N = 28$ ), for white sturgeon. Based on the biochemical study with white sturgeon larvae (Wang et al. 1987), the increase in wet weight during the endogenous feeding (days 0–10) was caused by an increase of moisture content, since the dry matter decreases during the endogenous feeding phase. At the feeding stage (age 10 d), mean weight and length were  $88.3 \pm 4.3$  mg and  $24.0 \pm 0.5$  mm ( $N = 27$ ), for green sturgeon, and  $41.9 \pm 2.4$  mg and  $18.4 \pm 0.5$  mm ( $N = 27$ ), for white sturgeon. Mean weights increased rapidly in both species during exogenous feeding (days 15–45), and green sturgeon were larger at each sampling time (Figure 3). At age 45 d, the weight and length of green sturgeon juveniles were  $2500 \pm 525$  mg and  $74.4 \pm 5.9$  mm ( $N = 27$ ), while the weight and length of the white sturgeon were  $1471 \pm 864$  mg and  $60.9 \pm 15.3$  mm ( $N = 29$ ). However, the specific growth rates during the exogenous feeding phase (15–45 d) were similar in both species,  $10.4\% d^{-1}$  and  $10.2\% d^{-1}$  for green and white sturgeon, respectively.

The analysis of weight-length relationship revealed two developmental periods of allometric growth: the yolk absorption phase, with low regression slopes, and the exogenous feeding phase, with higher slopes (Figure 4). The linear

equations for log-transformed variables are given below ( $W$ ,  $L$ , and  $R^2$  are body weight, total length and coefficient of determination):

Green sturgeon: age 0–6 d  $\log W = 0.118 + 1.267 \log L$ ,  $R^2 = 0.93$  ( $N = 115$ )

21–45 d  $\log W = -1.764 + 2.764 \log L$ ,  $R^2 = 0.99$  ( $N = 111$ )

White sturgeon: age 0–6 d  $\log W = -0.207 + 1.384 \log L$ ,  $R^2 = 0.93$  ( $N = 117$ )

15–45 d  $\log W = -1.869 + 2.795 \log L$ ,  $R^2 = 0.99$  ( $N = 146$ )

There was no significant difference in the regression line slopes for each developmental period between the two species, indicating similar patterns of allometric growth. Fish sampled at the onset of exogenous feeding (10 d in white and 10–15 d in green sturgeon) had a large variation in weight and length and did not fit either regression line (Figure 4, shown by larger symbols).

#### *Larval Behavior*

Green sturgeon larvae did not exhibit the pelagic swim-up behavior seen in other acipenserids. During the first 5 d post hatch, the green sturgeon larvae exhibited a strong tendency to clump together in large numbers at the bottom, around the edges of stones, polyvinyl chloride (PVC) pipes, the central drain pipe, or along the wall of the tank. They remained in clumps with limited movement during the night. Larvae began to display a nocturnal swim-up behavior at 6 d post hatch, when the rudiments of the pectoral and ventral fins were developed, dorsal and anal fin rays became apparent, yolk of the mid-intestine was depleted, and the mandible started rhythmic movement. Larvae clumped under the shade cloth during the day but swam actively during the night. These nocturnal behavior patterns persisted in green sturgeon from the onset of exogenous feeding to metamorphosis.

Unlike the green sturgeon, white sturgeon exhibited pelagic behavior during the first 5 d after hatching. The white sturgeon larvae swam up and out of the incubation jars upon hatching, dispersed throughout the water column in the rearing tank, and swam constantly during the day and night. They began to display nocturnal behavior, similar to green sturgeon, at 6 d post hatch, with the transition from the pelagic to a demersal swimming during the day and dispersal into the water column during the night. The white sturgeon swam at the bottom of the tank, aggregating in small groups, but never clumped as strongly as the green sturgeon. At the onset of exogenous

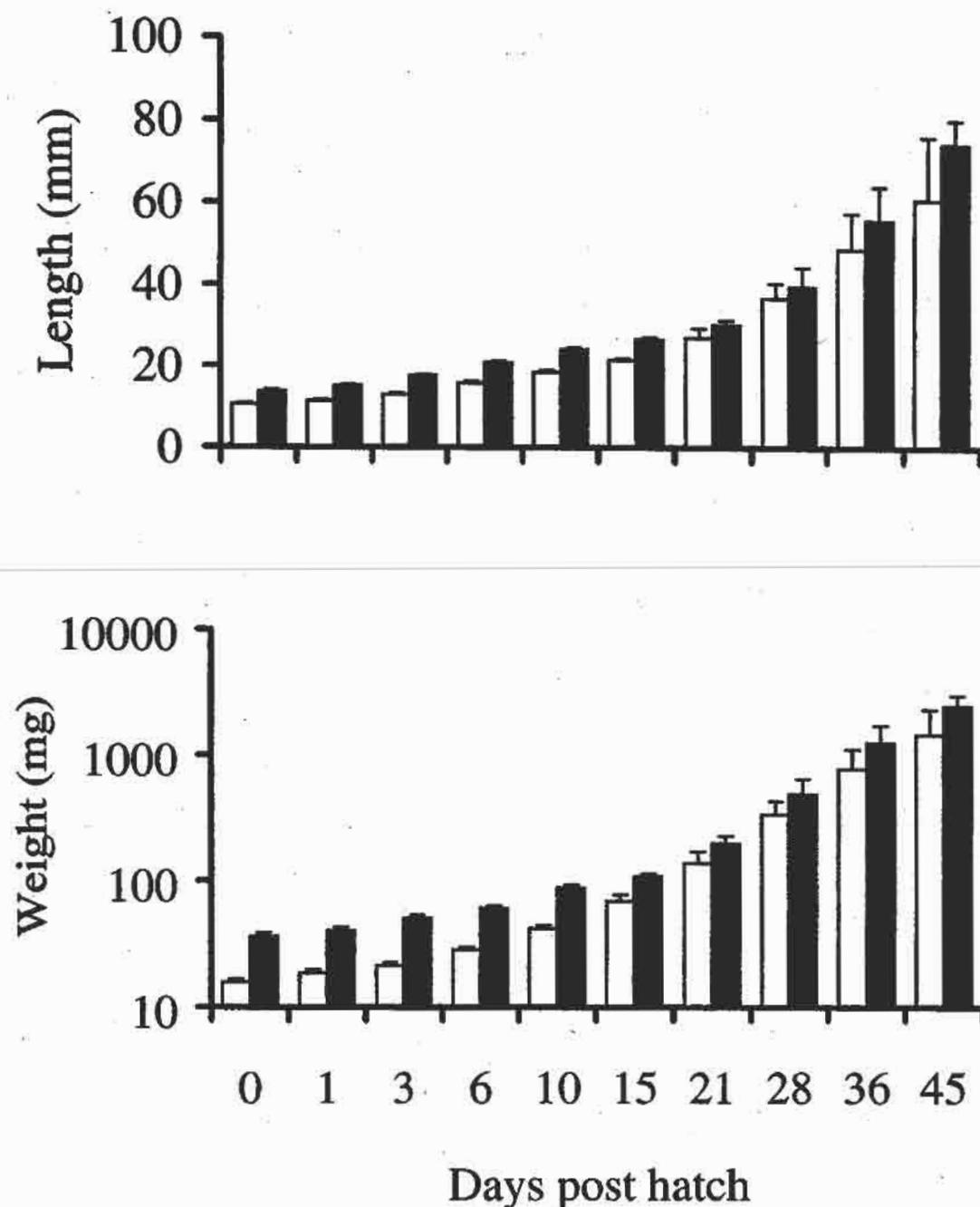


Figure 3. Length and weight (mean  $\pm$  SD) of white (open bars) and green (shaded bars) sturgeon from hatching to metamorphosis. Weight is shown in log-scale.

feeding, the clumping behavior disappeared, and larvae dispersed along the tank bottom.

#### Discussion

Embryos of green sturgeon exhibit the holoblastic pattern of development, similar to white stur-

geon (Beer 1981; Bolker 1993a, 1993b) and other acipenserids (Dettlaff et al. 1993). The rates of embryonic development and hatching time, at temperature 15.7°C, are similar in green and white sturgeon. Newly hatched larvae of green sturgeon are longer and heavier than larvae of white sturgeon, and they possess large reserves of endoder-

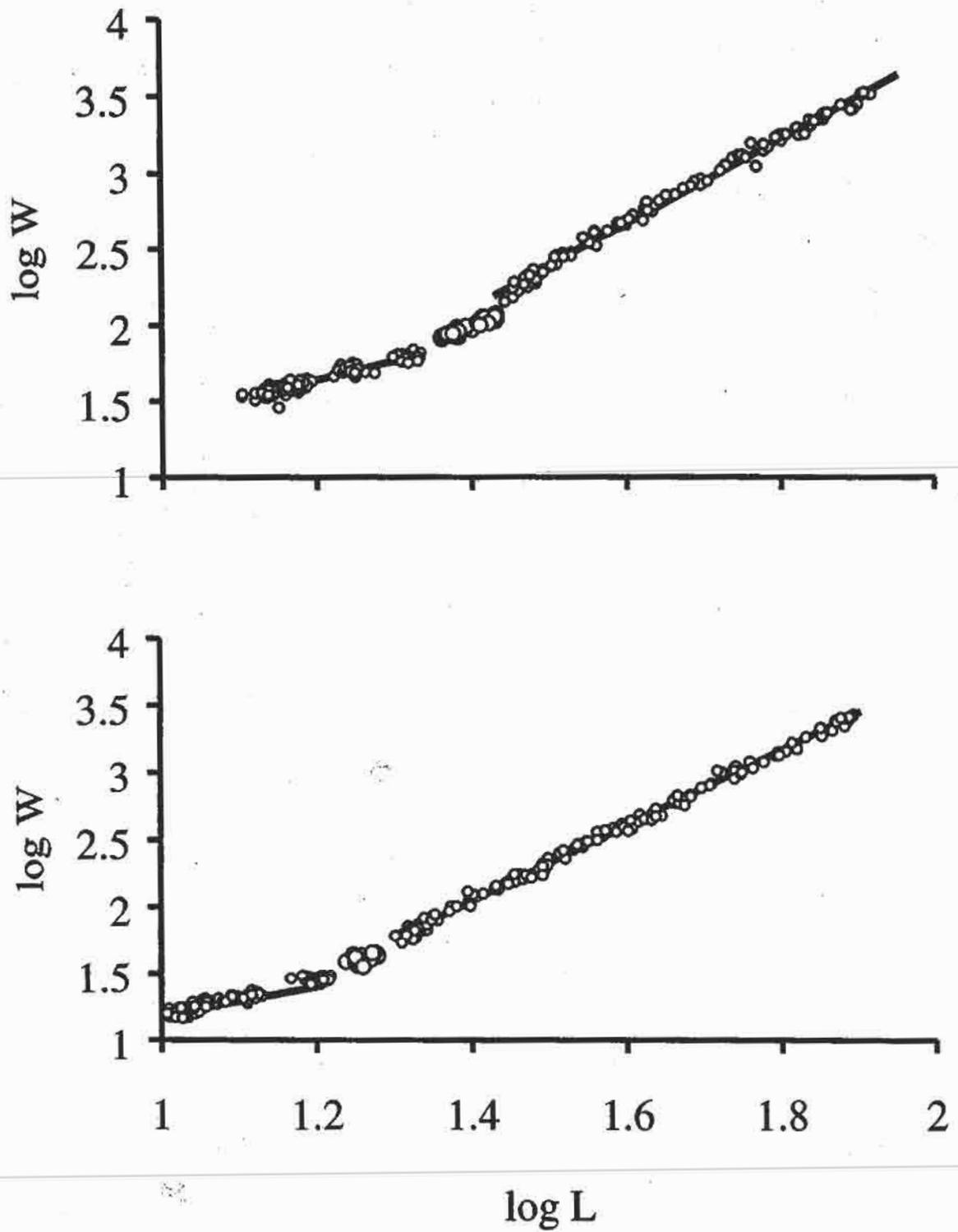


Figure 4. Weight-length relationships of green (top) and white (bottom) sturgeon (scatters and regression lines). The enlarged symbols (circle) show samples taken at the onset of exogenous feeding (not included in regression).

mal yolk. They can be distinguished from white sturgeon by their light pigmentation and the shape and size of the yolk sac. Juveniles of the two species are clearly distinguished by the number of lateral scutes, coloration, and the smooth skin in green sturgeon (versus rugged skin of white sturgeon).

While the fertilization and hatching rates were lower in green sturgeon (likely due to stress from capture and handling or delayed removal of eggs), the survival of larvae to metamorphosis was very high (93.3%). The large size and robustness of green sturgeon larvae contributed to their high survival rate in our previously reported hatchery trials (Van Eenennaam et al. 2001). Since the specific growth rates of green and white sturgeon, from the onset of exogenous feeding to metamorphosis, were similar, it appears that green sturgeon juveniles are larger due to the greater reserve of maternal yolk and, consequently, larger size at the onset of exogenous feeding.

Based on one progeny of each species, we present the first information on green sturgeon development and growth, which are comparable to those of white sturgeon under our specific culture conditions. While our study does not account for individual variation of egg size in the two species, the literature currently available does support significantly larger egg size in green sturgeon (Van Eenennaam et al. 2001), compared with white sturgeon (Lutes et al. 1987). However, egg size and color are known to vary greatly in sturgeon (Dettlaff et al. 1993); therefore, the egg pigmentation pattern needs to be verified, and the effects of egg size and maternal yolk reserves on growth of juveniles should also be further investigated.

Egg adhesiveness in sturgeon is another characteristic considered to be species-specific and associated with reproductive behavior, including selection of spawning substrate and hydrological environment. Based on studies of seven sturgeon species with different structure and adhesiveness of egg chorions, Vorobyeva and Markov (1999) concluded that the anadromous species, spawning under conditions of variable and generally slower river currents, had less adhesive eggs than the resident species spawning in strong water current. This appears to contradict our first (Van Eenennaam et al. 2001) and more recent (unpublished) observations on the poor adhesion of eggs in green sturgeon spawning in the fast flowing Klamath River. Our experience with the artificial spawning of wild green sturgeon indicates a con-

sistent weak adhesiveness of the fertilized eggs, and preliminary histological observations of the egg membranes revealed that the outer layer of chorion was approximately half the thickness of that in the white sturgeon. Vorobyeva and Markov (1999) also noted that the thickness of the chorion varied among the species with strongly or weakly adhesive eggs. The adhesiveness of eggs in sturgeon probably depends on the adhesive material secreted upon activation, the specific molecular structure of the outer chorion membrane, and the mode of egg attachment to a substrate. It is possible that green sturgeon eggs may not be attaching at all to the open substrate in the fast flowing Klamath River; instead, they may be trapped in the crevices of river bedrock or under gravel where the early development occurs. The pale coloration, limited mobility, and photophobic behavior of newly emerged green sturgeon larvae support this explanation.

We observed substantial differences in larval behavior between the two species. Unlike white sturgeon larvae, green sturgeon larvae do not swim up after hatching, similar to the observations of Artyukhin and Andronov (1990) on the Asian green sturgeon. It appears that green sturgeon larvae do not have an early pelagic phase, which facilitates larval dispersal and downstream migration to nursery grounds, as in white sturgeon of the Sacramento and Columbia rivers.

In conclusion, our study provides the first comparative information on the early development of North American green and white sturgeon. Sharing a holoblastic style of development, green sturgeon differ from white sturgeon by having larger eggs and larvae, weaker adhesiveness of fertilized eggs, and demersal larval behavior, suggesting a reproductive strategy different from that of white sturgeon, with regards to conditions of spawning rivers and larval nursery habitat. Unfortunately, neither spawning nor rearing habitats of green sturgeon are known at present time, and our laboratory results are not verified by field observations. Characterization of the reproductive habitat of green sturgeon is a priority for stock management and preservation of this species.

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## Histology of the developing digestive system and the effect of food deprivation in larval green sturgeon (*Acipenser medirostris*)

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### Abstract

The histological development of the digestive tract in hatchery-reared green sturgeon (*Acipenser medirostris*) larvae and the effects of food deprivation on the digestive system organization were studied from hatching until 31 days post-hatching (dph). At hatching, the larval digestive system consisted of two rudiments: a large endodermal yolk sac and a primordial hind-gut. During the endogenous feeding phase, the wall of the yolk sac differentiated into the stomach (glandular and non-glandular regions) and the anterior and intermediate intestine, while the hind-gut primordium differentiated into the spiral valve and rectum. At the onset of exogenous feeding (15 dph at 16 °C), the organization and cytoarchitecture of the digestive system in green sturgeon larvae was generally similar to those of juveniles and adults. Larvae deprived of food exhibited a progressive deterioration, with subtle pathological changes observed after 5-d starvation: shrinkage of digestive epithelia, tissue degeneration, and necrosis were observed at 10–15 d of starvation (30 dph). No changes were observed in the mucous secretion of different regions of the digestive tract of food-deprived larvae. The histological analysis of the larval digestive system may be used to evaluate the nutritional condition of larval green sturgeon in their nursery habitats in spawning rivers, which are affected by dams and flow diversions.

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### Résumé

**Histologie du système digestif en développement et l'effet de privation de nourriture chez les larves de l'esturgeon (*Acipenser medirostris*).** Le développement histologique du tractus digestif chez les larves d'esturgeon vert (*Acipenser medirostris*) élevées en éclosier et la privation de nourriture sur l'organisation du système digestif ont été étudiés de l'éclosion jusqu'à 31 jours après l'éclosion (j). À l'éclosion, le système digestif larvaire est caractérisé par un épais endoderme du sac vitellin et un intestin postérieur primaire. Durant la phase de nutrition endogène, la paroi du sac vitellin se différencie en estomac (régions glandulaire et non glandulaire) et en intestin antérieur et intermédiaire, tandis que l'intestin postérieur primaire se différencie en valve spiralée et en rectum. Au début de la phase de nutrition exogène (15 j à 16 °C), l'organisation et la structure cellulaire du système digestif chez les larves sont généralement similaire à celles des juvéniles et des adultes. Des larves privées de nourriture présentent une détérioration progressive, avec de légers changements pathologiques observés après 5 j de jeûne : retrécissement de l'épithélium digestif, dégénération du tissu et nécrose ont été observés à 10–15 j de jeûne (30 j après éclosion). Aucun changement n'a été observé dans la sécrétion des muqueuses des différentes régions du tractus digestif des larves privées de nourriture. L'analyse histologique du système digestif larvaire peut être utilisée pour évaluer les conditions nutritionnelles des larves de cet esturgeon présentes dans les fleuves, où s'effectue la reproduction, affectés de barrages ou soumis aux aléas des courants de marée.

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**Keywords:** *Acipenser medirostris*; Larvae; Digestive system; Histology; Starvation

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## 1. Introduction

Green sturgeon is the least studied species among North American acipenserids, and is considered a rare or vulnerable species in the United States and Canada (Birnstein, 1993) and an endangered species in Russia (Artyukhin and Andronov, 1990). In North America, the only known spawning populations of green sturgeon are in the Klamath, Rogue and Sacramento Rivers, all of which are affected by water projects. These river flows are largely controlled by dams and water diversion projects. The resulting significant reduction of flow (especially during the dry years) may result in changes of water quality (temperature and dissolved gases) and primary and secondary productions, which may affect the availability of food resources for larval and juvenile green sturgeon (Moyle et al., 1994). Predation is considered to be the main cause of mortality during the embryo and yolk-sac larval stages, whereas starvation plays an important role during transition to exogenous feeding, thus, increasing the vulnerability of larvae to predation (Kamler, 1992; Iguchi and Mizuno, 1999). Temperature may influence the time-interval for larvae to establish successful feeding, by changing the rate of metabolism and the pace at which the yolk reserves are consumed and first feeding occurs (Bisbal and Bengtson, 1995). Various, temperature-dependent periods of food deprivation may result in abnormal behaviour and development, including the degeneration of the alimentary tract and trunk musculature, reduced food utilization efficiency and feeding activity patterns (Heming et al., 1982).

The effects of experimental food deprivation on fish larval conditions have been described using morphometric and gravimetric (e.g. Elrich et al., 1976; Mookerji and Rao, 1999), biochemical (e.g. Robinson and Ware, 1988; Clemmesen, 1993; Suneetha et al., 1999), and histological (e.g. Oozeki et al., 1989; Theilacker and Watanabe, 1989; Green and McCormick, 1999) criteria, or a combination of those (Bisbal and Bengtson, 1995). The detection of starvation conditions is important for studies of both natural and cultured populations. However, such studies must be preceded by an experiment, where the starvation indicators are validated for fish of known nutritional history (Bisbal and Bengtson, 1995).

Acipenseriformes (sturgeon and paddlefish) differ from the modern teleosts by holoblastic cleavage, intracellular platelet yolk in the embryo, and by differentiation of the digestive system from the yolk-sac Anlagen (Dettlaff et al., 1993). Development of the digestive system has been studied in Russian sturgeon *Acipenser gueldenstaedti* (Dettlaff et al., 1993), white sturgeon *A. transmontanus* (Gawlicka et al., 1995) and Siberian sturgeon *A. baerii* (Gisbert et al., 1998, 1999). Deng et al. (2002) recently described early development in green sturgeon, however, there has been no comprehensive study on the digestive system in larval stages of this species.

Using the artificial spawning of green sturgeon (Van Eenennaam et al., 2001), we initiated the studies on cultured

fish to better understand the early life history and environmental physiology of this species. The objectives of this study are to characterize, using light microscopy, the development of the digestive system in green sturgeon larvae and to evaluate the effects of food deprivation on the histopathology of the digestive tract and accessory digestive organs from the onset of exogenous larval feeding.

## 2. Materials and methods

### 2.1. Supply and maintenance of fish

Green sturgeon gametes were obtained from one female (35 kg) and male (25 kg), caught in May of 2001 by gillnets and held in cages in the Klamath River (Weitchpec, California). Ovulation was induced by two intramuscular injections (0.6 and 20  $\mu\text{g kg}^{-1}$ , with 8-h interval) of GnRH $\alpha$  ([D-Ala<sup>6</sup>, Des-Gly<sup>10</sup>]-LH-RH Ethylamide). Domperidone (2 mg  $\text{kg}^{-1}$ ) was administered with the second injection. Spermiation was induced by a single injection of GnRH $\alpha$  (10  $\mu\text{g kg}^{-1}$ ). The ovulation occurred within 15 h after second injection at river temperature 18.4–19.8 °C. Gamete collection, artificial fertilization, and the silt treatment of fertilized eggs were conducted as described by Van Eenennaam et al. (2001). The eggs (fertilization rate 80% at second-third cleavage) were transported to UC Davis in oxygenated and cooled (13–14 °C) water and incubated at 14–15 °C for 7 d before hatching. One thousand newly emerged larvae were held in two circular tanks (120 l, ca. 500 larvae per tank) of the indoor semi-recirculation system and supplied with well water (dissolved oxygen at saturation, pH 7.8–8.2, constant 16 °C temperature, and 12L:12D artificial photoperiod). A small amount of food (semi-moist Silver Cup, Nelson & Sons, Utah, and live chopped Tubifex) was presented to one group (tank) at 12 days post-hatching (dph), while the other group was deprived of food during the entire experiment (31 dph). Initiation of feeding was determined by direct examination of larvae. Characteristics such as distended stomachs and characteristic internal coloration associated with the dry commercial diet and Tubifex acted as good indicators (Gisbert and Williot, 1997). Once the first feeding was detected at 14–16 dph, the larvae were fed *ad libitum* and uneaten feed, faeces, and mortalities were removed daily.

### 2.2. Fish growth measurements and histology procedures

Larvae were sampled ( $n = 10$ ) daily from hatching to 20 dph and then with 3-d intervals to 31 dph. At sampling, fish were euthanized (overdose of tricaine methanesulphonate), and fixed in phosphate-buffered formalin. Large sampling mortality did not allow for a survival analysis. After 1 month of storage in formalin, larvae were measured (total length TL,  $\pm 0.01$  mm, stereoscopic microscope with a camera lucida and a digital pad Micro-Plan II, Laboratory Computer systems, Inc.), weighed (BW,  $\pm 0.01$  mg, digital microbalance), and five specimens from each sample were dehy-

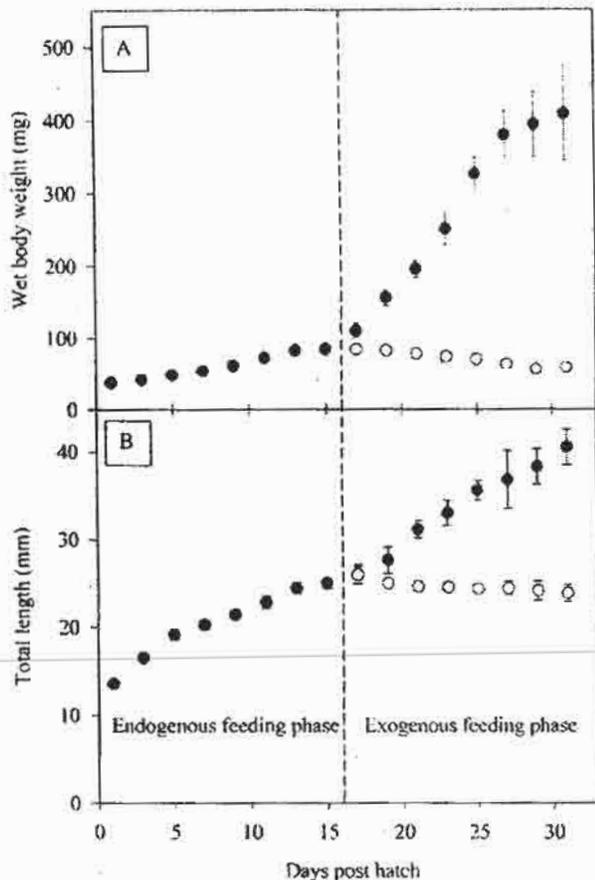


Fig. 1. Wet weight (A) and total length (B) (means  $\pm$  S.D.,  $n = 10$ ) of fed (●) and starved (○) green sturgeon larvae.

drated in graded ethanol, embedded in paraffin, and sectioned at 4–6  $\mu\text{m}$  (LKB Historange Microtome). Sections were stained by haematoxylin and eosin (HE) for histological observations, periodic acid-Schiff (PAS) for neutral mucosubstances, and Alcian Blue (AB) at pH 2.5, 1.0 and 0.5 for carboxyl-rich and sulphated (weakly and strongly ionized) glycoconjugates and sialic acid (HCl hydrolysis + AB, pH 2.5) (Gisbert et al., 1999). Melanin pigment granules were identified as previously reported (Gisbert and Sarasquete, 2000). Height of the epithelial cells from different regions of the digestive tract was measured at 600 $\times$  under a microscope with an ocular micrometer (Bisbal and Bengtson, 1995), and compared among feeding and starving groups, using a Student's *t*-test ( $P < 0.05$ ).

### 3. Results

Food deprivation during 15–31 dph resulted in a loss of body wet weight and no increase of length in the food-deprived group (Fig. 1). Unfed larvae continued swimming along the bottom and walls of the holding tank, exhibited food searching behaviour, and were reactive to external stimuli. However, most food-deprived larvae died between

28 and 31 dph, whereas no mortality was observed in green sturgeon larvae that were fed.

#### 3.1. Histodifferentiation of the digestive system in fed larvae

At hatching, the larval digestive system was represented by two rudiments, a large endodermal yolk sac and a primordial hind-gut, neither were open to the exterior (mouth and anus were not differentiated). The endodermal yolk sac (volume  $16.9 \pm 2.9 \text{ mm}^3$ ,  $n = 20$ ) was filled with yolk platelets and lined with a simple, squamous epithelium, which would differentiate into the walls of stomach and intestine (Fig. 2A). The hind-gut, containing a small amount of yolk, appeared as an undifferentiated straight and narrow rudiment, which would differentiate into the spiral valve and rectum. The accessory digestive organs (liver and pancreas) were absent at hatching. Histological differentiation of the digestive system after hatching is described below.

##### 3.1.1. Buccopharynx

At hatching, the buccopharynx was closed and its lumen was filled with small yolk platelets (Fig. 2A). Clusters of basophilic cells (future gill arches) were seen in a circular position in the posteroventral region of the buccopharynx (Fig. 2B). Between 1 and 2 dph, the mouth opened with two differentiated oral valves that were composed of a stratified squamous epithelium in differentiation. The buccopharyngeal mucosa consisted of a stratified squamous epithelium with connective tissue fibres (Fig. 2C). Epithelial cells contained supranuclear eosinophilic (HE) and PAS-positive yolk platelets and some melanin granules (bleachable with hydrogen peroxide), which disappeared at age 4–5 dph. Ciliated cells (scattered through buccopharyngeal epithelium) were present from hatching until 12–14 dph (Fig. 3A). First goblet cells (unreactive to stain) appeared at 6 dph. At age 7–8 dph, they were stained by the AB (pH 2.5, 1.0, 0.5) and PAS stains, indicating the presence of neutral and acidic (carboxylated and sulphated) glycoconjugates (Table 1). The number and size of goblet cells in the buccopharyngeal epithelium increased as larvae developed. Taste bud cells (basophilic) were differentiated between 8 and 9 dph, and taste buds were fully developed at 10–11 dph. Differentiation of canine-like teeth proceeded from the base of the buccopharyngeal epithelium at 6–7 dph (Fig. 3B), with the teeth protruding into the oral valves and pharyngeal lumen at age 11–12 dph (larval dentition was present at 31 dph). Between 11 and 12 dph, the ventral and dorsal fungiform and filiform papillae developed in the anterior and central part of the buccopharynx, respectively (Fig. 3C). Numerous mucous cells and taste buds developed in the surface of the papillae.

##### 3.1.2. Oesophagus

The oesophagus was not differentiated to 6 dph, and the posterior region of buccopharyngeal cavity remained filled with the residual yolk. At 7 dph, the primordial oesophagus wall consisted of a pseudostratified columnar epithelium

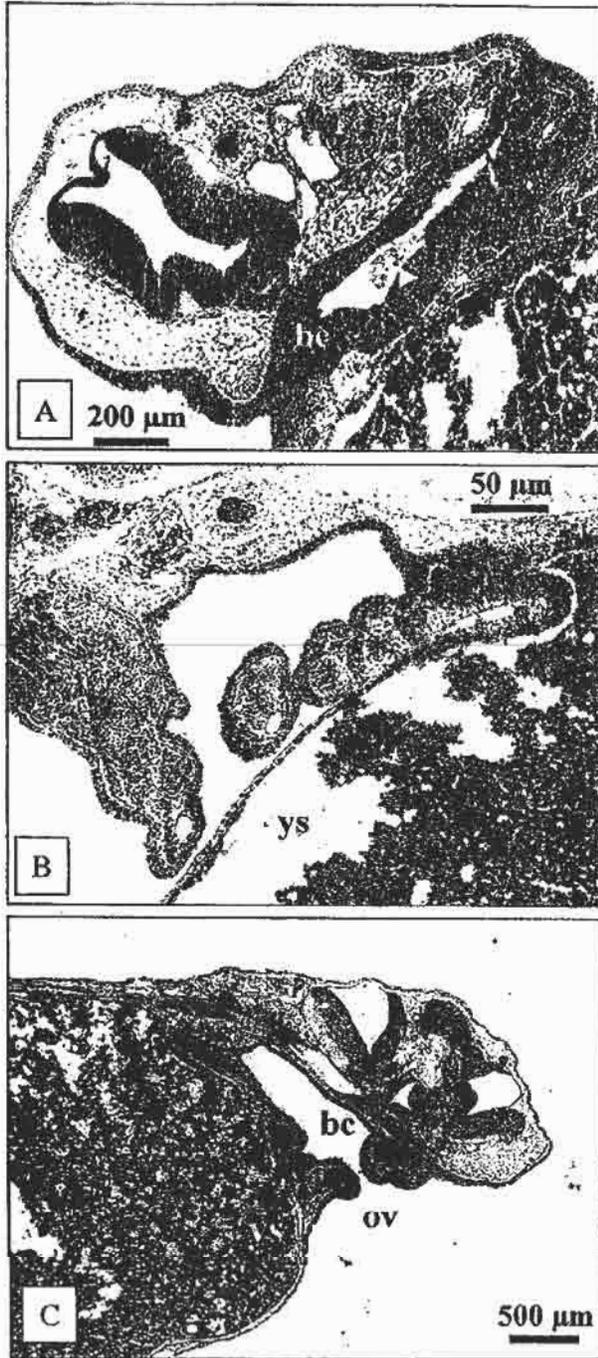


Fig. 2. (A) Head of a green sturgeon larva at hatching: note the closed buccopharynx (bc) and the buccopharyngeal epithelium containing yolk inclusions (white arrow head). (B) Differentiation of gill arches (asterisks) in the posteroventral region of the buccopharyngeal mucosa of a newly hatched larva. (C) Differentiation of oral valves (ov) and mouth opening at 2 dph; note the posterior pharynx filled with yolk (arrow) and the large yolk sac (ys).

with numerous cells containing yolk inclusions and melanin granules (Fig. 4A), which disappeared between 8 and 9 dph.

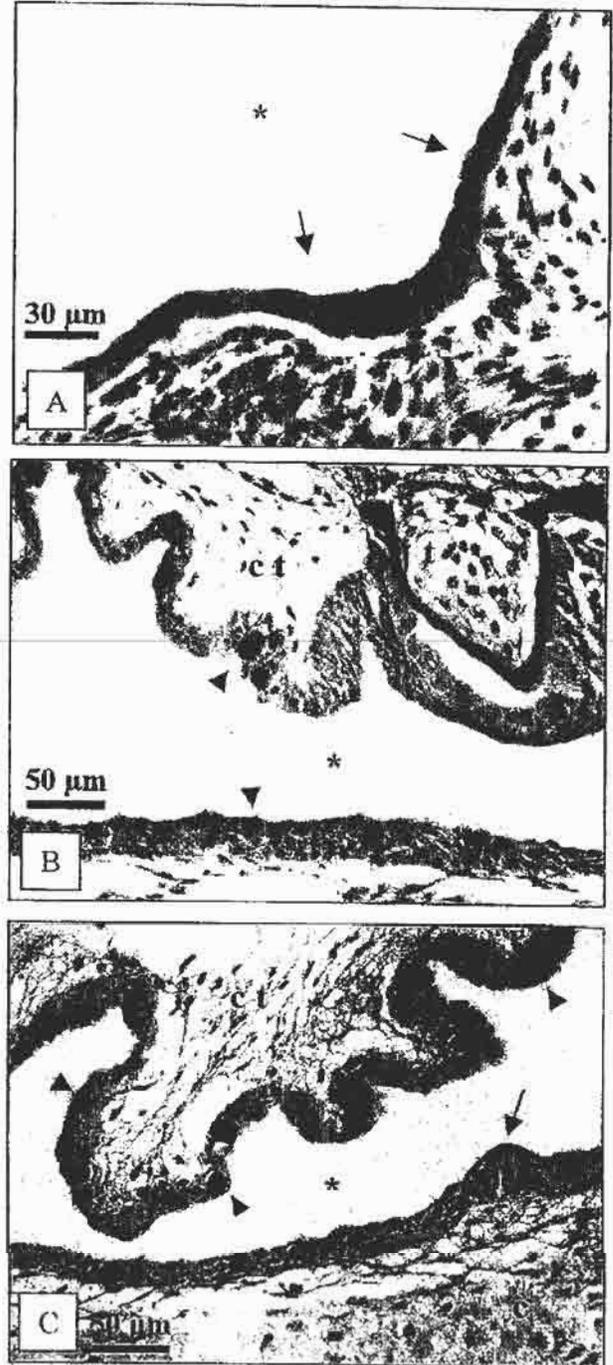


Fig. 3. (A) Ciliated cells (arrows) in the buccopharyngeal epithelium at 6 dph. (B) Details of the stratified squamous epithelium with functional goblet cells (arrow heads) lining the buccopharynx at 7 dph. Note the presence of a tooth (t) in differentiation before protruding into the buccopharyngeal lumen (\*) and the connective tissue (ct) surrounding the buccopharyngeal epithelium. (C) Details of pharyngeal papillae with goblet cells (arrow heads) at 12 dph. Note the presence of a taste bud (arrow) in the ventral buccopharyngeal epithelium and the presence of the Meckel's cartilage (c).

Differentiation of the oesophagus proceeded from the posterior region to buccopharynx. At 10 dph, the oesophagus wall

Table 1

Distribution of acid and neutral glycoproteins produced by goblet cells in different regions of the digestive tract during early development of fed green sturgeon larvae

	Buccopharynx	Oesophagus	Stomach		Intestine		
			Cardiac	Pyloric	Anterior	Intermediate	Spiral valve
Neutral glycoproteins (PAS)	3	3	3	2	2	2	3
Carboxyl-rich glycoproteins (sulphated or not) (AB pH 2.5)	2	2	0	0	2–3	2–3	2–3
Sulphated proteins (weakly ionized) (AB pH 1.0)	2	2	0	0	2	2	2
Sulphated glycoproteins (strongly ionized) (AB pH 0.5)	1	2	0	0	1–2	1–2	2
Sialic acid (HCl hydrolysis-AB pH 2.5)	0	1	0	0	2	2	2

Results are reported considering the intensity of the histochemical reactions: 0, negative; 1, weak; 2, moderate; 3, intense.

was composed of a mucosa with a *lamina propria* (loose connective tissue and a layer of musculature), a submucosa (connective tissue fibres with some blood vessels), and a serosa lined by a thin layer of squamous epithelium. Two regions of the oesophagus were distinguished by histological characteristics of the epithelia. The anterior region was lined with two cell layers: the inner layer of a simple, cuboidal epithelium and the outer layer of a simple, columnar epithelium with abundant goblet cells staining for neutral mucosubstances (PAS), carboxylated and sulphated acidic mucins (AB pH 2.5 and AB pH 1.0, 0.5), and for sialic acid (HCl hydrolysis + AB pH 2.5) (Fig. 4B, Table 1). The posterior region was lined with a ciliated columnar epithelium with very few goblet cells (Fig. 4B). Some mucosal folds were detected in the region connecting the oesophagus and glandular stomach.

### 3.1.3. Stomach

At hatching, the large endodermal yolk sac of green sturgeon was surrounded by a thin squamous basophilic epithelium. Two furrows (invaginating epithelium) appeared at 2 dph in the dorsal and ventral posterior regions of the yolk sac (Fig. 5A), merging and dividing the yolk sac into two compartments between 3 and 4 dph (Fig. 5B). The anterior wall of the furrow lined with a squamous epithelium became the ventral lining of the stomach, while its posterior wall lined with a columnar epithelium became the dorsal lining of the intestine.

The pyloric (non-glandular) stomach started differentiation at 6 dph in the anterior ventral region of the yolk sac from a fold of stratified squamous epithelium (Fig. 5C) and was well differentiated at 11–12 dph, with mucosal folds surrounded by a prominent *tunica muscularis* (Fig. 6A). The epithelial lining of the pyloric lumen consisted of ciliated columnar cells with supranuclear vacuoles containing eosinophilic (HE) and neutral (PAS-positive, AB pH 2.5, 1.0 and 0.5-negative) mucosubstances (Table 1). A wall of the pyloric stomach was composed of submucosa with connective fibres (AB pH 2.5 and 1.0-positive), some blood vessels, circular muscle fibres, and a thin serosa with basophilic squamous cells (Fig. 6B). The organ was separated from the anterior intestine by the pyloric sphincter (Fig. 6A). Platelet yolk was present in the lumen of pyloric stomach until the age 14 dph.

The cardiac (glandular) stomach started to differentiate at 8 dph, with cytoarchitectural changes (squamous to columnar cells) in the epithelium of the yolk-sac. Gastric glands in the cardiac stomach wall were not detectable by the PAS staining at 10 dph, but they were prominent at 12 dph, as the multicellular tubular glands composed of a single-type secretory cells with eosinophilic and PAS-positive apical borders and the secretory products containing neutral (PAS-positive) mucosubstances (Fig. 6C, Table 1). These glands were surrounded by compact layers of connective tissue stained for acidic mucins (AB pH 2.5, 1.0 and 0.5), smooth circular musculature, and a thin serosa. The number of gastric glands and thickness of mucosa layers increased during larval feeding phase (15–31 dph), while their histochemical properties remained the same. The platelet yolk was present in the glandular stomach until 14 dph.

### 3.1.4. Anterior and intermediate intestine

Differentiation of the intestinal wall started at 2–3 dph, progressing in a posteroanterior direction. However, the anterior region of the intestine was filled with yolk and did not differentiate until 7 dph (Fig. 5A). The differentiation of the intestinal mucosa was concomitant with the disappearance of yolk in the supranuclear vacuoles of epithelial cells. The mucosa had generally similar histological structure along the length of intestine, with the exception of number and size of intestinal folds, which were less abundant and smaller in the posterior region (Fig. 7A). During yolk resorption, supranuclear lipidic vacuoles in the cells of the intestinal epithelium increased in size and number, and were present until 16–17 dph (Fig. 7B).

The first goblet cells appeared at age 6–10 dph, in the posterior and anterior regions of the intestine, respectively. The number of goblet cells increased with differentiation of the mucosa, and they were more abundant in the posterior region. Goblet cells contained carboxylated and sulphated glycoconjugates (AB-positive at pH 2.5; 1.0, 0.5) and sialic acid (HCl hydrolysis + AB pH 2.5). Most goblet cells exhibited dark-blue staining (AB and PAS positive staining), but some exhibited magenta or purple staining, suggesting the presence of acid mucosubstances in the majority of cells and the neutral (magenta) or neutral and acid (purple) glycoconjugates in some of the cells (Table 1).

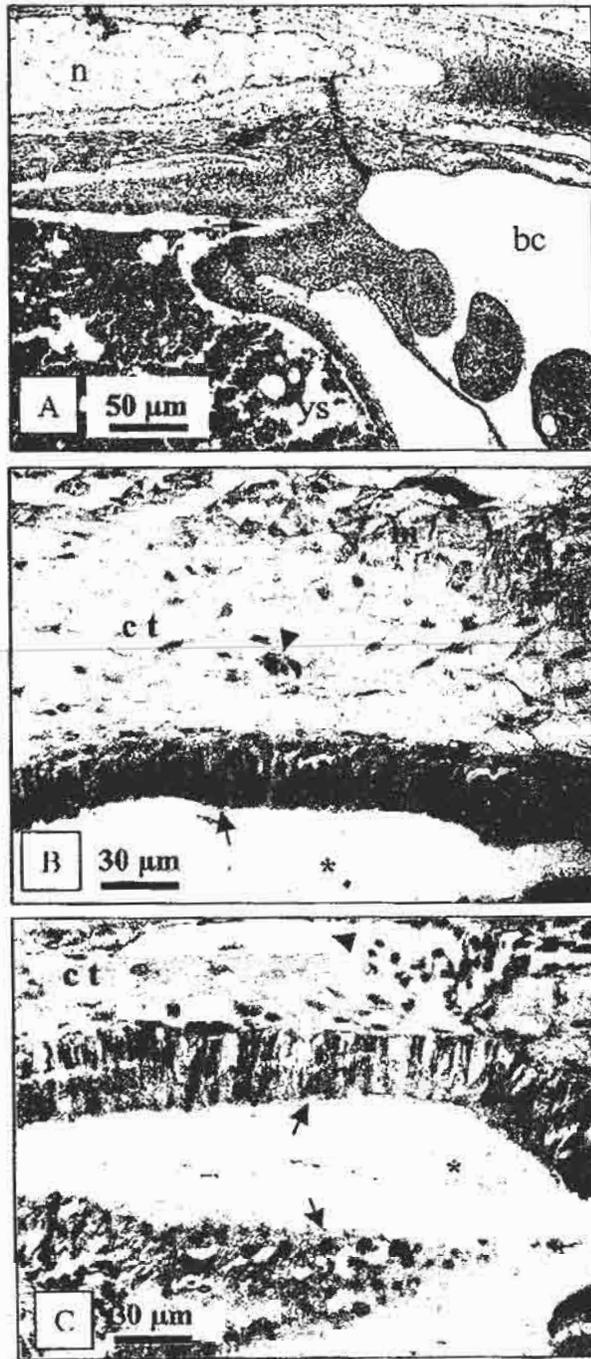


Fig. 4. (A) Primordial oesophagus (arrow) with epithelial cells containing yolk inclusions at 7 dph. (B) Anterior region of the oesophagus lined by a columnar epithelium with abundant goblet cells (arrow) at 7 dph. (C) Posterior region of the oesophagus with few goblet cells (arrows). Blood vessel (arrow head), buccopharynx (bc), connective tissue (ct), muscular layer (m), notochord (n), oesophageal lumen (asterisk), yolk sac (ys).

### 3.1.5. Spiral valve and rectum

Differentiation of spiral valve (posterior intestine) occurred soon after hatching (1–2 dph) (Fig. 5A). The lumen of spiral valve was lined with a simple ciliated columnar epithelium containing yolk inclusions in the supranuclear vacuoles (Fig. 7C). The first goblet cells appeared at 2 dph, and they

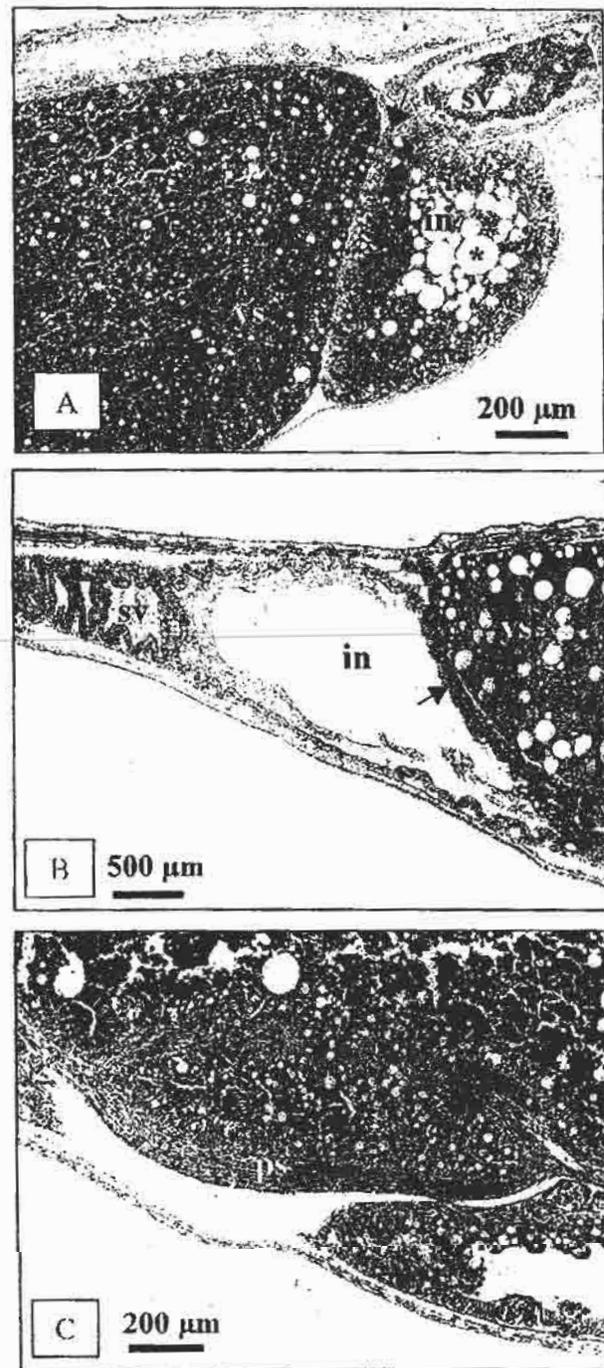


Fig. 5. (A) Division of the yolk sac (ys) by a furrow (arrow) separating it from the intestine (in) and spiral valve (sv) at 2 dph. Note lipid inclusions (asterisk) in the yolk and the rudimentary intestine and spiral valve filled with yolk. (B) Yolk sac (anlagen of the stomach), intestine (in), and spiral valve (sv) of a green sturgeon larva at 4 dph. The arrow indicates the furrow dividing the future stomach filled with yolk and the intestine. (C) Differentiation of pyloric stomach (ps) at 6 dph, from a fold of stratified squamous epithelium surrounded the yolk sac.

lium containing yolk inclusions in the supranuclear vacuoles (Fig. 7C). The first goblet cells appeared at 2 dph, and they

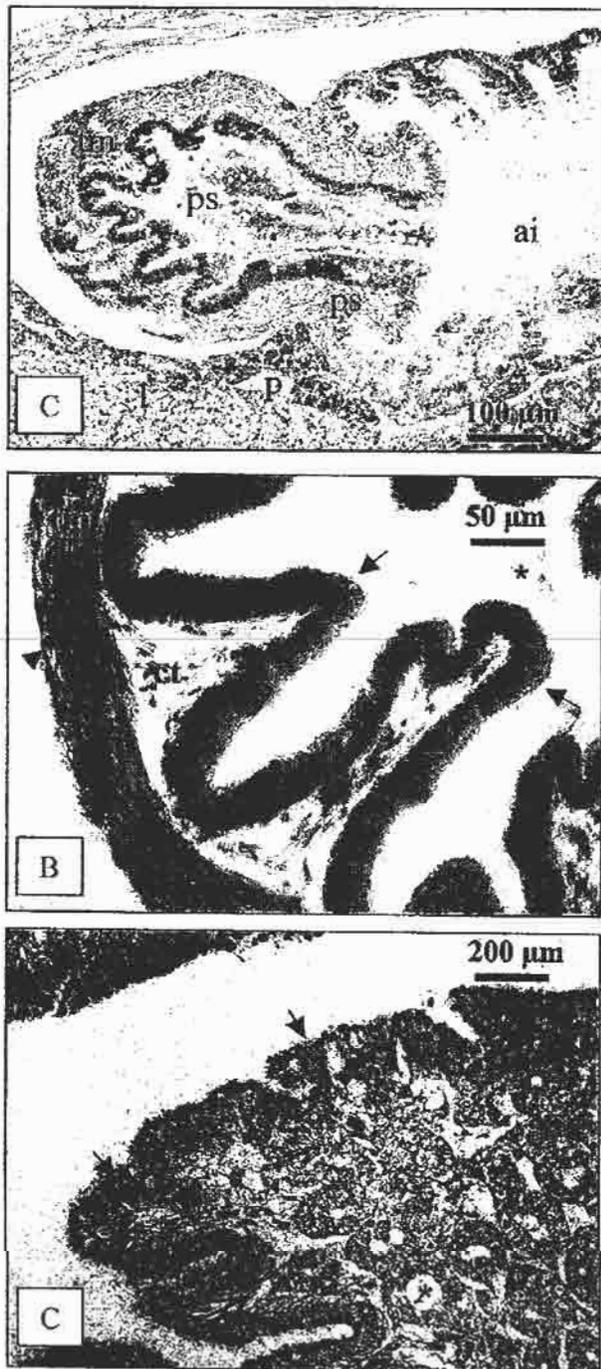


Fig. 6. (A) General view of the pyloric stomach (ps) at 15 dph. Anterior intestine (ai), liver (l), pancreas (p), pyloric sphincter (ps), *tunica muscularis* (m). (B) Details of the pyloric stomach with prominent mucosal folds (arrows) surrounded by a *tunica muscularis* (tm). Blood vessel (arrowhead), connective tissue (ct), pyloric lumen (asterisk). (C) Cardiac stomach with multicellular tubular glands (asterisk) with eosinophilic apical borders (arrows) secreting neutral mucosubstances.

were similar in their histochemical properties to those in the anterior and intermediate intestine (Table 1). The lumen of the spiral valve was initially filled with yolk but, as larvae

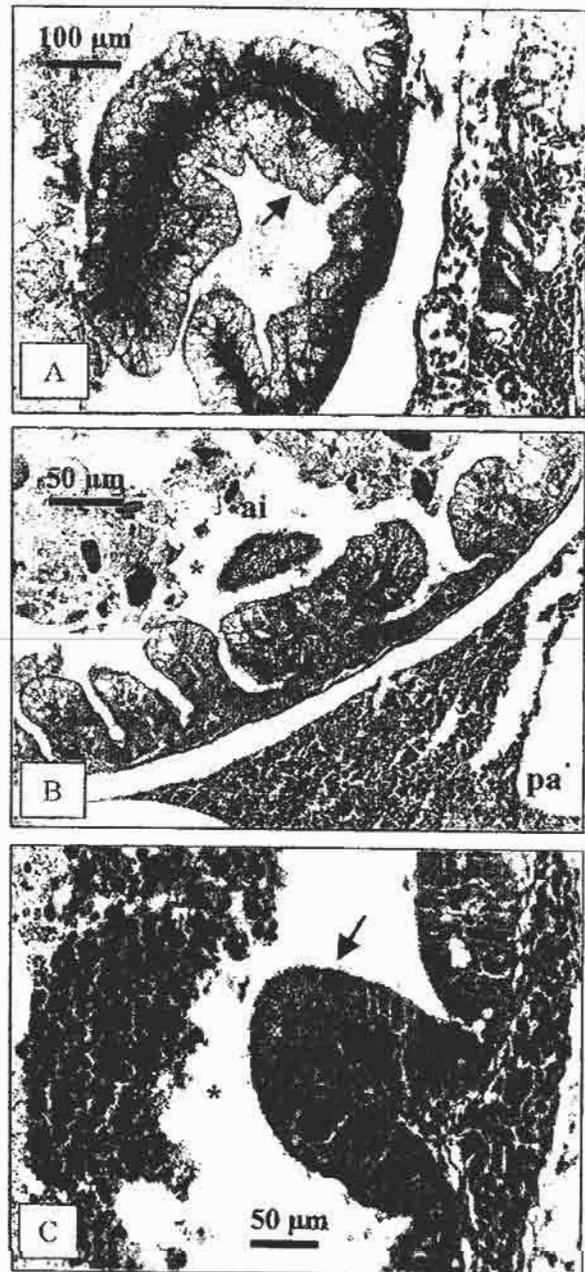


Fig. 7. (A) Epithelium (arrow) of the intermediate intestine at 14 dph, with the large supranuclear lipid vacuoles. (B) Anterior intestine (ai) and pancreas (pa) at 20 dph. (C) Enteroocytes (arrow) of the spiral valve containing yolk platelets in supranuclear vacuoles, at 2 dph; note the presence of free yolk in the intestinal lumen. Intestinal lumen (asterisk).

developed, it became devoid of yolk and accumulated a dark pigment (Fig. 5A). The posterior region of a hind-gut primordium differentiated into the rectum at 5 dph. The rectal mucosa was lined with a ciliated columnar epithelium containing few goblet cells (Fig. 7B). The anus opened at 9–10 dph, after the complete resorption of the residual yolk in the spiral valve.

### 3.1.6. Liver and pancreas

The liver rudiment appeared at 2 dph in a ventral portion of endodermal yolk sac, and the rudiment of exocrine pancreas developed dorsally to the furrow dividing yolk sac into the stomach and intestine. At 4 dph, polygonal hepatocytes containing large supranuclear lipidic vacuoles (not stained by HE, PAS and AB) and glycogen inclusions (PAS and diastase-PAS positivity), were arranged along hepatic sinusoids (Fig. 8A). The lipidic vacuolization and density of glycogen granules in hepatocytes increased with larval development, especially in feeding larvae. Exocrine pancreatic cells were arranged in acini at age 4–5 dph, around the small intercellular lumina. Acinar cells had eccentric (basal) nuclei and strongly basophilic cytoplasm. Zymogen granules were detected (HE and PAS) in acinar cells before the onset of first feeding (at 14 dph) (Fig. 8C).

### 3.2. Effect of food deprivation on digestive system

The digestive system of food-deprived larvae exhibited a progressive deterioration, starting after 5 d of starvation. Starvation-induced changes in tissues and cytoarchitecture of the digestive system were summarized in Table 2 and Fig. 11.

In the buccopharynx, filiform and fungiform papilla decreased in size and number between 2 (17 dph) and 16 (31 dph) days of starvation (Fig. 9A), while the ventral papillae were gradually resorbed and disappeared entirely at 16 d of starvation (31 dph). The epithelium became thinner and taste buds and larval teeth strongly protruded into the lumen. The cytoplasm of epithelial cells became hyaline after 5 d of fasting (20 dph) and pycnotic nuclei were seen at 14 d (29 dph). The histochemical properties of goblet cells in buccopharynx did not change throughout the starvation period.

Similar to the buccopharyngeal epithelium, epithelial cells of the oesophagus shrunk at 5–10 d of starvation (Fig. 11). From day 5 of fasting (20 dph) to the end of the study, the oesophageal mucosa and submucosa decreased in thickness, due to the shrinkage of the smooth muscle and connective tissue fibres. Scattered pycnotic nuclei in the oesophageal epithelium were observed at 10 d of starvation, and they were abundant after day 16 of starvation (31 dph). The histochemical properties of the goblet cells (secretion of neutral and acidic mucins with sialic acid) did not change throughout the starvation period.

Epithelial cells of the cardiac and pyloric stomach shrunk after 5–10 d of starvation (Fig. 11), and their brush borders lost their smooth appearance as starvation progressed. Scattered pycnotic nuclei were seen in the pyloric stomach after 10–12 d of starvation (25–27 dph). Mucosal folds of the pyloric stomach flattened and disappeared after 15–16 d of starvation (30–31 dph) (Fig. 9B). At the end of food deprivation period, the smooth muscle fibres separated to form intercellular spaces, and fibroblasts became atrophic or necrotic, resulting in the shrinkage of the stomach submucosa (Fig. 10A). In the cardiac stomach, gastric glands collapsed and their lumen almost disappeared, pycnotic nuclei were

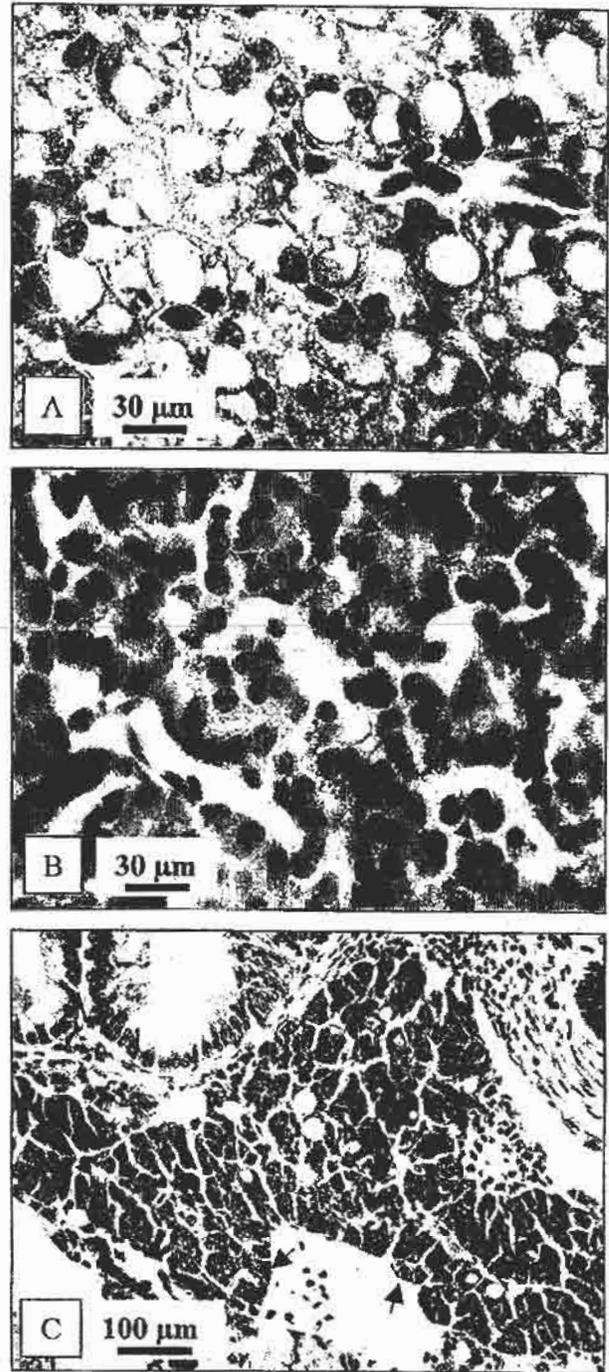


Fig. 8. (A) Liver of a feeding green sturgeon larva at 25 dph, with polygonal hepatocytes arranged along the sinusoids and containing large lipid vacuoles. (B) Liver of a larva starved for 12 d (27 dph); note large intercellular spaces, collapsed cytoplasm (arrow head) and pycnotic nuclei of hepatocytes (arrows). (C) Exocrine pancreas.

seen in the gastric glands after the 13-d starvation period (28 dph) (Fig. 9C). No changes in histochemical properties of the glycoconjugates of the different regions of the stomach were observed throughout the 16-d fasting period.

Table 2  
Summary of histological changes in the digestive system of food-deprived green sturgeon larvae (starvation day 0 = 15 dph)

Starvation days	Buccopharynx	Oesophagus	Stomach	Intestine	Liver	Pancreas
0	Squamous stratified epithelium, taste buds, goblet cells, canine teeth, filiform and fungiform papillae	Differentiated mucosa, submucosa, serosa, secretory, and food transport regions	Compact muscle fibres and connective tissue; gastric glands and epithelial cells with microvilli	Numerous mucosal folds, enterocytes with smooth microvilli; lipidic vacuoles in enterocytes	Polygonal hepatocytes arranged along sinusoids with large lipidic vacuoles and glycogen inclusions	Exocrine pancreatic cells with peripheral nuclei, basophilic cytoplasm, arranged in acini; zymogen granules present
5	Reduction in size and number of dorsal and ventral papillae	Slight decrease in epithelial cells height	Microvilli are separated; no changes in mucosa and submucosa	Disappearance of lipidic vacuoles, decreased height of enterocytes	Lipidic vacuoles disappear, glycogen decreases; hepatocytes with large cytoplasm and peripheral nuclei	Shrinkage of acinar cells and enlarged intercellular spaces
10	Thinning of mucosa, shrinkage of epithelial cells, protrusion of taste buds and teeth	Thinning of mucosa, submucosa, pycnotic nuclei; disarray of microvilli	Pycnotic nuclei in pyloric region; degeneration of connective tissue in submucosa	Flattened mucosal folds and detached microvilli; strong secretion of acid mucosubstances (AB pH 2.5, 1, 0.5 positive)	Glycogen granules disappear, hepatocytes shrink, some with pycnotic nuclei	Acinar cells with darkly pigmented nuclei, condensed cytoplasm; zymogen granules present
15	Resorption of ventral papillae, numerous pycnotic nuclei	Collapsed cytoplasm and numerous pycnotic nuclei in epithelial cells	Collapsed gastric glands, no pyloric folds, disarrayed microvilli, separated muscle fibres	Disappearance of mucosal folds; autolytic processes and epithelial desquamation; melanin plug is present	Large intercellular spaces, collapsed cytoplasm, atrophic tissue	Disarray of acinar structure, tissue degeneration; zymogen granules present

Changes in the intestinal mucosa were seen after 8–10 d of starvation (23–25 dph), including a flattening of folds in the intestine and spiral valve and shrinking of epithelial cells (Fig. 10B). Enterocytes exhibited collapsed hyaline cytoplasm and darkly pigmented elongated nuclei at 10–12 d of starvation (25–27 dph). After 14 d of starvation (29 dph), the brush borders of enterocytes were not smooth and detached from their apical borders. Pycnotic nuclei were also apparent in the intestinal mucosa after 13 d of starvation (28 dph), and desquamation processes of the intestinal mucosa were observed after 16 d (31 dph). In contrast to fed larvae, the intestinal lumen of food-deprived larvae contained a large amount of mucosubstances, mainly acidic mucins (AB pH 2.5, 1.0 and 2.5). Food-deprived larvae had the melanin plug in the spiral valve in the advanced stages of starvation (Fig. 10C).

Lipidic vacuoles and glycogen granules in hepatocytes decreased after only 2 d of starvation (17 dph) and disappeared after 5 d of starvation (20 dph), but cells retained their normal appearance (prominent eosinophilic cytoplasm, polygonal shape, and organization along hepatic sinusoids). Hepatocytes with pycnotic nuclei appeared after 10 d of starvation (25 dph) and increased in number thereafter. The cytoplasm of hepatocytes collapsed and the intercellular spaces increased in size, giving a disordered appearance to the hepatic tissue (Fig. 8B). Food deprivation resulted in a disarray of the acinar structure of the pancreas, acinar cells shrunk, developed darkly pigmented nuclei, condensed cytoplasm, and apical zymogen granules (PAS-positive). At the end of starvation period, the exocrine pancreas lost its acinar organization.

#### 4. Discussion

Anatomically and histologically, the development of the digestive system in larval green sturgeon was similar to other acipenserids (Dettlaff et al., 1993; Gawlicka et al., 1995; Gisbert et al., 1998; Boglione et al., 1999), except for the greater amount of yolk and slightly slower rate of development (Deng et al., 2002). The histological differentiation of the alimentary canal in green sturgeon proceeded from the posterior to anterior, with the spiral valve differentiating at 2 dph and the gastric stomach at 14 dph, just before first feeding. At the onset of exogenous feeding, the general anatomy and histology of larval digestive system was similar to that in juvenile or adult sturgeon species (Dettlaff et al., 1993; Gisbert et al., 1998, 1999). The buccopharynx was lined with a stratified squamous epithelium with numerous fungiform and filiform papillae, epidermal teeth and taste buds. The lumen of the oesophagus was lined with ciliated and mucous cells, with abundant goblet cells secreting neutral and acidic mucosubstances in the anterior region, and the ciliated epithelium performing food transport function in the posterior region. The epithelium of cardiac stomach was composed of cuboidal cells, with numerous simple and tubular gastric glands. The neutral secretory products present in this region of the digestive tract may serve to protect the epithelium of the stomach from auto-digestion processes caused by hydrochloric acid and enzymes produced in gastric glands (Gisbert et al., 1999). The pyloric stomach was lined with a simple columnar ciliated epithelium containing supra-nuclear vacuoles filled with neutral mucosubstances and organized in folds surrounded by a prominent *tunica muscu-*

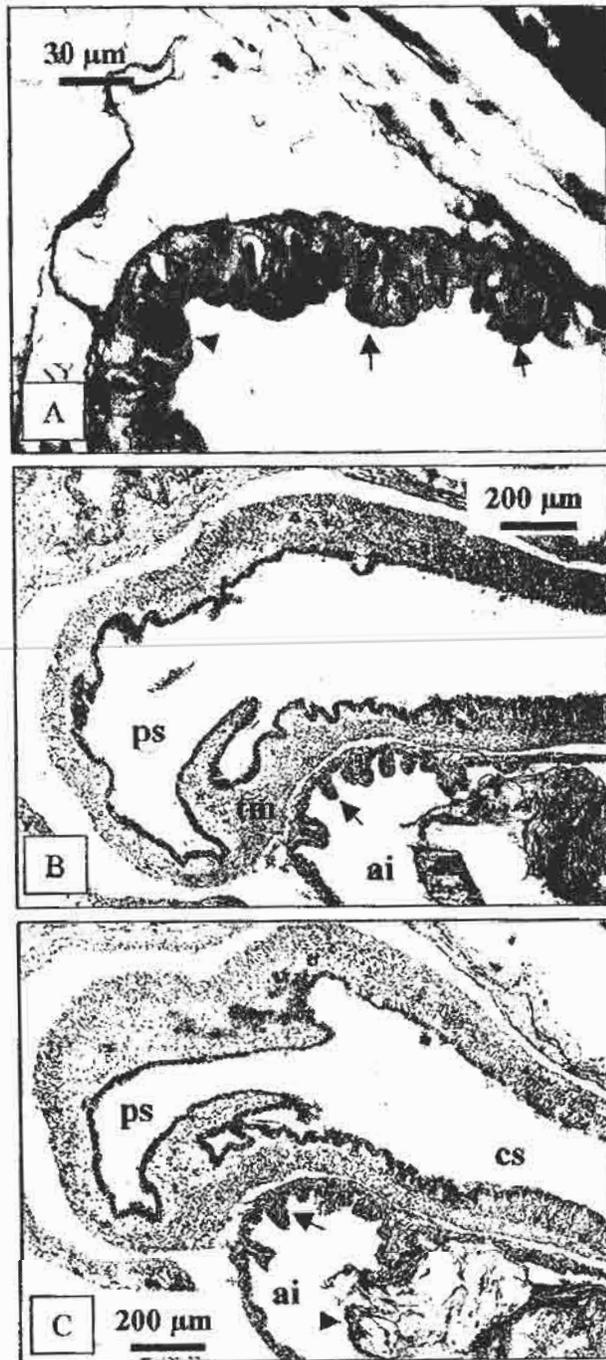


Fig. 9. (A) Resorption of dorsal pharyngeal papillae in a larva starved for 12 d (27 dph): note the presence of functional goblet cells (arrow head) and pycnotic nuclei (arrows) in the buccopharyngeal epithelium. (B) Pyloric stomach of a larva starved for 15 d (30 dph); note the thinning of the mucosa and *tunica muscularis* (tm) and disappearance of pyloric folds. (C) Cardiac stomach (cs) and anterior intestine (ai) of a larva fasted for 13 d (28 dph); note the reduction of folding and thinning of the mucosa (arrow).

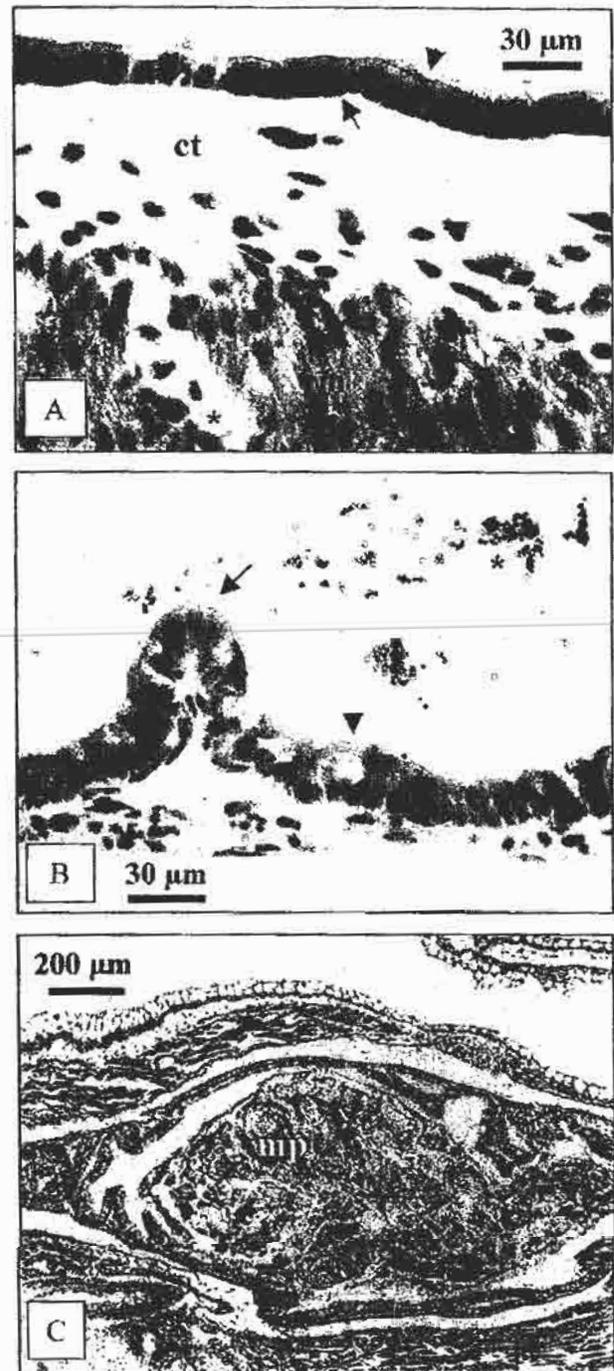


Fig. 10. (A) Details of the pyloric stomach of a larva starved for 12 d (27 dph) showing the enterocytes with a collapsed cytoplasm (arrow) and disarrangement of their brush border (arrow head). Note the onset of separation of muscle fibres (asterisk) in the *tunica muscularis* (tm) and the lax connective tissue (ct). (B) Details of the anterior intestine mucosa of a larva fasted for 13 d (28 dph); note the presence of goblet cells (arrow head), the disarrangement of the enterocytes' brush border (arrow) and the presence of melanin granules (asterisk). (C) Spiral valve of a larva starved for 12 d (27 dph); note the presence of the melanin plug (mp), the thinning of the mucosa and reduction in folding.

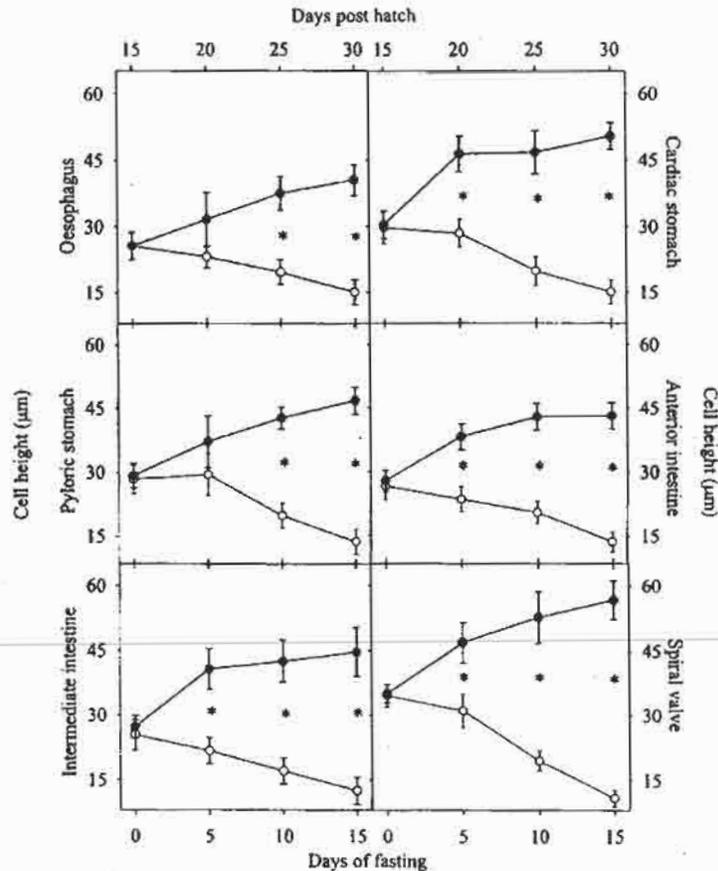


Fig. 11. Height (means  $\pm$  S.D.,  $n = 10$ ) of the epithelial cells in different regions of the digestive tracts of fed (●) and starved (○) green sturgeon larvae. Asterisks indicate significantly different means ( $P < 0.05$ ).

*laris*. The secretion of neutral mucosubstances, in conjunction with thick mucosa of pyloric region, may serve to protect the underlying layers from chemical and physical damages during trituration processes (Gisbert et al., 1998). The histological organization of the intestine was generally similar in different regions, with a simple columnar ciliated epithelium and numerous goblet cells that secreted neutral and acidic mucosubstances. At the onset of feeding, the cells of intestinal mucosa were filled with large lipid vacuoles that gradually disappeared after onset of feeding. The accumulation of lipids in the intestinal mucosa occurring during endogenous feeding phase may explain the ability of sturgeon larvae to survive long periods of food deprivation in laboratory experiments (Gisbert and Williot 1997; Gisbert et al., 1998).

While the green sturgeon larvae stayed alive during a prolonged period of food deprivation, starvation had a marked effect on the histological organization of the digestive system. In the endogenous feeding phase of sturgeon larval development, the proteins and carbohydrates of yolk were utilized for growth and metabolic energy, while lipids stored in the liver and intestinal epithelium served as an energy source for 16–17 dph (Wang et al., 1987; Gisbert et al., 1999). When lipid reserves were exhausted, the body tissues of food-deprived larvae were catabolized, resulting in

a progressive degeneration of the digestive tract and accessory organs. Histopathological changes in the digestive system of food-deprived green sturgeon larvae were similar to those observed in larval teleosts, including: (a) changes in the liver organization, decrease in glycogen and lipids stored in hepatocytes (Margulies, 1993; Green and McCormick, 1999; Crespo et al., 2001); (b) reduction in the height of enterocytes (Margulies, 1993; Bisbal and Bengtson, 1995; Theilacker and Porter, 1995; Green and McCormick, 1999; Gwak et al., 1999), and (c) degeneration of the exocrine pancreatic tissue (Yúfera et al., 1993; Gwak et al., 1999; Crespo et al., 2001).

In teleost species, liver glycogen and lipids are the first energy sources mobilized by fasted larvae (O'Connell and Paloma, 1981). The mobilization of these nutrients under the conditions of continued fasting results in the reduction of energy available to larvae (Green and McCormick, 1999). Similar results that were associated with a moderate to severe deterioration of hepatocytes of green sturgeon larvae starved for 10–15 d were observed under current experimental conditions. Proteolysis of the intestinal mucosa is also an important response to starvation. For this reason, the enterocyte height has been used as a reliable indicator of starvation or sub-optimal feeding in teleosts (Theilacker and Watanabe, 1989; Theilacker and Porter, 1995; Bisbal and Bengtson,

1995; Theilacker et al., 1996; Green and McCormick, 1999). Enterocyte degeneration implies a reduction of the absorption surface area, particularly in the spiral valve, which is the main site of nutrient absorption in sturgeons (Gawlicka et al., 1995; Gisbert et al., 1999). This fact may compromise the digestive capabilities of re-feeding larvae and directly affect their growth and survival. The intestine is not the only region, where proteolytic processes take place during starvation. Degeneration and separation of muscle fibres in the oesophagus and stomach indicate catabolic processes to provide the energy for starving larvae when food supplies are limited (Green and McCormick, 1999). Histopathological changes in digestive mucosa, pancreas and liver, caused by starvation, may also affect food digestion in green sturgeon larvae, which resumed feeding. Pancreatic enzymes appear to be particularly sensitive to food deprivation in teleost larval fish (Zambonino Infante and Cahu, 2001). Gwak et al. (1999) reported the decline of trypsin and amylase activities in starving *Paralichthys olivaceus* to very low levels, which was associated with a reduction of pancreatic volume and partial necrosis of the exocrine pancreas. In our study, we observed a progressive degeneration of exocrine pancreas due to starvation, but zymogen granules were still present in larvae starved for 10–15 d, as it has been described in teleost species (Yúfera et al., 1993). While, the stomach tissues deteriorated progressively with starvation in green sturgeon larvae, the presence of mucosubstances in pyloric region and intestinal lumen seemed to suggest that gastric glands were still secreting pepsinogen and hydrochloric acid (Buddington and Doroshov, 1986), hence the secretion of this mucous may protect the digestive mucosa from auto-digestion.

In conclusion, the histological differentiation of digestive system in green sturgeon followed patterns as reported for other acipenserids. Some chronological differences in development could be related to the rearing temperature and greater reserve of yolk in green sturgeon larvae. Food deprivation resulted in progressive deterioration of the digestive system, with the first pathological signs after 5 d of starvation, followed with severe atrophic changes in the digestive organs after 10–15 d of starvation. The histological analysis of larval digestive system may provide sensitive indicators of the nutritional condition of green sturgeon larvae, and can be used in stock management and studies on nursery habitat affected by water projects.

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1 **Effect of incubation temperature on green sturgeon embryos, *Acipenser***  
2 ***medirostris***

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6  
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8 **Key words:** Acipenseridae, temperature tolerance, egg mortality, embryo development, hatching,  
9 deformities

10 **Synopsis**

11 Regulation of river flow and the amount of winter rainfall are the major factors affecting the water  
12 temperature of the spawning grounds, for green sturgeon in the Klamath River. During the primary  
13 spawning period of green sturgeon, mid-April to June, the water temperature may vary from 8 to 21°C. To  
14 estimate the potential implications of this modified thermal regime, we examined the survival and devel-  
15 opment in three progeny groups of green sturgeon embryos from zygote to hatch, at constant incubation  
16 temperatures (11–26°C). Temperatures 23–26°C affected cleavage and gastrulation and all died before  
17 hatch. Temperatures 17.5–22°C were suboptimal as an increasing number of embryos developed abnor-  
18 mally and hatching success decreased at 20.5–22°C, although the tolerance to these temperatures varied  
19 between progenies. The lower temperature limit was not evident from this study, although hatching rate  
20 decreased at 11°C and hatched embryos were shorter, compared to 14°C. The mean total length of hatched  
21 embryos decreased with increasing temperature, although their wet and dry weight remained relatively  
22 constant. We concluded that temperatures 17–18°C may be the upper limit of the thermal optima for green  
23 sturgeon embryos, and that the river thermal regime during dry years may affect green sturgeon repro-  
24 duction.

26 **Introduction**

27 The green sturgeon, *Acipenser medirostris*, is an  
28 anadromous species inhabiting the North Ameri-  
29 can Pacific Ocean, from the Aleutian Islands to  
30 California. Despite its wide geographic distribu-  
31 tion and some significant commercial landings  
32 (Houston 1988), the green sturgeon is considered a  
33 vulnerable species in the United States and Can-  
34 ada (Moyle et al. 1994, Campbell 1997). A closely  
35 related Asian green sturgeon (Sakhalin sturgeon,  
36 *A. mikadoi*) is similar in morphology (North et al.  
37 2002) but was recently separated as a species based  
38 on the cell DNA content and cytochrome-b gene  
39 sequence (Birstein & DeSalle 1998). The Sakhalin

sturgeon is an endangered species in Russia and 40  
Japan, but the 2001 petition to list the American 41  
green sturgeon under the U.S. Endangered Species 42  
Act was unsuccessful. After reviewing the best 43  
available scientific and commercial information 44  
for green sturgeon the National Marine Fisheries 45  
Service determined that listing the green sturgeon 46  
as threatened or endangered is not warranted, at 47  
this time.<sup>1</sup> 48

Unfortunately there has been a scarcity of re- 49  
search on green sturgeon life history, habitat, and 50  
stock abundance. Although some recent studies 51

<sup>1</sup> U.S. Federal Register Vol. 68, No. 19, pp. 4433–4441; 29  
January 2003.

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52	have provided new information on green sturgeon	abe & Tracy 1994, Bruch & Binkowski 2002,	102
53	karyotype (Van Eenennaam et al. 1999), repro-	Perrin et al. 2003) species.	103
54	ductive biology (Van Eenennaam et al. 2001, Deng	The successful spawning and early development	104
55	et al. 2002), adult movement (Erikson et al. 2002),	form the basis for sturgeon recruitment. Since the	105
56	and physiology (Gisbert et al. 2001, Gisbert &	temperature range permitting normal development	106
57	Doroshov 2003, Lankford et al. 2003), many as-	is critical in fish reproduction, the primary objec-	107
58	pects of green sturgeon biology remain obscure.	tive of this research was to determine the effects of	108
59	Knowing the temperature tolerance of green	egg incubation temperature on green sturgeon	109
60	sturgeon embryos may assist in protection of this	embryo survival to hatch. This study provides the	110
61	species.	first information on development and survival of	111
62	Reproduction and stock recruitment are the	green sturgeon embryos incubated, from zygote to	112
63	most vulnerable phases of sturgeon life history	hatch, at different constant temperatures under	113
64	(Dettlaff et al. 1993). Green sturgeon spawn in the	laboratory conditions.	114
65	Klamath, Rogue, and Sacramento Rivers, all of		
66	which have flow regimes affected by water projects		
67	(Moyle et al. 1994). The Klamath River is a major	<b>Materials and methods</b>	<b>115</b>
68	site of green sturgeon reproduction in the Pacific		
69	Northwest (Moyle et al. 1994, Van Eenennaam	<i>Broodfish and spawning</i>	<i>116</i>
70	et al. 2001) and the spawning run starts in April		
71	and extends through June, with the peak and	We obtained mature green sturgeon on 12 May	117
72	duration dependent on river flow and water tem-	2000 (one female and two males, river temperature	118
73	perature. Seven hydroelectric dams and numerous	12–13°C) and on 26 April 2002 (two females and	119
74	irrigation diversions built between 1906 and 1962	two males, 12–14°C) from the Yurok Tribe gillnet	120
75	greatly affect the river flow and anadromous fish	fishery in the lower Klamath River (Van Een-	121
76	migration, particularly during the 'dry' years	ennaam et al. 2001). After 1–5 days holding in	122
77	(Service 2003).	river cages, we transported the fish to the Center	123
78	The variation in annual precipitation and flow	for Aquatic Biology and Aquaculture (CABA,	124
79	regulation results in large fluctuations of river	University of California, Davis) and placed them	125
80	temperature (10–23°C) during the spawning run of	for spawning in 4 m diameter flow-through tanks.	126
81	green sturgeon (unpublished data for the Klamath	We also collected semen from one naturally mil-	127
82	River at Weitchpec, California). This has raised	ting male in 2002 and stored it in plastic bags with	128
83	concerns regarding the survivability of green	oxygen on wet ice. We weighed brood fish	129
84	sturgeon embryos and the effect of controlled flow	(±0.1 kg), measured their length (±0.5 cm), and	130
85	on natural recruitment.	collected small samples of ovarian follicles	131
86	In laboratory studies with sympatric white	(n = 15–20) by catheter to determine oocyte	132
87	sturgeon, <i>A. transmontanus</i> , temperatures between	diameter and polarization index, PI (Van Een-	133
88	14 and 16°C were optimal and temperatures above	ennaam et al. 2001), using a dissecting scope with	134
89	20°C were lethal for the embryos during develop-	camera lucida and a digital image-analyzing tablet	135
90	ment from fertilization to hatch (Wang et al. 1985,	with computer interface (±0.01 mm).	136
91	1987). Studies with the Caspian beluga, <i>Huso huso</i> ,	The hormonal induction of spawning followed	137
92	ship, <i>A. nudiiventris</i> , Russian sturgeon, <i>A. gue-</i>	the methods of Van Eenennaam et al. (2001), with	138
93	<i>ldenstaedtii</i> , and sevruga, <i>A. stellatus</i> , revealed	slight modifications. Males received a single	139
94	generally similar temperature effects on the early	injection (10 µg kg <sup>-1</sup> ) of mammalian GnRHα [D-	140
95	life stages (Nikol'skaya & Sytina 1978, Igumnova	Ala <sup>6</sup> , Des-Gly <sup>10</sup> ] - LH-RH Ethylamide (Peninsula	141
96	& Dubinin 1987, Sytina & Shagaeva 1987).	Laboratories, Belmont, California). An additional	142
97	Observations on natural spawning, documented	male green sturgeon, captured in the Sacramento	143
98	by the collection of eggs and larvae, indicate that	River as a yolk sac larva and reared to full sexual	144
99	the majority of sturgeons reproduce within the	maturity at the CABA facilities, was induced to	145
100	temperature range of 10–20°C, including Eurasian	spermiate in 2002. The females received two	146
101	(Dettlaff et al. 1993) and North American (McC-	GnRHα injections: priming (1 µg kg <sup>-1</sup> ) and	147

148	resolving (19 $\mu\text{g kg}^{-1}$ ), with a 12-h interval. In	
149	addition, the females received 1–3 $\text{mg kg}^{-1}$ of the	
150	dopamine antagonist domperidone (Research	
151	Diagnostics, Flanders, New Jersey). All injections	
152	were intramuscular and given underwater to min-	
153	imize handling stress.	
154	Broodfish holding temperature during spawning	
155	ranged 13.1–14.6°C. We periodically examined the	
156	female holding tank for released eggs, starting 12 h	
157	after the second injection. We collected milt with a	
158	60 ml plastic syringe and a 4 cm long vinyl cath-	
159	eter inserted into the urogenital pore (Conte et al.	
160	1988) and sperm was evaluated for the percent and	
161	duration of motility (Van Eenennaam et al. 2001).	
162	We extracted the ovulated eggs surgically (Conte	
163	et al. 1988), from fish under anesthesia (MS-222).	
164	Artificial fertilization followed techniques	
165	developed for white sturgeon (Conte et al. 1988).	
166	We fertilized ovulated eggs in bowls, using 4 l of	
167	diluted semen (1:100 or 200 with hatchery water)	
168	per 1 l of ova. After fertilization, we gently mixed	
169	the eggs in a suspension of Fuller's Earth (100–200	
170	mesh size, Sigma Chemical Co., St. Louis, Mis-	
171	souri) for de-adhesion, and two subsamples (ca. 350	
172	eggs) of each progeny were incubated at 15.5°C	
173	(Deng et al. 2002) to determine 'control' fertility at	
174	the 4-cell stage (Dettlaff et al. 1993).	
175	We incubated small batches of eggs in 15 l tanks	
176	(28 cm diameter, 35 cm deep, flow rate 1.5–	
177	2.0 l $\text{min}^{-1}$ ) housed in six temperature-controlled	
178	recirculation systems each with a YSI thermostat,	
179	chiller, heater, biological filter, and aeration.	
180	During the course of the experiments, dissolved	
181	oxygen was 95–100% air saturation, pH ranged	
182	8.5–8.6, and ammonia nitrogen did not exceed	
183	0.05 ppm. Temperature variation in each system,	
184	measured twice a day with a certified calibrated	
185	thermometer (National Institute of Standards and	
186	Technology), did not exceed $\pm 0.1^\circ\text{C}$ . We used	
187	artificial photoperiod 16L: 8D and protected the	
188	tanks with shade-cloth covers that blocked 80% of	
189	the light. Two experiments were conducted, one	
190	during 2000 (range-finding) and another in 2002	
191	(upper temperature tolerance).	
192	<i>Experiment 1 (2000)</i>	
193	We incubated the eggs of one female, fertilized at	
194	14°C with pooled semen of two Klamath males, in	
195	six treatments (11, 14, 17, 20, 23, 26°C) with four	
	replications. The 60 ml egg batches (ca. 700 eggs)	196
	were placed in 1.5 l half-filled crystallizing dishes	197
	floating in the 14°C treatment tanks. We trans-	198
	ferred dishes stepwise to warmer or cooler treat-	199
	ments, at a rate of temperature change of $4^\circ\text{C h}^{-1}$ ,	200
	and submerged them on the tank bottoms. Each	201
	tank received flowing water (1.5 l $\text{min}^{-1}$ ) via tygon	202
	tubing positioned at the inner edge of the crystal-	203
	lizing dish, ensuring continuous exposure of the	204
	embryos to fresh water. We removed and counted	205
	the unfertilized (not cleaving) and dead (opaque)	206
	eggs at stages 5–6 (4–8 cells), 22–23 (closure of	207
	neural tube), and 36 (hatch, stages as in Dettlaff	208
	et al. 1993). We recorded the time of 50% hatch,	209
	counted the hatched embryos, and sampled 10	210
	embryos from each tank for length ( $\pm 0.01$ mm),	211
	live weight ( $\pm 0.1$ mg), and dry weight ( $\pm 0.01$ mg,	212
	desiccated 15 h at $100^\circ\text{C}$ ). The length of bent lar-	213
	vae (prevalent disorder at higher temperatures)	214
	was measured by tracing specimens along the	215
	curved notochord with the light cursor of the	216
	digital image-analyzer. Due to a technician error,	217
	the embryos hatched in replicate tanks were	218
	pooled within each treatment, thus we recorded	219
	the proportions of abnormal embryos in pooled	220
	samples.	221
	<i>Experiment 2 (2002)</i>	222
	We incubated the eggs of two females in six	223
	treatments (16, 17.5, 19, 20.5, 22, 23.5°C) with five	224
	replications for each progeny. The eggs of Female	225
	1 were fertilized with semen of one Klamath male	226
	with the best sperm motility, and the eggs of Fe-	227
	male 2 with pooled semen of three males (from	228
	Klamath and Sacramento rivers). The egg transfer,	229
	incubation, and sampling were similar with	230
	Experiment 1, except for lower egg densities in	231
	crystallizing dishes (20 ml egg volume, ca. 240 eggs	232
	per tank) and a fertilization temperature of $16^\circ\text{C}$ .	233
	We recorded percent abnormalities in each tank	234
	and photographed representative hatched embryos	235
	under a dissecting scope.	236
	<i>Data analyses</i>	237
	We used JMP statistical software (Version 4, SAS	238
	Institute, Cary, North Carolina) for data analysis.	239
	The arcsine transformation of percent survival	240
	data was necessary to normalize distributions. We	241

242 transformed zero percent survival data as an arc-  
 243 sine  $(1 - 4n^{-1})^{0.5}$ , where 'n' was the number of  
 244 stocked eggs (Zar 1984). The normality of trans-  
 245 formed data was confirmed by the Shapiro-Wilk  
 246 test. We used two-way ANOVA with repeated  
 247 measure (tanks within the treatment) and Tukey  
 248 HSD test to test the effect of temperature on sur-  
 249 vival to cleavage, neurulation and hatching. The  
 250 egg source (female) effect was included for  
 251 Experiment 2. One-way ANOVA and Tukey test  
 252 were used for all other data. Pooled proportions of  
 253 the abnormal embryos in Experiment 1 were  
 254 compared by contingency tables. The accepted  
 255 significance level was  $p < 0.05$ . Data in figures  
 256 and text are untransformed means and standard  
 257 deviations.

## 258 Results

### 259 Spawning

260 Mature females were significantly larger than  
 261 males (student *t*-test, for both years of observa-  
 262 tions combined) (Table 1). The fully grown oo-  
 263 cytes of three females ranged 4.17–4.45 mm in  
 264 mean diameter and 0.03–0.04 in PI (distance from  
 265 the germinal vesicle to the animal pole is 3–4% of  
 266 egg diameter). These low PI values indicate the  
 267 advanced stage of oocyte development and matu-  
 268 rational competence in acipenserids (Dettlaff et al.  
 269 1993). All broodfish responded to hormonal  
 270 treatment, with a latent period from the resolving  
 271 injection to ovulation of 14–17 h. The number of

ova collected from each female ranged 88–133 272  
 thousand. All males had milt with a high per- 273  
 centage of motile sperm (>80%) and extended 274  
 duration of motility (>4.5 min), although the milt 275  
 stored for 24 h on ice and the fresh milt collected 276  
 from the domestic male had about 10% less motile 277  
 sperm and 1–2 min shorter motility. Mean fertil- 278  
 ization rates in controls were 41% in 2000, and 279  
 56% (Female 1) and 62% (Female 2) in 2002. 280

### Experiment 1 (2000)

281  
 282 Due to the difficulty of sorting through the large  
 283 quantity of eggs in each tank, only the obvious  
 284 unfertilized eggs (with no cleavage at all) were  
 285 counted as 'mortalities' at the 4-cell stage, and the  
 286 eggs with mosaic (part of the egg does not divide)  
 287 and parthenogenetic (activated unfertilized egg  
 288 with irregular furrows) cleavage (Dettlaff et al.  
 289 1993) were kept in each tank until neurulation. As  
 290 a result, cleavage survival was not estimated  
 291 properly (ranged 64–69% in the experiment, com-  
 292 pared to 41% in the fertilization control) and was  
 293 excluded from data analysis.

294 ANOVA revealed a significant ( $p < 0.0001$ )  
 295 effect of incubation temperature on survival to  
 296 neurulation and hatching; 36–40% of embryos (a  
 297 majority of fertilized eggs) survived to neurulation  
 298 in temperatures 11–20°C, but only 16% survived in  
 299 23°C, and none of the embryos survived in the  
 300 26°C treatment (Figure 1), where the cleavage was  
 301 abnormal and ceased before gastrulation. Survival  
 302 to hatch was the highest in the 14 and 17°C  
 303 treatments (39 and 36%) and lowest in the 11 and

Table 1. Reproductive characteristics of green sturgeon broodstock used for spawning.

Date of capture	12 May 2000		25–26 April 2002		
	F	M ( $n = 2$ )	F1	F2	M ( $n = 3$ )
Sex (F/M)					
Fork length, cm	166	156 ± 2	185	179	148 ± 7
Weight, kg	38	24 ± 1	50	52	24 ± 4
Oocyte PI, ratio	0.03	–	0.04	0.03	–
Oocyte D, mm	4.17	–	4.34	4.45	–
Latent period, h	17	–	14	14	–
Ova collected, ×10 <sup>3</sup>	88	–	113	109	–
Sperm motility, %	–	95 ± 3	–	–	89 ± 7
Motility duration, s	–	387 ± 9	–	–	320 ± 60
Control fertility, %	41 ± 3	–	56 ± 1	62 ± 1	–

Data for males are means and SD. The oocyte polarization index (PI) and diameter (D) are means of 15–20 eggs. Latent period is time from the resolving GnRH<sub>a</sub> injection to ovulation. Control fertility was based on two subsamples at the 4–8 cell stage.

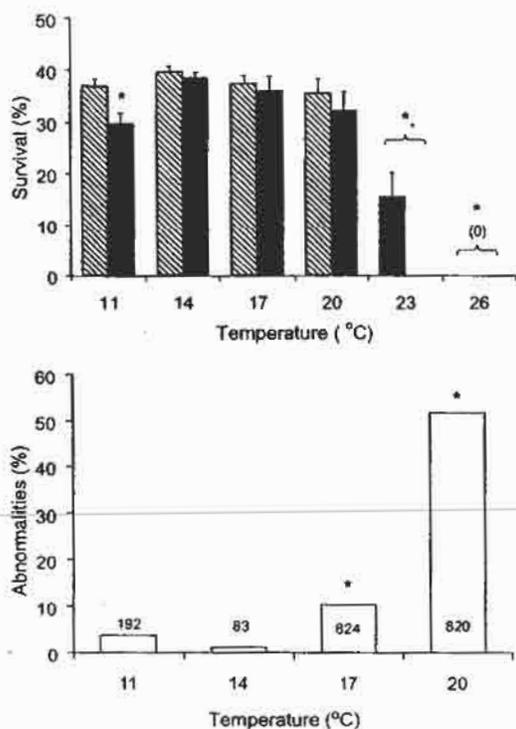


Figure 1. Top: survival (mean, SD) of green sturgeon embryos at neurulation (shaded bars,  $n = 4$ ) and hatch (solid bars,  $n = 4$ ) in Experiment 1. Asterisks indicate significantly different sample means, for stages among the treatments ( $p < 0.05$ ). Bottom: proportions of hatched abnormal embryos in pooled samples (asterisk denote different proportions,  $p < 0.05$ , numbers are total counts).

304 20°C treatments (30 and 32%), with detected sig-  
 305 nificant difference in hatch-rate between the 11 and  
 306 14°C treatments. No embryos survived to hatch at  
 307 23°C. The proportions of deformed hatched em-  
 308 bryos were low at 11 and 14°C (3.7 and 1.2%) but  
 309 increased at 17 and 20°C (10.3 and 51.6%, Fig-  
 310 ure 1). The deformities were similar to those ob-  
 311 served in the 2002 experiment.

312 Embryos hatched in the 11–20°C treatments did  
 313 not differ in dry weight (15.0–15.1 mg), but were  
 314 significantly longer at 17 and 14°C ( $12.5 \pm 0.29$   
 315 and  $11.8 \pm 0.12$  mm) compared to the 11 and 20°C  
 316 treatments ( $10.9 \pm 0.08$  and  $11.0 \pm 0.07$  mm). The  
 317 percent dry weight was highest at 14°C ( $46.8\% \pm$   
 318  $0.46$ ) and lowest at 20°C ( $44.1\% \pm 0.47$ , signifi-  
 319 cantly different).

#### Experiment 2 (2002)

320

321 Lower egg densities in this experiment facilitated  
 322 identification and accurate counts of unfertilized  
 323 eggs. The percent of cleavage in treatments 16–  
 324 22°C were similar to control fertilization rates (52–  
 325 56% vs. 56% control for progeny 1, and 60–62%  
 326 vs. 62% control for progeny 2). ANOVA revealed  
 327 a significant interaction between the treatment and  
 328 egg source ( $p < 0.0001$ ), with a higher survival to  
 329 neurulation and hatch at 20.5 and 22°C in the  
 330 progeny of Female 2. The effect of treatment was  
 331 analyzed separately for each progeny (Figure 2).

332 The incubation at 23.5°C decreased cleavage  
 333 survival and resulted in 100% mortality before  
 334 neurulation in both progenies. Treatment 22°C  
 335 decreased neurulation survival in progeny 1 and  
 336 survival to hatch in both progenies, with the only  
 337 0.3% hatch in progeny 1 (all deformed embryos)  
 338 and 32% in progeny 2 (78.4% deformed embryos).

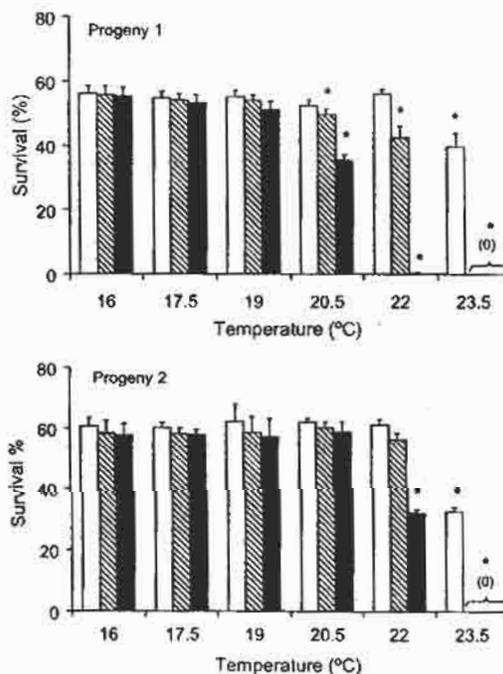


Figure 2. Survival (mean, SD) of green sturgeon embryos at cleavage (open bars,  $n = 5$ ), neurulation (shaded bars,  $n = 5$ ), and hatch (solid bars,  $n = 5$ ) in Experiment 2. Asterisks denote significantly different treatment means ( $p < 0.05$ ).



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339 Treatment 20.5°C decreased survival to neurula-  
 340 tion and hatch in progeny 1, but not in progeny 2.  
 341 The proportions of deformed hatched embryos  
 342 increased with temperature in both progenies, and  
 343 were higher in progeny 1 (Figure 3). In both  
 344 progenies, the highest hatching success (56.2 and  
 345 60.6%) and the lowest proportions of deformed  
 346 embryos (1.0 and 0.3%) were in 16°C treatment.  
 347 The abnormalities at the elevated temperatures  
 348 were predominantly (>80%) body deformations,  
 349 such as the bent and shortened posterior trunk and  
 350 tail regions, including lordosis and kyphosis; ede-  
 351 ma and the more severe developmental defects  
 352 such as an underdeveloped head, trunk, and tail  
 353 regions were less common (Figure 4).

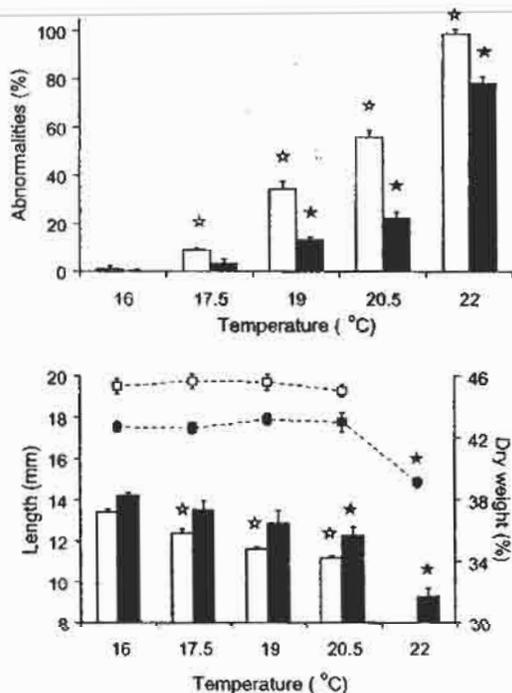


Figure 3. Top: proportions (mean, SD) of hatched abnormal green sturgeon larvae in Experiment 2, for progenies 1 (open bars,  $n = 5$ ) and 2 (solid bars,  $n = 5$ ). Stars indicate significantly different ( $p < 0.05$ ) means among the treatments within each progeny. Bottom: hatched larvae length (mean and SD, open and solid bars for progenies 1 and 2,  $n = 5$ ) and dry weight (open and solid squares for progenies 1 and 2,  $n = 5$ ). Stars denote different treatment means within each progeny ( $p < 0.05$ ).

In progeny 1, the length of hatched embryos  
 354 decreased at higher temperatures, from 13.4 mm  
 355 at 16°C to 11.2 mm at 20.5°C. The live weight  
 356 (40.0–40.5 mg) and percent dry weight (45.1–  
 357 45.8%) were not affected by temperature. A similar  
 358 relationship was observed in progeny 2 within the  
 359 range of 11–20.5°C, but the embryos hatched at  
 360 22°C were shorter (9.3 mm) and had a lower  
 361 (39.2%) dry weight (Figure 3). The hatchery control  
 362 larvae (pooled progenies) were similar in  
 363 length ( $13.9 \pm 0.12$  mm), live weight ( $40.4 \pm$   
 364  $0.28$  mg) and dry weight ( $45.1 \pm 0.28\%$ ), to the 16  
 365 and 17.5°C treatment larvae. The longer larvae in  
 366 the female 2 progeny were likely due to the larger  
 367 egg size in this female (Table 1).  
 368

The relationship between median time of hatch  
 369 and incubation temperatures 11–22°C (observa-  
 370 tions from both experiments) was best represented  
 371 by an exponential equation (Figure 5) and was  
 372 similar to the relationship for stage 35 (beginning  
 373 of hatch) of white sturgeon reported by Wang  
 374 et al. (1985).  
 375

## Discussion

We provide the first information on the effect of  
 377 incubation temperature on green sturgeon em-  
 378 bryos. Temperatures 23°C and above resulted in  
 379 total mortality before hatch. Suboptimal tempera-  
 380 tures (17.5–22°C) reduced the number of normal  
 381 embryos and decreased hatching success, but the  
 382 responses to suboptimal temperature varied be-  
 383 tween progenies. This study did not reveal the low  
 384 temperature limit for green sturgeon embryos, but  
 385 the lowest experimental temperature of 11°C de-  
 386 creased hatching success and produced smaller  
 387 embryos at hatch. Our study suggests that green  
 388 and white sturgeon embryos have similar thermal  
 389 optima, upper temperature limits and tempera-  
 390 ture-dependent rates of development (Wang et al.  
 391 1985). Given the same geographic range and  
 392 spawning season of these two Pacific Coast spe-  
 393 cies, these similarities are not surprising. Although  
 394 white and green sturgeons may reproduce in the  
 395 same river (e.g. Sacramento River), they appear to  
 396 maintain reproductive isolation by selecting dif-  
 397 ferent spawning sites within one watershed (Deng  
 398 et al. 2002). White sturgeon dominate the spawn-  
 399 ing run of the Sacramento river while the opposite  
 400

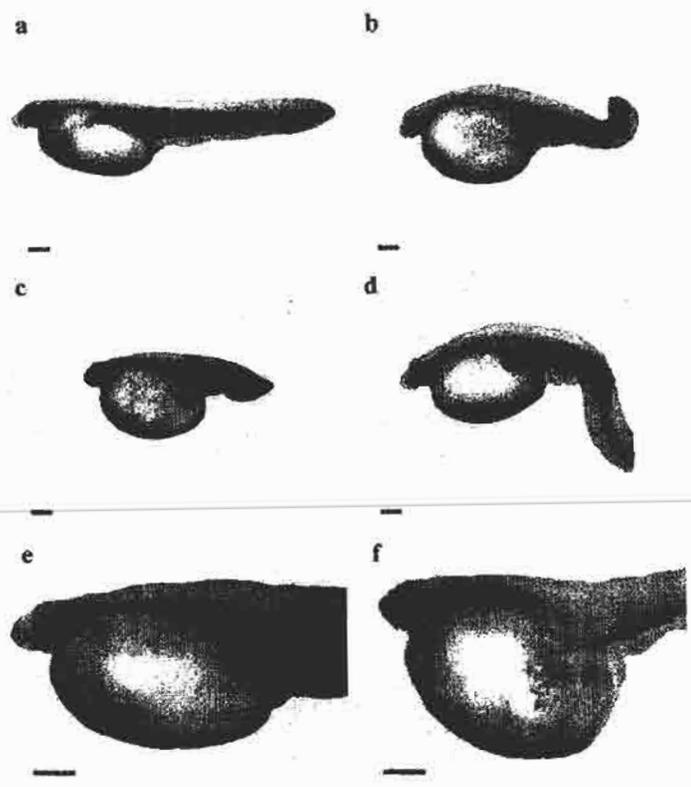


Figure 4. Representative abnormalities observed at hatch included kyphosis (b), lordosis (d), the more severe underdeveloped head, trunk, and tail regions (c), and edema (f). A representative normal embryo is included for comparison (a, e). Scale bars are 1 mm.

401 is true for smaller fast-flowing Klamath and Ro-  
 402 gue rivers. There is no published information on  
 403 the occurrence of hybrids between these two spe-  
 404 cies, and the natural hybridization appears to be  
 405 either rare or not adequately investigated (T. Rien  
 406 & J. North, Oregon Department of Fish & Wild-  
 407 life, D. Erickson, Wildlife Conservation Society,  
 408 Oregon, personal communication). We have not  
 409 investigated artificial hybridization between green  
 410 and white sturgeon. However, Kolman et al.  
 411 (1999) described hybrids between the Asian green  
 412 sturgeon and Siberian sturgeon (*A. baerii*) pro-  
 413 duced in the hatchery.  
 414 The effect of temperature on different stages of  
 415 embryo development was not fully revealed by this  
 416 study, since the abnormalities at cleavage and  
 417 neurulation were not characterized. Significant

418 proportions of embryos survived to cleavage at 23  
 419 and 23.5°C, but abnormal cleavage could lead to  
 420 gastrulation arrest and, consequently, mortality  
 421 before neurulation (Sytina & Shagaeva 1987).  
 422 Dettlaff et al. (1993) reported abnormal, mosaic  
 423 cleavage in beluga (*H. huso*) eggs obtained from  
 424 females maturing at the upper temperature limit  
 425 for spawning. The mosaic cleavage pattern and the  
 426 resulted deformed embryos were explained by the  
 427 damaging effect of holding temperature on the  
 428 ooplasm and the inhibition of the cortical reaction  
 429 at fertilization in the areas associated with undi-  
 430 vided portions of the egg (Dettlaff et al. 1993). The  
 431 complete block of egg cleavage in acipenserids was  
 432 reported at temperatures outside the spawning  
 433 range, e.g. at  $\geq 28^{\circ}\text{C}$  in stellate sturgeon, *A. stell-*  
 434 *atus* (Sytina & Shagaeva 1987).

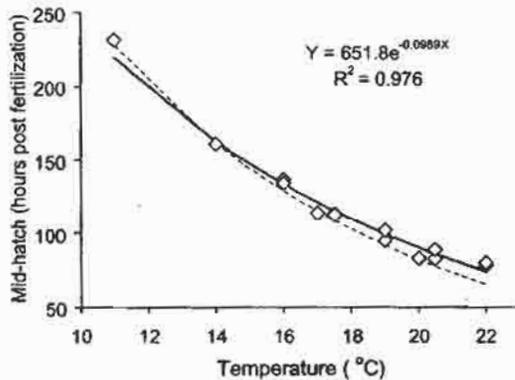


Figure 5. Rate of development to mid-hatch (hours post fertilization) of green sturgeon embryos at different temperatures. The data are from observations in both experiments, and the best fit relationship is shown by the solid line and an exponential equation. For comparison the exponential equation for white sturgeon (dashed line, Wang et al. 1985) is included.

435 The deformities of green sturgeon embryos  
 436 hatched at elevated temperatures were similar to  
 437 those described and illustrated for Russian sturgeon  
 438 *A. gueldenstaedti* by Dettlaff et al. (1993).  
 439 These investigators also noted the relationship  
 440 between the rate of deformities and egg quality (up  
 441 to 25–30% in poor quality eggs and few in high  
 442 quality eggs) and the effect of high incubation  
 443 temperature ('at times, higher than 26°C', Dettlaff  
 444 et al. 1993, p. 138) on the increase, up to 90%, of  
 445 distinct structural defects. The prevailing abnormality  
 446 in our study was a curved notochord, downwards (lordosis)  
 447 or upwards (kyphosis), which affected mobility of hatched embryos.  
 448 The notochord development and the osmotic mechanism  
 449 of its elongation, straightening and stiffening (as in the  
 450 amphibian embryo, Adams et al. 1990) might be affected  
 451 by high incubation temperatures. The shortening of the  
 452 embryo body, decrease in percent dry weight, and edema  
 453 were observed in green sturgeon embryos hatched at  
 454 22°C (Figure 3). The reduced mean length of embryos  
 455 hatched at 17.5–20.5°C is more likely to reflect the  
 456 effect of temperature on somitogenesis (Brooks &  
 457 Johnston 1994).  
 458 Factors affecting temperature tolerance of fish  
 459 embryos may include egg quality (Dettlaff et al. 1993),  
 460 as well as thermal experience of the parents

(Hubbs & Bryan, 1974). Embryos of *A. stellatus*,  
 463 obtained from spawners during different periods  
 464 of migration into the Volga River exhibited varying  
 465 tolerance to upper temperature, with the differences  
 466 of 2–3°C in lethal, sublethal, and suboptimal  
 467 temperatures between the early and late spawning  
 468 run (Sytina & Shagaeva 1987). In our study, broodfish  
 469 were obtained from the same area of the Klamath  
 470 River and at similar river temperatures. Egg quality  
 471 was likely the main factor affecting the different  
 472 responses of two progenies in the 2002 experiment,  
 473 with lower egg fertility and consistently higher  
 474 proportions of abnormal embryos in all temperature  
 475 treatments in the female 1 offspring.

476 Our data show that temperatures above 20°C  
 477 are most likely detrimental for green sturgeon  
 478 reproduction. While the temperatures between  
 479 17.5 and 19°C may result in satisfactory hatching  
 480 success, the percent of abnormal hatched embryos  
 481 increases within this range and it can be hypothesized  
 482 that the resulting offspring are subject to higher  
 483 mortality rates. Although there are undoubtedly  
 484 some females that produce embryos that can tolerate  
 485 elevated river temperatures better than others, based  
 486 on this study it seems that temperatures 17–18°C  
 487 may be the upper limit of the optimal thermal range  
 488 for green sturgeon development, wherein neither  
 489 hatching success nor normal development are  
 490 affected by temperature.

491 Klamath River temperature profiles were provided  
 492 by the Yurok Tribe fishery biologists using data  
 493 loggers located at approximately river kilometers  
 494 (rk) 56 and 66 (Figure 6). The river temperature  
 495 on the spawning grounds is affected by flow  
 496 releases from the Iron Gate dam (rk 306), the  
 497 amount of heating and cooling in transit to the  
 498 spawning areas (below Ishi Pishi Falls, rk 107),  
 499 thermal loading from other streams and rivers,  
 500 and the amount of precipitation during the winter  
 501 and spring. The predominant spawning period,  
 502 from mid-April to the end of May (Figure 6),  
 503 was based on the time when the tribal fishers  
 504 collect approximately 80% of their annual catch  
 505 (J. Van Eenennaam, unpublished data). In the past,  
 506 the Iron Gate Dam on the Klamath River had  
 507 Federal Energy Regulatory Commission (FERC)  
 508 stipulated minimum flows that decreased during  
 509 the time of sturgeon spawning (1300 cfs during  
 510 April, 511 512

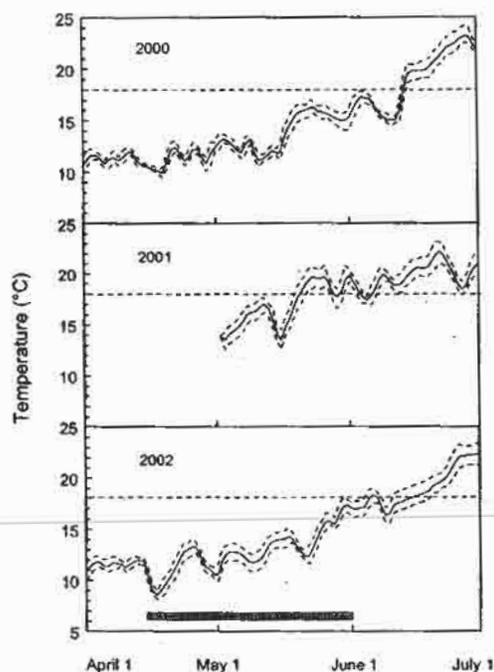


Figure 6. Klamath river temperature profiles for years 2000–2002 (daily means, minimum and maximum recorded temperatures at approximately river kilometer 56 and 66). The horizontal slashed bar indicates the time of the predominant spawning migration over a 5-year period (1999–2003).

513 1000 cfs during May, and 710 cfs during June).<sup>2</sup>  
 514 However, since 1996, the United States Bureau of  
 515 Reclamation annual Klamath Project Operations  
 516 Plans has dictated flow releases that have generally  
 517 exceeded the required FERC instream flows.  
 518 During relatively normal rainfall years, such as  
 519 2000 and 2002, the potential for embryos being  
 520 exposed to high water temperatures was relatively  
 521 low. However, during dry years, such as 2001,  
 522 green sturgeon may be exposed to water temper-  
 523 atures that would have a detrimental effect on  
 524 developing embryos. In reality the elevated temper-  
 525 atures during 2001 truncated the spawning run  
 526 and we were unable to obtain broodfish for  
 527 spawning that year. We cannot conclude if the low

<sup>2</sup> Pacificorp Klamath hydroelectric project. FERC Project No. 2082. Chapter 10. Klamath River below iron gate dam. Available from the Internet URL <http://www.pacificorp.com/File/File16145.pdf>.

528 water temperature during mid-April, 2002 had any  
 529 effect on sturgeon spawning or embryo survival.  
 530 Indeed, it may have shifted spawning to slightly  
 531 later in the season.

532 The effect of regulated water flow on parameters  
 533 other than temperature, such as decreases in tur-  
 534 bidity and river level, could also affect the  
 535 spawning run and the reproductive success. Stur-  
 536 geons require specific current velocity, bottom  
 537 substrate, riparian habitat, water temperature and  
 538 turbidity for spawning, and terrestrial nutrients for  
 539 survival of larvae and juveniles (Dettlaff et al.  
 540 1993, Bemis & Kynard 1997, Coutant 2004).  
 541 Changes in the hydraulic regimes of rivers effected  
 542 by hydroelectric construction and diversions have  
 543 made them either unsuitable or inadequate for  
 544 sturgeon natural recruitment. Investigation and  
 545 protection of sturgeon spawning habitat seems to  
 546 be the most important task for fishery manage-  
 547 ment programs, to ensure the long-term survival of  
 548 the few remaining species, including green stur-  
 549 geon.

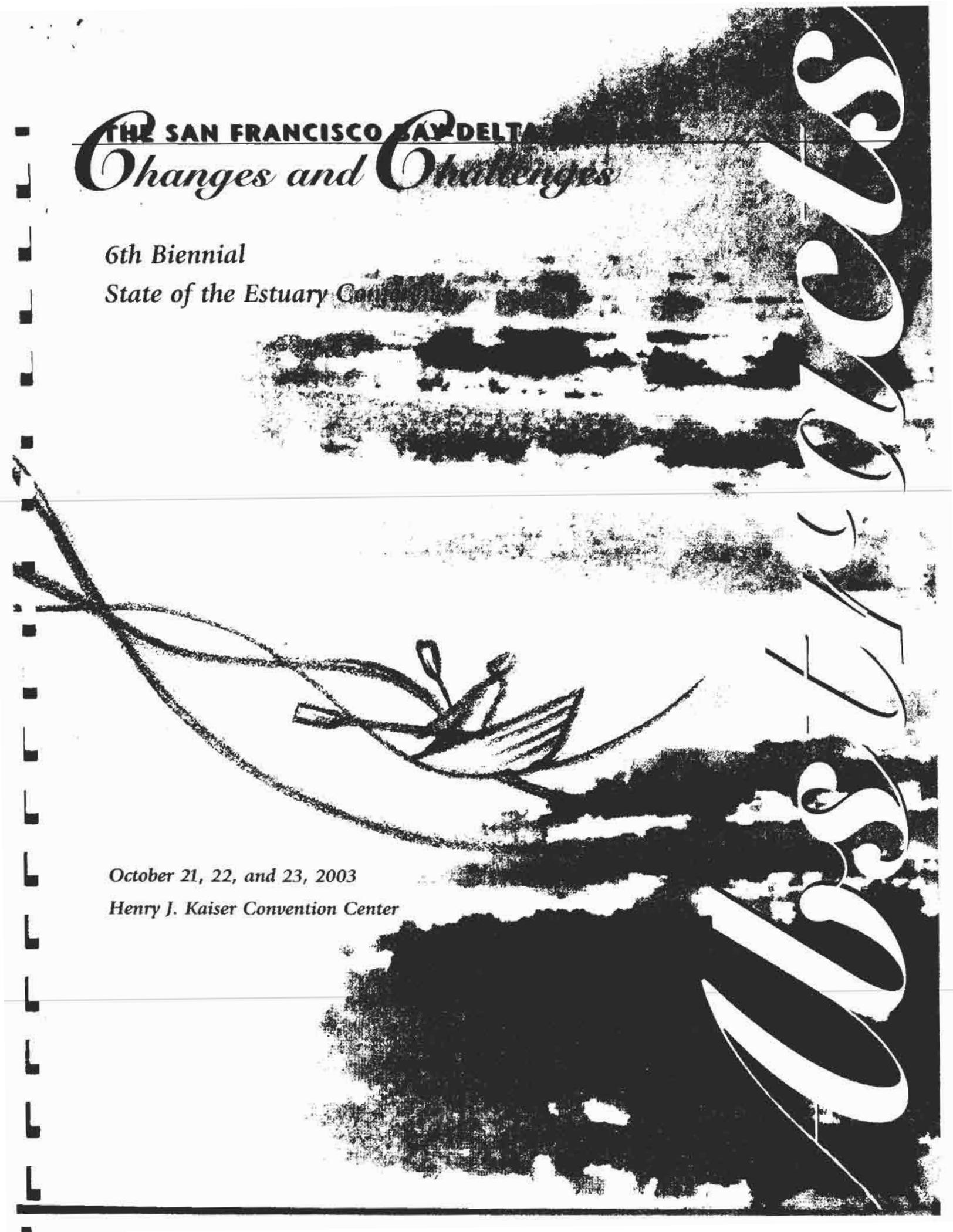
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*TEMPERATURE TOLERANCE AND THE HEAT-SHOCK PROTEIN RESPONSE IN LARVAL GREEN STURGEON (ACIPENSER MEDIROSTRIS) - C-GS-14*

Although the ability to tolerate and adapt to a range of water temperatures can be of great importance for the success of wild populations of green sturgeon, little is known about temperature stress in these fish. To detect and quantify thermal stress, this study investigated heat-shock protein (hsp) expression levels in newly hatched larval sturgeon exposed to a range of water temperatures during yolk-sac absorption. Hsps are intracellular proteins important in protecting organisms against the cytotoxic consequences of protein denaturation and play a major role in thermotolerance and thermoprotection. Wild broodfish were obtained from the Yurok Tribe gillnet fishery on the lower Klamath River, transported to the University of California, Davis, within 24 hour of capture, and placed into 4 m diameter circular flow-through tanks. After ovulation, spermiation and fertilization, eggs were incubated to hatching at  $16.5 \pm 1^\circ\text{C}$  in upwelling incubating jars. Hatched larvae were exposed to 18, 20, 22, 24, 26, and  $28^\circ\text{C}$  in 4 replicate flow-through tanks per treatment using six water recirculating systems. Mortality was recorded daily, and samples (10 larvae) for hsp analysis were collected and snap-frozen before, 24 h and 96 h after begin of temperature exposure and immediately after yolk sack absorption. Heat-shock proteins were analyzed by Western blotting.

# P R O G R A M



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## Reproduction and culture of green sturgeon (*Acipenser medirostris*)

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The anadromous green sturgeon is considered vulnerable in the United States and Canada, with the only known spawning populations in the Klamath, Rogue and Sacramento Rivers. Since reproduction is a critical factor in sturgeon stock management, our objectives have been to characterize the Klamath River broodstock and develop culture techniques for conservation-oriented research. We sampled 128 broodfish, over a four year period (1999-2002), during the spring (April-June) spawning run in the lower Klamath River. Broodfish ranged 14-40 years in age and 145-242 cm in total length, with a bimodal distribution of sexes (older and larger females). The condition factor was lower than in white and Atlantic sturgeons, associated with the long-tapered body shape in green sturgeon. Mean gonadosomatic index averaged 13% in females and 5% in males, and individual fecundity averaged 152,000. Green sturgeon have large eggs (twice by volume and weight compared to white sturgeon), and all females had oocytes in an advanced stage of germinal vesicle migration (polarization index  $<0.08$ ), indicating spawning readiness. Methods have been established for handling and transport of broodfish, hormonal induction of spawning, *in vitro* fertilization, embryo incubation, larval and juvenile rearing.

**Biological Assessment of Green Sturgeon in the Sacramento-San Joaquin Watershed, Phases 3 and 4, Task 3.** Josh Israel and Bernie May, Department of Animal Science, UC Davis, Davis, CA 95616

## **Introduction**

Green sturgeon, *Acipenser medirostris*, are a long-lived, migratory species found along the west coast of North America from Central California to Alaska. While often inhabiting bays and estuaries within this range, currently *A. medirostris* is only known to spawn in the Sacramento, Klamath, and Rogue Rivers. Analyses completed as part of Phases 3 and 4 have determined there to be more than one genetically distinct breeding population of green sturgeon. In addition, an unusual geographic pattern of genetic differentiation has been detected that possibly supports one of these populations relying more heavily on estuarine habitat for overwintering. This and other possible hypotheses regarding the detected patterns will be further explored once additional microsatellite DNA markers are adequately described.

The primary focus of Task Three, Phases 3 and 4 has been to complete an initial survey of genetic differentiation in green sturgeon and increase the number of microsatellite DNA markers available for differentiating these populations. A portion of the results and discussion in this report are abbreviated versions of those presented in the article "Geographic patterns of genetic differentiation among western U.S. collections of North American green sturgeon (*Acipenser medirostris*)." This paper is scheduled to appear in the August 2004 volume of the North American Journal of Fisheries Management. Presentations were made at the 2002 Calfed Science Conference, 2003 State of the Estuary Conference, and to the Yurok Tribal Council and Red Bluff Fish and Wildlife Office concerning the information developed in Phases 3 and 4.

## **Methods and Results**

**Additional Samples.** Green sturgeon samples have been collected by personnel from the California Department of Fish and Game (CDFG), Yurok Tribal Fisheries Program (YTFP), Oregon Department of Fish and Wildlife (ODFW), Washington Department of Fish and Wildlife (WDFW), USFWS-Red Bluff Fish and Wildlife Office (RBFWO), University of Idaho (UI) and University of California, Davis (UCD) and sent to the Genomic Variation Laboratory (GVL) at UCD. Table 1 contains the cumulative information on green sturgeon in the GVL's California Imperiled Fish Tissue Archive through 2003, including collecting organization, year of collection, and number of individuals. Tissue samples preserved in 95% ethanol have yielded higher DNA concentrations than dried fin samples, therefore, all sampling kits now recommend the ethanol preservation method. The current sampling protocol being shared with collaborators is attached (Appendix 1).

**DNA extraction.** During Phases 3 and 4, DNA from *A. medirostris* samples was isolated using a Qiagen DNeasy™ tissue kit. Sample DNA concentrations were quantified by comparison with human genomic DNA standards imaged on the FluorImager.

**Table 3:** Additional microsatellite DNA markers currently being attempted for use in this study. PCR conditions are described in Table 2.

	PCR Conditions			
	Touchdown	Promega <i>Taq</i>	Faststart <i>Taq</i>	
<i>An</i> 0	1.75 MgCl	-	-	Zane et al. 2002
<i>An</i> 20	1.5 MgCl	-	-	Zane et al. 2002
<i>An</i> 76	-	-	No modifications	Zane et al. 2002
<i>AfuG</i> 34	1.75 MgCl	-	-	Welsh et al. 2003
<i>AfuG</i> 229	No modifications	-	-	Welsh et al. 2003
<i>AfuG</i> 234	No modifications			Welsh et al. 2003
<i>AfuG</i> 237	No modifications, optimized	-	-	Welsh et al. 2003

**Green Sturgeon Genetic Sample Collection Protocol**  
**95% Ethyl Alcohol in 2ml tubes**

- **Gloves are not needed for handling the tubes, but precautions should be taken to avoid contaminating samples.**
- **To avoid contamination, rinse profusely with water and wipe dry the knife, scissor, or razor used for cutting tissue between samples.**
- **The target size for a pelvic or anal fin piece is 1 by 1 cms. That is about the size of your smallest fingernail.** 
- **When a sample is collected record the sample number and date on the tube label. Labels can be written on with indellible pen (Sharple works best) or pencil (#3 works best).**
- **When a sample is collected record the following information on the data sheet: sample number, date, total length size, location of capture, and other comments you think might be important.**
- **Place fin tissue in envelopes to dry as a backup if you run out of the EtOH tubes. Wait 24 hours to stack the envelopes so the tissue can dry quickly.**
- **Contact Josh Israel at UC Davis for more tubes or other questions.**  
**[jisrael@ucdavis.edu](mailto:jisrael@ucdavis.edu)**  
**(530)752-6351**

**Thanks for your assistance.**

## Papers and Presentations

- Israel, J.A., M. Blumberg, J. Cordes and B. May. 2004 "Geographic patterns of genetic differentiation among western U.S. collections of North American green sturgeon (*Acipenser medirostris*)". *North American Journal of Fisheries Management*, In press.

**Abstract.** The population structure of green sturgeon (*Acipenser medirostris*) has not previously been evaluated, although commercial and bycatch harvests may be impacting multiple populations of this species. Molecular markers were used to distinguish between green and white sturgeon and to examine the genetic variation among green sturgeon collected from four locations along the west coast of the United States between San Pablo Bay, CA and the Columbia River, WA. Verification that putative green sturgeon samples were not white sturgeon was accomplished using a diagnostic restriction fragment length polymorphism in the mitochondrial Cytochrome *b* locus. Genetic variation in green sturgeon was investigated using six microsatellite loci developed from published primers. Allele frequencies,  $F_{ST}$ , and observed heterozygosities were determined at three disomic and three tetrasomic loci amplified from fin tissue samples. Pairwise  $F_{ST}$  values of 0.005 and -0.002 indicated no genetic differentiation between the Klamath and Rogue Rivers or the Columbia River and San Pablo Bay collections, respectively. All other  $F_{ST}$  values among pairs of collections ranged between 0.06 and 0.08 and indicated a significant difference between collections from the Rogue River and Klamath River compared to the San Pablo Bay and Columbia River collections. These results and highly significant *G*-tests of genotypic differences between these pairs of collections support the existence of genetic structuring in this species along the Pacific coast. These data suggest that not all spawning populations have been identified and additional genetic research will be necessary to adequately describe the population structure of green sturgeon before effective management plans can be developed.

- Israel, J.A. and B.P. May. 2003. "The origins of San Pablo Bay green sturgeon: Where are they from?" Poster, State of the Bay Conference, Oakland, CA.

The origin of green sturgeon (*Acipenser medirostris*) that congregate in San Pablo Bay and the Delta during the summer and early fall remains unidentified. Our recent genetic analysis has shown significant population differentiation between fish from San Pablo Bay and samples from Klamath and Rogue River spawning adults. Green sturgeon have inhabited Pacific coastal marine waters for about 2 millions years, although reproducing populations are only found on the Sacramento, Klamath and Rogue Rivers. As home to a southern green sturgeon population, the Sacramento River plays an unknown, but potentially important role in the evolution of population structure in green sturgeon. Physical and biological differences between the Sacramento River and the Klamath and Rogue Rivers may explain the unusual observed pattern of population differentiation.

Considerable gaps exist in manager's knowledge of green sturgeon migration, although generally some number occupy coastal estuaries between San Pablo Bay, CA and the

Fraser River, BC in the summer. Genetic analysis determined that green sturgeon collected in the Columbia River estuary were distinct from reproducing fish in the Klamath and Rogue Rivers, yet not different than San Pablo Bay samples. While uncommonly taken in commercial and sports fisheries in Californian waters, green sturgeon are captured in larger numbers in Oregon and Washington's coastal estuaries. If the Sacramento River is the origin of San Pablo Bay green sturgeon, a closer examination of out-of-state harvest impacts will be critical for adequately managing a discrete southern population of this species. Understanding the origin of green sturgeon from San Pablo Bay will provide a basis for evaluating the relationships and possible admixture of stocks within Pacific Northwest coastal estuaries.

- Israel, J.A. and B.P. May. 2003. "The origins of San Pablo Bay green sturgeon: Where are they from?" Invited Presentation, Red Bluff Fish and Wildlife Office, Red Bluff CA.
- Israel, J.A. and B.P. May. January. 2002. "Application of genetics markers for species identification and population structure analysis of green sturgeon (*Acipenser medirostris*)." Presentation, CALFED Science Conference, Sacramento CA.

Green sturgeon (*Acipenser medirostris*) are a long-lived, anadromous species, with known breeding populations in the Klamath, Sacramento, Rogue, and Umpqua Rivers, although other populations may still exist. Currently, juvenile green sturgeon are found in many bays between Monterey, CA and the Fraser River, British Columbia. A sportfishery exists for green sturgeon in the Columbia River estuary and off the coast of Oregon.

The application of a PCR-RFLP marker for the identification of green and white sturgeon allowed accurate determination of species from putative *A. medirostris* eggs and larvae. Microsatellite DNA markers from other *Acipenser* species were optimized to analyze genetic variation in samples from the Klamath, Sacramento, Rogue and Columbia Rivers. Both disomic and tetrasomic loci were used in the analysis. Allele frequencies were evaluated to determine stock structure among component populations. Differences between the Columbia River sportfishery and Klamath River breeding stock suggest that *A. medirostris* caught in the Columbia constitute a mixed stock. Future work will focus on using microsatellite markers for mixed stock analysis of ocean fisheries, providing information for the management of the species. These results are crucial to the debate over the listing of the green sturgeon.

- Israel, J.A. and B.P. May. January. 2002. "Relationships of Green Sturgeon (*Acipenser medirostris*) from the Rogue and Klamath Rivers: Identifying Distinct Populations." Presentation, Yurok Tribal Council, Eureka CA.





1 **Abstract.** The population structure of green sturgeon (*Acipenser medirostris*) has not previously  
2 been evaluated, although commercial and bycatch harvests may be impacting multiple  
3 populations of this species. Molecular markers were used to distinguish between green and  
4 white sturgeon and to examine the genetic variation among green sturgeon collected from four  
5 locations along the west coast of the United States between San Pablo Bay, CA and the  
6 Columbia River, WA. Verification that putative green sturgeon samples were not white sturgeon  
7 was accomplished using a diagnostic restriction fragment length polymorphism in the  
8 mitochondrial Cytochrome *b* locus. Genetic variation in green sturgeon was investigated using  
9 six microsatellite loci developed from published primers. Allele frequencies,  $F_{ST}$ , and observed  
10 heterozygosities were determined at three disomic and three tetrasomic loci amplified from fin  
11 tissue samples. Pairwise  $F_{ST}$  values of 0.005 and -0.002 indicated no genetic differentiation  
12 between the Klamath and Rogue Rivers or the Columbia River and San Pablo Bay collections,  
13 respectively. All other  $F_{ST}$  values among pairs of collections ranged between 0.06 and 0.08 and  
14 indicated a significant difference between collections from the Rogue River and Klamath River  
15 compared to the San Pablo Bay and Columbia River collections. These results and highly  
16 significant *G*-tests of genotypic differences between these pairs of collections support the  
17 existence of genetic structuring in this species along the Pacific coast. These data suggest that  
18 not all spawning populations have been identified and additional genetic research will be

- 1 necessary to adequately describe the population structure of green sturgeon before effective
- 2 management plans can be developed.

1           Green sturgeons (*Acipenser medirostris*) are a long-lived, anadromous Acipenseridae  
2 inhabiting the Pacific Ocean between the Aleutian Islands, Alaska and northern Baja California,  
3 Mexico (Moyle 2001, Figure 1). Little is known about green sturgeon life history (Emmett et al.  
4 1991). Juveniles are believed to migrate out to sea after spending one to three years in freshwater  
5 (Nakamoto et al. 1995). During this time they are believed to feed on benthic invertebrates  
6 (Moyle 2001) and exhibit nocturnal behavior (Cech et al. 2000). The length of time juvenile  
7 green sturgeons remain in coastal estuaries is unknown. During the late summer and early fall,  
8 subadult and non-spawning adult green sturgeon concentrate in Pacific coastal estuaries north of  
9 San Francisco Bay (Emmett et al. 1991), although the reason for this behavior remains unknown.  
10 Adult green sturgeon are uncommon in the San Francisco Bay system (Ayles 1854; Schaffter  
11 and Kolhorst 1999), and are infrequently encountered in freshwater on the Fraser and Skeena  
12 Rivers of British Columbia (Scott and Crossman 1973). They appear to be the most common  
13 sturgeon in Willapa Bay, WA (Emmett et al. 1991). Columbia River green sturgeon concentrate  
14 in the estuary, and have been observed up to Bonneville Dam though no evidence exists for  
15 spawning in this system (Rien et al. 2000). The population of origin for these fish remains  
16 unknown. Miller (1971) tagged 54 San Pablo Bay green sturgeon in 1967-1968, and 3 of 5  
17 recovered tags occurred in coastal estuaries, including two at the mouth of the Columbia River  
18 and one in Gray's Harbor, WA. Also, the recovery of a San Pablo Bay tagged green sturgeon in

1 Willapa Bay, WA this past summer (Matt Howell, WDFW, personal communication) supports a  
2 migratory connection between northern and southern green sturgeon aggregations.

3 Known green sturgeon spawning is limited to the Sacramento and Klamath Rivers in  
4 California and the Rogue River in Oregon (Moyle 2001). Spawning populations are believed  
5 extirpated on the Eel, South Fork Trinity, and San Joaquin Rivers in California (Moyle 2001).  
6 Adults are believed to spawn every three to five years and reside in marine water between  
7 spawning runs (Adams et al. 2002) that occur during the summer. Estimated ages of  
8 reproductive female green sturgeon from the Klamath River ranged from 25 to 32, while male  
9 green sturgeon used in the same artificial spawning ranged from 18 to 30 (Van Eenennaam et al.  
10 2001).

11 In Canada, green sturgeon are recognized as rare by the Committee on the Status of  
12 Endangered Wildlife (Houston 1988), and were recently listed as a candidate species under the  
13 United States Endangered Species Act by the National Marine Fisheries Service (NMFS). A  
14 substantial decline in the total annual catch of these fish from estuaries and the ocean fishery has  
15 been observed over the last fifteen years due to regulatory changes, yet the potential for  
16 differential impact on multiple populations due to harvesting has not been evaluated (Adams et  
17 al. 2002). Sturgeons are known to have strong homing capabilities and spawning site fidelity  
18 (Bemis and Kynard 1997), but the natal rivers of non-spawning green sturgeon aggregations  
19 remain unknown.

1 No prior studies have evaluated the potential for stock structure in green sturgeon,  
2 although molecular analyses have demonstrated population structure exists in other North  
3 American sturgeons including lake sturgeon *A. fulvescens* (McQuown et al. 2002), Atlantic  
4 sturgeon *A. oxyrinchus* (King et al. 2001), and white sturgeon *A. transmontanus* (Smith et al.  
5 2002). Clearly, insight into potential population structure in green sturgeon is necessary for  
6 formulation of adequate management strategies. Unfortunately, work has been hindered by the  
7 inability to distinguish green sturgeon from the sympatric white sturgeon at all life history stages  
8 and a limited knowledge about green sturgeon spawning habitat and migration patterns. This  
9 study was undertaken with two main objectives in mind. The first was to develop an  
10 interspecific molecular marker that differentiated green and white sturgeons at all life history  
11 stages. The second was to test the hypothesis that non-spawning aggregations and spawning  
12 populations of green sturgeon comprise a single reproductive population. To test this hypothesis  
13 we used microsatellite DNA markers to investigate genetic differentiation among samples  
14 collected from two non-spawning aggregations (San Pablo Bay and Columbia River) and two of  
15 the three known spawning populations (Klamath and Rogue Rivers) of green sturgeon.

16

17

## Methods

18

19

**Samples.** Green sturgeon samples were collected from four locations along the western coast of the United States (Figure 1). Throughout the paper, the term “sample” is used to

1 identify a tissue sample collected from a single individual green sturgeon. The word “collection”  
2 is reserved for describing a group of samples that were are taken from the same place over the  
3 same period of time. The term “population” describes green sturgeon collections containing  
4 reproductively mature samples and are from rivers with known spawning activity. Adult fish  
5 were captured in freshwater from the Klamath and Rogue River populations. Table 1 contains  
6 collection location, collecting organization, year of collection, collection size, and life history  
7 stage of green sturgeon samples included in this analysis. Tissue samples included dry fin clips  
8 and wet tissue samples preserved in 95% ethanol stored at room temperature.

9 ***DNA Extraction.*** Samples from the Klamath and Columbia Rivers were extracted using  
10 a standard phenol-chloroform procedure (Sambrook et al. 1989). DNA from the Rogue River  
11 and San Pablo Bay were isolated using a Qiagen DNeasy™ tissue kit. Two µL of each sample  
12 were evaluated for degradation by electrophoresis on a 0.7% agarose gel for 50 minutes at 100V.  
13 Gels were fluorescently labeled with 1% Gelstar (BioWhitaker Molecular Applications,  
14 Rockland, ME) and visualized on a Molecular Dynamics 595 Fluorimager.

15 ***Species Identification Study.*** A restriction map was created using DNA Club (Molecular  
16 Biology Shortcuts, www.justbio.com) from Cytochrome *b* sequences in green and white  
17 sturgeon (Birstein and DeSalle 1997) and examined for restriction site polymorphisms between  
18 the two species. Green and white sturgeon samples from multiple locations were PCR-amplified  
19 with primers H15149 (Kocher et al. 1989) and L14724 (Meyer et al. 1990). Each reaction

1 mixture contained 2.0 $\mu$ L DNA (~15ng/ $\mu$ L), 2 $\mu$ L of Promega 10x buffer (Madison, WI), 2 $\mu$ L  
2 each of forward and reverse primer (10 $\mu$ M), 1.6 $\mu$ L of MgCl (25mM), 2 $\mu$ L of dNTPs(2.5mM)  
3 and 0.08 $\mu$ L of Promega Taq polymerase. Filtered, deionized water was added to each mixture to  
4 bring the total volume to 20 $\mu$ L. Following PCR (Table 2), 10  $\mu$ L of product were digested at  
5 40°C for six hours with 0.1 $\mu$ L Ssp1 restriction enzyme (New England BioLabs, Beverly, MA),  
6 1.2  $\mu$ L 10X buffer, and 0.7  $\mu$ L dH<sub>2</sub>O. Both undigested and digested PCR samples were run on a  
7 5% acrylamide denaturing gel for 1.5 hours at 35W. Gels were stained with a Fluorescein-  
8 Agarose overlay (Rodzen et al. 1998) and visualized with a Molecular Dynamics 595  
9 Fluorimager.

10 ***Microsatellite Population Structure Study.*** Samples from at least two of the  
11 geographically distinct green sturgeon collections were amplified with a touchdown PCR  
12 protocol (Table 2) using a total of 86 microsatellite primer pairs originally developed in  
13 shovelnose sturgeon (McQuown et al. 2000), lake sturgeon (May et al. 1997; Welsh et al 2003),  
14 or Atlantic sturgeon (King et al. 2001; T.L. King, U.S. Geological Survey, personal  
15 communication). Each reaction mixture contained 4.8  $\mu$ L filtered, deionized H<sub>2</sub>O, 1.0 $\mu$ L DNA  
16 (~15ng/ $\mu$ L), 1 $\mu$ L of Promega 10x buffer, 1 $\mu$ L each of forward and reverse primer (10 $\mu$ M),  
17 0.3 $\mu$ L of MgCl (25mM), 0.8 $\mu$ L of dNTPs (2.5mM) and 0.075 $\mu$ L of Promega Taq polymerase.  
18 Of the 86 primer pairs attempted, four promising systems [*Aox* 27 (King et al. 2001), *AfuG* 43  
19 and *AfuG* 135 (Welsh et al. 2003), and *Sp1* 106 (McQuown et al. 2000)] were selected that were

1 readily amplifiable, did not amplify multiple loci or difficult to interpret stutter bands, and did  
2 not contain null alleles. To increase the number of usable markers, primers were redesigned to  
3 increase sequence specificity in two [*Spl* 120 and *Spl* 101 (McQuown et al. 2000)] additional  
4 promising loci. Bands were cut out and sequenced (Davis Sequencing, Davis, CA), sequences  
5 were compared for homology, and primers were designed for distinct locus sequences. A  
6 modified forward primer (5'-TTA AAG AGG ATT GAA TAG CCT AAT-3') was used with the  
7 *Spl* 120 reverse primer (McQuown et al. 2000) to amplify a disomic locus. The original *Spl* 101  
8 locus (McQuown et al. 2000) was modified with primers of the following sequences: forward  
9 (5'-GGA AAT TTG ACA AAT CAC ACC C-3') and reverse (5'-AAA GCA CAT CAG TTA  
10 AAA GGT CAA-3') to yield a scorable locus. These loci were renamed *Spl* 120b and *Spl* 101b,  
11 respectively, for this study. PCR conditions and mixtures were optimized for each microsatellite  
12 system (Table 2). *Spl* 106 did not require modification of the PCR reaction mixture which  
13 contained 4.7  $\mu$ L filtered, deionized H<sub>2</sub>O, 1.0  $\mu$ L DNA (~15ng/ $\mu$ L), 0.8  $\mu$ L of Promega 10x buffer,  
14 1  $\mu$ L each of forward and reverse primer (10  $\mu$ M), 0.5  $\mu$ L of MgCl (25mM), 0.8  $\mu$ L of  
15 dNTPs(2.5mM) and 0.1  $\mu$ L of Promega Taq polymerase. PCR reaction mixtures with Faststart™  
16 Taq polymerase (Roche, Indianapolis, IN) contained 4.8  $\mu$ L filtered, deionized H<sub>2</sub>O, 1.0  $\mu$ L DNA  
17 (~15ng/ $\mu$ L), 1.0  $\mu$ L of Roche 10x buffer with MgCl, 1  $\mu$ L each of forward and reverse primer  
18 (10  $\mu$ M), 1.0  $\mu$ L of dNTPs(2.5mM) and 0.1  $\mu$ L of Faststart™ Taq polymerase. PCR products  
19 were run on a 5% acrylamide denaturing gel for 50 minutes to 1.5 hours at 50 W. The gels were

1 stained with a Fluorescein-Agarose overlay (Rodzen et al. 1998) and scanned with a Molecular  
2 Dynamics 595 FluorImager. Allele sizes were designated using FragmeNT Analysis 1.1  
3 (Molecular Dynamics, Sunnyvale, CA) with a 400 basepair (bp) commercial ladder (The Gel  
4 Company, San Francisco, CA) and an allelic ladder.

5 *Statistical Analyses.* Allele and genotype frequencies, as well as genotypic  
6 disequilibrium at the three disomic loci, were calculated using the program GENEPOP 3.1  
7 (Raymond & Rousset 1995). Significance of observed genotypic differentiation was tested with  
8 an unbiased estimate of the  $P$ -value of a log-likelihood ( $G$ ) based exact test (Goudet 1996).  
9 Observed genotype frequencies were tested for deviation of each collection from Hardy-  
10 Weinberg expectations for the three disomic loci using Genes in Populations (Program designed  
11 by B. May and C. Krueger, written in C by W. Eng and E. Paul, unpublished). Significance was  
12 determined by log-likelihood  $G$ -test and chi square test ( $P < 0.05$ ) (Sokal and Rohlf 1981).  
13 Observed heterozygosities were found for data from three loci displaying disomic banding  
14 patterns. The number of alleles sampled and pairwise  $F_{ST}$  tests for differentiation were  
15 computed with FSTAT (Goudet 2001). Pairwise  $F_{ST}$  values were evaluated for significance  
16 using the log-likelihood  $G$ -test (Goudet et al. 1996). This test statistic evaluated significance  
17 based on the  $P$ -value of all loci estimated with a standard Bonferroni correction (Rice 1989)  
18 following 120 permutations.

19

## Results

1  
2       ***Species Identification Study.*** A 467 bp fragment was amplified in the Cytochrome *b*  
3 locus of putative green sturgeon. Using DNA Club, the restriction enzyme *Ssp1* was determined  
4 to distinguish between green and white sturgeon by producing a single unique cut in green  
5 sturgeon mtDNA that resulted in two fragments of 296 bp and 195 bp. This marker was used to  
6 confirm the identity of putative green sturgeon in the collections. One white sturgeon individual  
7 in the San Pablo Bay collection and two in the Rogue River collection were identified and  
8 eliminated from the remainder of the study.

9       ***Microsatellite Population Structure Study.*** Three of the microsatellite loci (*AfuG* 43, *Spl* 101b,  
10 and *Spl* 106) used in this study displayed banding patterns characteristic of tetrasomic loci. The  
11 remaining three loci *Spl* 120b, *AfuG* 135, and *Aox* 27 displayed disomic banding patterns. Table  
12 3 shows sample sizes (*n*), observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosities, and allele  
13 frequencies for the six microsatellite loci and four geographically distinct collections used in this  
14 study. Number of alleles per locus ranged from six (*Spl* 106 and *Aox* 27) to 20 (*Spl* 101b), and  
15 heterozygosities from three loci varied among collections from 0.564 (Rogue River) to 0.734  
16 (Klamath and Columbia Rivers). The three disomic loci met Hardy-Weinberg expectations in all  
17 collections at all loci except for the Columbia River collection, which had an excess of  
18 heterozygotes at the *AfuG* 135 locus. No significant linkage disequilibrium was detected among  
19 three disomic loci in each of the four collections or across loci. The Klamath collection had the

1 greatest number of alleles (53) at all loci while the Columbia collection showed the lowest  
2 overall number of alleles (n=44). Of the 65 alleles observed, 27 occurred at frequencies of  
3  $\leq 0.05$  in all collections. *Aox* 27 showed the least variability with the most common alleles  
4 ranging in frequency from 0.43-0.75. *Spl* 1101 displayed the greatest variability with the most  
5 common allele ranging in frequency from 0.28 to 0.35.

6 *G*-tests of genotypic differentiation among collections indicated highly significant  
7 ( $P \leq 0.0001$ ) differences at all six loci between a group containing the Klamath and Rogue Rivers  
8 samples when compared to the collections from San Pablo Bay and the Columbia River.  
9 Likewise, significant pairwise  $F_{ST}$  differences ( $P \leq 0.008$ ) existed when comparing the Klamath  
10 and Rogue River collections to the collections from the Columbia River and San Pablo Bay  
11 (Table 4). Significant differences were not detected between the Klamath and Rogue River  
12 collections or the Columbia River and San Pablo Bay collections.

13

14

#### Discussion

15 Variation at six microsatellite loci showed significant differentiation between green  
16 sturgeon collections along the west coast of the United States. Pairwise  $F_{ST}$  values showed a  
17 moderate level of differentiation between collections from the Columbia River and San Pablo  
18 Bay in comparison to populations from the Klamath and Rogue Rivers. These data also  
19 indicated a highly significant difference in genotype frequencies between the San Pablo Bay and

1 Columbia River collections compared to the Klamath and Rogue River populations. The  
2 analysis does not support the hypothesis that the San Pablo Bay and Columbia River collections  
3 are derived from the Klamath and Rogue River populations. The lack of significant linkage  
4 disequilibrium and Hardy-Weinberg equilibrium being observed in the San Pablo Bay collection  
5 suggests that this group of samples is not a mixed stock. Linkage disequilibrium due to an  
6 excess of heterozygotes at one of three loci in the Columbia River collection suggests these  
7 samples may represent a mixed stock not solely derived from the same population as San Pablo  
8 Bay. These data indicate the likely existence of one or more additional spawning populations  
9 responsible for this aggregation. Tag and recapture data support the genetic data presented that  
10 green sturgeons from the Columbia River and San Pablo Bay are not different. Also, recapture  
11 data support the genetic distinctiveness of Klamath or Rogue River green sturgeons from these  
12 estuarine aggregations since no tag recoveries have indicated exchange of fish between these  
13 locations. Since samples of juvenile or adult green sturgeon from freshwater on the Sacramento  
14 River were not analyzed, the unique variation observed in the San Pablo Bay and Columbia  
15 River collections cannot be attributed to the Sacramento River spawning stock. Our results  
16 support the existence of distinct populations of green sturgeon, but do not offer clear insight into  
17 reasons for the observed variation.

18 Multiple factors may potentially contribute to the observed differences between the  
19 Klamath and Rogue River populations compared to the collections from San Pablo Bay and the

1 Columbia River. Multiple studies have confirmed sturgeon's fidelity to spawning sites (Hatin et  
2 al.2002, Hildebrand et al. 1999, Auer 1996). These studies, as well as limited information on  
3 green sturgeon (Erickson et al. 2002, Kurt Brown, CDFG, personal communication), suggest  
4 green sturgeon spawning habitat is distinctive within the river systems they use for spawning.  
5 Because of the distances between river mouths supporting Sacramento River and Klamath  
6 Mountains Province reproducing populations, the genetic differentiation observed between  
7 collections may be caused by geographic isolation of populations. This explanation is also  
8 supported by the proximity and non-differentiation between the Rogue and Klamath River  
9 populations.

---

10 Reproductive populations of green sturgeon may represent distinct ecotypes. The  
11 Klamath and Rogue Rivers drain coastal mountains of the Pacific mid-coastal ecoregion (Abell  
12 et al. 2000), while the Sacramento River empties interior tributaries of the Sierra Nevada through  
13 the Central Valley ecoregion (Abell et al. 2000, Omernik 1986). Artyukhin and Andronov  
14 (1990) suggested that green sturgeon evolved in association with "reproduction in fast-flowing  
15 small rivers with a relatively short lowland reach and with spawning grounds quite close to the  
16 river mouth." This qualitatively describes the Klamath and Rogue Rivers when compared to the  
17 Sacramento River. On the Klamath and Rogue Rivers, habitats in a constrained channel with a  
18 bedrock or large cobble substrate and swift velocities that are likely preferred for green sturgeon  
19 spawning occur throughout the lower 100 miles of these rivers. However, on the Sacramento

1 River, this type of habitat is limited throughout the entire system and spawning adults must  
2 ascend more than 200 miles of river to find small reaches of this type of habitat close to Red  
3 Bluff, CA. The Klamath and Rogue rivers have been noted for their high productivity  
4 (Erickson et al 2002, Kesner and Barnhardt 1972), and this factor contributed to the presence of  
5 the unique "half-pounder" life history in steelhead (Kesner and Barnhardt 1972) found in these  
6 rivers. For this reason, juvenile green sturgeon may be rearing in freshwater portions of the  
7 Klamath and Rogue Rivers, instead of their small estuaries (769 and 627 acres, respectively).  
8 Conversely, juvenile green sturgeon from the Sacramento River may be using the highly  
9 productive San Francisco estuary as a nursery grounds. The San Francisco estuary (of which San  
10 Pablo Bay is a part) is 400 square miles and the Columbia River estuary is 101,750 acres; and  
11 both are characterized by extensive shallow-water habitats. Age-to-growth curves are different  
12 between green sturgeon from the Klamath River and San Pablo Bay (Adams et al. 2002) and  
13 may signify a difference in growth that may be influenced by feeding or migratory behavior. If  
14 juvenile green sturgeon become specialists for resources distinct to their natal drainages, their  
15 migration patterns may reflect a desire to find similar sources of food. This may contribute to  
16 the lack of differentiation between green sturgeon samples collected from the Columbia River  
17 and San Pablo Bay.

18           Green sturgeon of Klamath and Rogue River origin may undertake a migratory pattern  
19 different from those in San Pablo Bay and the Columbia River. The potential for green sturgeon

1 to learn migrational patterns may play a role in explaining the observed nonclinal structuring of  
2 the collections. Green sturgeon longevity, seasonal aggregation in estuaries, and electrosensory  
3 capacity may support behavior that would allow informational transfer between different age  
4 groups. Lucas and Baras (2001) suggested informational transfer to be important for spatial  
5 mapping and learning of migration routes, particularly if the knowledge of an older individual  
6 may influence foraging movements. If green sturgeon follow learned migratory patterns, as  
7 exhibited by other fish species (Lucas and Baras 2001), it would support the idea that subadult  
8 and adult green sturgeon in the Columbia River are primarily from a reproductive population  
9 common to the San Pablo Bay samples. If learned behavior is an important factor in explaining  
10 the observed structuring of green sturgeon populations, managers have yet to identify the  
11 migration pattern of green sturgeon from the Klamath and Rogue Rivers.

12 Geological events in the Klamath and Rogue Rivers may have eliminated free passage to  
13 spawning habitats in these coastal basins and contributed to the differentiation of reproductive  
14 populations. As recently as the 1980's, green sturgeon faced migration obstacles that limited  
15 access at low flows beyond a debris slide near Coon Creek Falls on the lower Klamath River  
16 (Adair et al. 1985). If ancient or unrecorded barriers existed for long periods, the Sacramento  
17 River may have constituted the single spawning population. As green sturgeon recolonized  
18 unoccupied coastal drainages, they found habitat that favored high survival and rapid population  
19 growth from a small number of colonizers. Genetic drift from a small pool of founding

1 genotypes and gene flow between the newly established Klamath and Rogue River populations,  
2 because of their proximity, could explain the observed non-significant differentiation of these  
3 collections and their significant differentiation from the San Pablo Bay samples. The size of the  
4 Columbia River's mouth and estuary permitted accessibility from the ocean and likely kept the  
5 river open during periods when smaller coastal basins to the south were inaccessible (McPhail  
6 and Lindsay 1986), allowing migratory green sturgeon to concentrate in the early summer and  
7 late fall in an estuary similar to their natal system's estuary.

8         The recent decision by NMFS to recognize Northern and Southern green sturgeon distinct  
9 population segments is an important first step towards delineating green sturgeon stocks;  
10 however, the complexities of their migration and population structure are not adequately  
11 reflected in these initial delineations (NMFS 2003). These data suggest that detection of  
12 population structure is possible with a limited number of microsatellites. However, additional  
13 microsatellite loci should be used to complete a genetic stock identification program and  
14 quantify the potential contribution of distinct population segments to mixed stocks found in the  
15 ocean and estuaries. This would provide managers with information on migratory patterns and a  
16 more comprehensive understanding of the stock complexity of green sturgeon. Additional  
17 samples from the Sacramento River and other putative breeding locations will be required to  
18 understand the composition of green sturgeon aggregations like those found in San Pablo Bay  
19 and the Columbia River. The marker described here, which distinguishes green and white

1 sturgeon samples, will allow us to accurately assess the presence or absence of green sturgeon  
2 from larval samples and spawning activity in basins where both species are sympatric. This  
3 marker could also have forensic uses to verify if caviar or meat samples are from white or green  
4 sturgeon.

5         Currently, management of green sturgeon sportfisheries along the west coast are based on  
6 the status and population estimates of white sturgeon (B. James, Washington Department of Fish  
7 and Game; T. Rien, Oregon Department of Fish and Wildlife; and R. Schaffter, California  
8 Department of Fish and Game, personal communications). Until the status of green sturgeon  
9 spawning populations are better understood, fishing regulations should discriminate their harvest  
10 from that of white sturgeon. Development of additional microsatellite markers will allow for an  
11 evaluation of whether the distinct populations of green sturgeon have been impacted in the recent  
12 past by population bottlenecks, estimate effective population sizes of reproducing populations,  
13 and quantify estimates of gene flow between drainages. This information will be essential for  
14 understanding the biology of the green sturgeon, identifying the need for adaptive management  
15 to adequately protect the genetic diversity of these fish, and developing management plans that  
16 integrate genetic considerations into their formulation.

17

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1  
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1 Table 1. Green and white sturgeon samples used in this study.

Sturgeon species	Location	Collecting Agency	Year of collection	Samples used in analysis	Life History Stage
Green	San Pablo Bay	Calif. Department of Fish & Game	2001	46	Subadult, Adult
Green	Klamath River	Yurok Tribal Fisheries Program	1998	66	Adult
Green	Rogue River, OR	Oregon Department of Fish & Wildlife	2000	34	Adult
Green	Columbia Estuary	Washington Department of Fish & Wildlife	1995	32	NA
White	Sacramento River	Calif. Department of Fish & Game	1998	8	Subadult

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3

1 Table 2. Conditions of different polymerase chain reaction (PCR) protocols used for  
 2 amplification of microsatellite and RFLP loci used in this study. Modifications of the reaction  
 3 mixture for individual systems are listed below.

Protocols:

<u>Touchdown</u>	<u>Promega Taq</u>	<u>Faststart™ Taq</u>	<u>RFLP</u>
95°C 1min	95°C 1min30s	95°C 4min	94°C 1min30s
15 cycles of	35 cycles of	30 cycles of	35 cycles of
95°C 30s	95°C 1min	95°C 30s	95°C 30s
65°C 1min	56°C 45s	56°C 30s	52°C 30s
(-1°C/cycle)			
72°C 1min	72°C 2min	72°C 1min	72°C 1min
15 cycles of			
95°C 30s	72°C 5min	72°C 5min	72°C 5min
50°C 1min			
72°C 1min			

Modifications to above protocols for specific loci:

<u>Locus</u>				
<i>Aox 27</i>	1.25 mM MgCl	-	-	-
<i>AfuG 43</i>	-	1.75 mM MgCl, T <sub>A</sub> =59°C	-	-
<i>AfuG 135</i>	-	2.0 mM MgCl, T <sub>A</sub> =52°C	-	-
<i>Spl 101b</i>	-	T <sub>A</sub> =54°C	-	-
<i>Spl 120b</i>	-	-	2.0 mM MgCl	-

4

1 Table 3. Allele frequencies, sample sizes (n), and number of gene doses at six microsatellite loci  
 2 in four collections of green sturgeon. Observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosities are  
 3 given at the three microsatellite loci displaying disomic banding patterns. The number of gene  
 4 doses is in parenthesis under the locus name.

Locus	Alleles (bp)	Collections			
		Klamath	Rogue	San Pablo Bay	Columbia
<b><i>Spl 101b</i></b> (4 doses)	163	-	-	0.006	-
	167	-	-	0.051	0.056
	171	-	-	-	0.016
	175	0.005	0.016	-	0.008
	179	0.009	-	0.019	0.032
	183	0.186	0.172	0.038	0.04
	187	0.1	0.094	0.103	0.04
	191	0.282	0.328	0.353	0.315
	195	0.105	0.062	0.122	0.081
	199	0.114	0.141	0.09	0.161
	203	0.045	0.031	0.051	0.032
	207	0.091	0.086	-	0.024
	211	0.023	0.031	0.051	0.065
	215	0.027	0.031	0.038	0.048
	219	0.014	0.008	0.006	0.056
	223	-	-	0.013	-
	229	-	-	0.019	-
	231	-	-	0.026	0.008
	235	-	-	0.006	0.016
	239	-	-	0.006	-
	$H_o$	0.718	0.750	0.747	0.787
	$H_e$	0.840	0.820	0.832	0.820
	n	110	64	79	64
<b><i>Spl 106</i></b> (4 doses)	230	-	-	0.012	-

	234	0.076	0.098	0.018	0.023
	238	0.237	0.288	0.024	0.032
	240	0.004	0.030	0.024	-
	242	0.679	0.583	0.888	0.921
	246	0.004	-	0.035	0.024
	<b>n</b>	224	168	132	128
<b>Aox 27</b>					
(2 doses)	124	-	-	0.012	-
	132	0.473	0.439	0.679	0.758
	136	0.268	0.394	0.048	0.048
	140	0.205	0.121	0.262	0.194
	144	0.045	0.045	-	-
	160	0.009	-	-	-
	<b>H<sub>o</sub></b>	0.589	0.727	0.429	0.452
	<b>H<sub>e</sub></b>	0.660	0.635	0.469	0.386
	<b>n</b>	112	66	84	62
<b>AfuG 43</b>					
(4 doses)	192	0.035	0.029	0.011	-
	196	-	0.007	-	-
	204	0.395	0.309	0.344	0.375
	208	0.281	0.412	0.506	0.453
	212	0.096	0.041	0.078	0.055
	216	0.004	0.015	-	-
	220	0.066	0.059	0.044	0.07
	224	0.088	0.118	0.017	0.039
	228	0.035	0.008	-	0.008
	<b>n</b>	228	136	180	128
<b>AfuG 135</b>					
(2 doses)	220	-	0.015	0.013	0.018
	224	0.017	-	-	-
	228	0.095	0.258	0.488	0.614
	232	0.034	-	-	-
	236	0.069	0.03	-	-
	240	0.034	0.015	0.013	-
	244	0.095	0.121	-	-
	248	0.164	0.076	0.087	0.07
	252	0.25	0.152	0.188	0.175
	256	0.147	0.167	0.162	0.088

	260	0.06	0.121	0.013	-
	264	0.034	0.045	0.038	0.035
	<b>H<sub>o</sub></b>	0.897	0.909	0.625	0.500
	<b>H<sub>c</sub></b>	0.859	0.844	0.691	0.585
	<b>n</b>	116	66	80	57
<b><u>Sp/ 120b</u></b>					
(2 doses)	228	-	0.029	-	-
	232	0.017	0.029	-	0.031
	236	0.025	0.088	0.023	-
	240	0.042	0.015	0.307	0.344
	244	0.195	0.176	0.057	0.094
	248	0.042	0.015	0.034	0.016
	252	0.102	0.132	0.227	0.125
	256	0.314	0.309	0.284	0.266
	260	0.059	0.059	0.068	0.078
	264	0.136	0.132	-	0.031
	268	0.042	0.015	-	-
	272	0.017	-	-	0.016
	288	0.008	-	-	-
	<b>H<sub>o</sub></b>	0.864	0.765	0.750	0.781
	<b>H<sub>c</sub></b>	0.825	0.825	0.764	0.778
	<b>n</b>	118	68	88	64
<b>Average H<sub>c</sub></b>	0.734	0.734	0.597	0.734	
<b>Average H<sub>o</sub></b>	0.668	0.654	0.492	0.654	

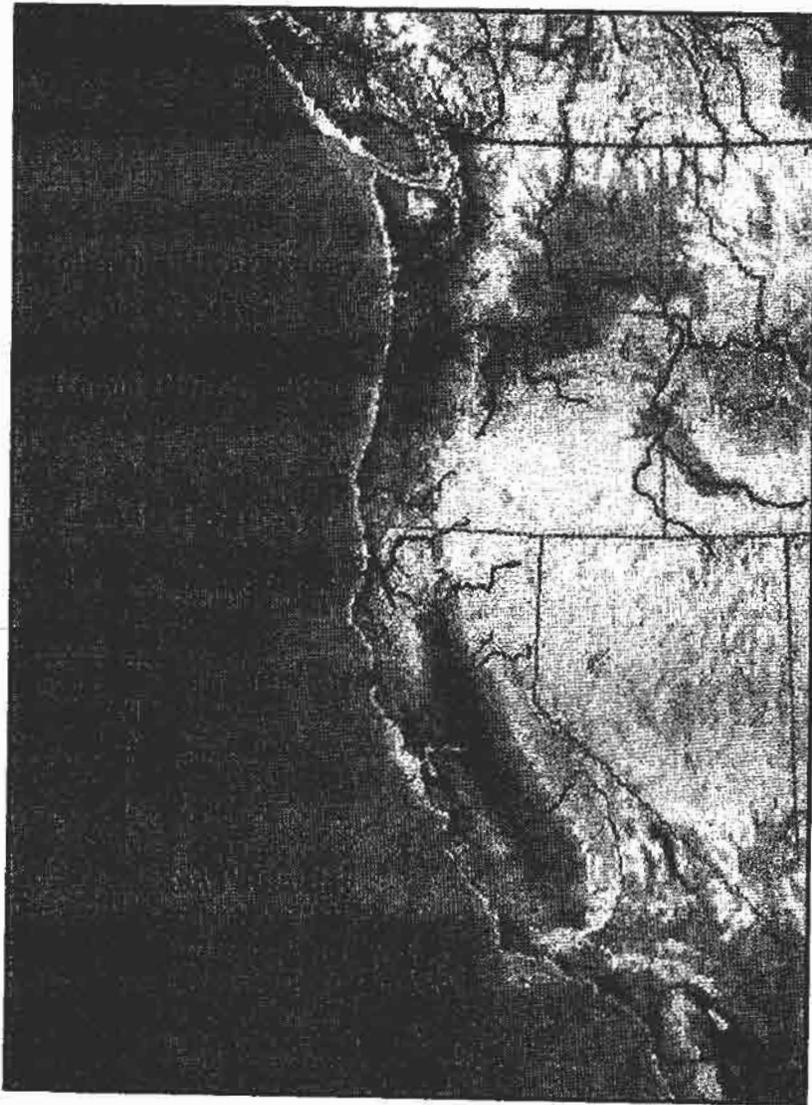
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- 1 Table 4. Population pairwise  $F_{ST}$  values for four collections of green sturgeon are given above  
 2 the diagonal; below are the associated  $P$ -values (Bonferroni adjusted,  $\alpha=0.008$ ). Significant  
 3 values are marked with an asterisk.

Population	Klamath	Columbia	San Pablo Bay	Rogue
Klamath		0.074	0.061	0.005
Columbia	0.008*		-0.002	0.078
San Pablo Bay	0.008*	0.992		0.065
Rogue	0.017	0.008*	0.008*	

- 1 Figure 1- Locations of collection sites of green sturgeon samples analyzed in this study. Green
- 2 sturgeon are believed to have been extirpated in italicized river systems (Moyle 2002).

1 Figure 1.  
2  
3





**Biological Assessment of Green Sturgeon in the Sacramento-San Joaquin  
Watershed, Phases 3-4**

Anadromous Fish Recovery Program, Agreement # 11332-1-G005

**Task 4, Telemetric Studies of Green Sturgeon in San Pablo Bay**

A. Peter Klimley, John T. Kelly, Carlos E. Crocker

The objectives of Task 4 were to determine the directions and rates of movement of adult/subadult green sturgeon (GS) in San Pablo Bay and the relative importance of temperature, salinity, and water current direction using ultrasonic telemetry. GS is a long-lived, iteroparous, anadromous, native fish that occurs in low numbers in the San Francisco Estuary. Adults spawn in freshwater rivers in Oregon and California including the Sacramento River (Moyle et al. 1995). Larvae develop within these freshwater systems, and remain in the estuaries for between one and four years before migrating to the ocean (Beamesderfer & Webb 2002). Adults move into estuaries in the spring, and sexually reproductive individuals continue into natal rivers in late spring/early summer to spawn. Post spawning adults return to the estuary, before migrating back to the ocean in late fall and early winter. Sub-adult fish also are thought to also enter estuaries during summer and fall months. Little is known about green sturgeon distribution within the San Francisco Estuary and what, if any, physical parameters (e.g. temperature, dissolved oxygen [DO], salinity) influence their movements. The goal of this project was to provide a greater understanding of these movements by means of tracking with ultrasonic telemetry.

GS were captured by trammel net in San Pablo Bay. Fish were surgically implanted with high power, depth sensing ultrasonic transmitters (Vemco V22XP) and released at the point of capture. The tagged fish were tracked using the manual "ground-zero" method described by Nelson (1987). Briefly, this method entails following the fish in a boat using a directional hydrophone to locate the tag signal. The boat is maneuvered as close to the fish as possible, and the location of the boat is recorded at regular intervals via a GPS-equipped laptop PC, which also decodes and stores the depth data telemetered by the tag. Only one fish can be tracked at a time in this method, so tracking efforts focused on that individual until it either could not be relocated or the tag died (expected tag life 10+ days). Track timing and duration were constrained by weather conditions on the bay. *Unsafe conditions* were common in the afternoon and evening, limiting tracking during these times. At hourly intervals during each track, the salinity, temperature, and DO profiles of the water column were sampled to a maximum depth of 25 m with a Hydrolab Surveyor II.

Five sub-adult and one adult GS (gender unknown) were tracked in the San Francisco Estuary for 2-16 hours a day over periods ranging from 1-12 days during the fall months of 2001 and 2002 (Fig. 1). Movements can be categorized as either directional or non-directional, with the latter observed more frequently, accounting for 60.8% of observations. Fish moving non-directionally (e.g., Fig. 1:GS6) were observed to remain on or near the bottom (Fig. 2), moving slowly or even not at all (Fig. 4) and making

frequent changes in direction (Fig. 5a). Directional movements (e.g. Fig. 1:GS5) were typified by continuous and active swimming (Fig. 4) near the surface (Fig. 3) while holding a steady course for many consecutive hours (Fig. 5b). The fish were typically observed to move in the same direction as the prevailing current, though swimming actively, perhaps engaging in selective tidal stream transport. A manuscript detailing orientation with respect to current is forthcoming. GS ranged widely across physical parameters, and were found at depths of between 0.7 m and in excess of 15 m, temperatures between 14.5-20.8 °C, salinities between 8.8-32.1 ppt, and DO contents between 6.5-9.0 mgO<sub>2</sub>/L. Activity appears to be independent of light level with no discernable crepuscular, nocturnal, or diurnal changes in movement. A manuscript describing green sturgeon movements and relationships to physical parameters is in preparation.

GS make use of much of the San Francisco Estuary, and occasionally make significant large scale movements throughout the region. At the start of the project, it was assumed that these movements would be influenced by physical parameters such as temperature, salinity, and DO with the fish seeking preferred conditions. However, the estuary is vertically well mixed and there are no apparent patterns to sturgeon preferences. When the conditions experienced by the fish at the time of each Hydrolab profile are compared to the mean values of the water column at that time, it is apparent that sturgeon movements are no different than would be expected if they were moving at random. There was no difference between fish and mean column values for temperature, salinity, dissolved oxygen.

GS clearly move for some reason, shifting from localized, non-directional "milling" to large scale directional movements. We do not know what the fish were doing while "milling" but foraging is suspected. Reasons for directional movements likely vary with both maturity of the fish and time of year. Movements by sub-adults (e.g. Fig. 1:GS3) and pre and post spawn adults may be related to ranging between foraging sites, while late season movements such as that exhibited by GS5 (Fig. 1) are likely the initiation of migratory behavior.

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## **Publications and Presentations**

Movement of green sturgeon in the Greater San Francisco Bay Region. Presentation to Yurok Tribal Council. Arcata, CA, January 2002.

Movement of green sturgeon in the Greater San Francisco Bay Region. Presentation to green sturgeon research planning meeting. Portland, OR, July 2003.

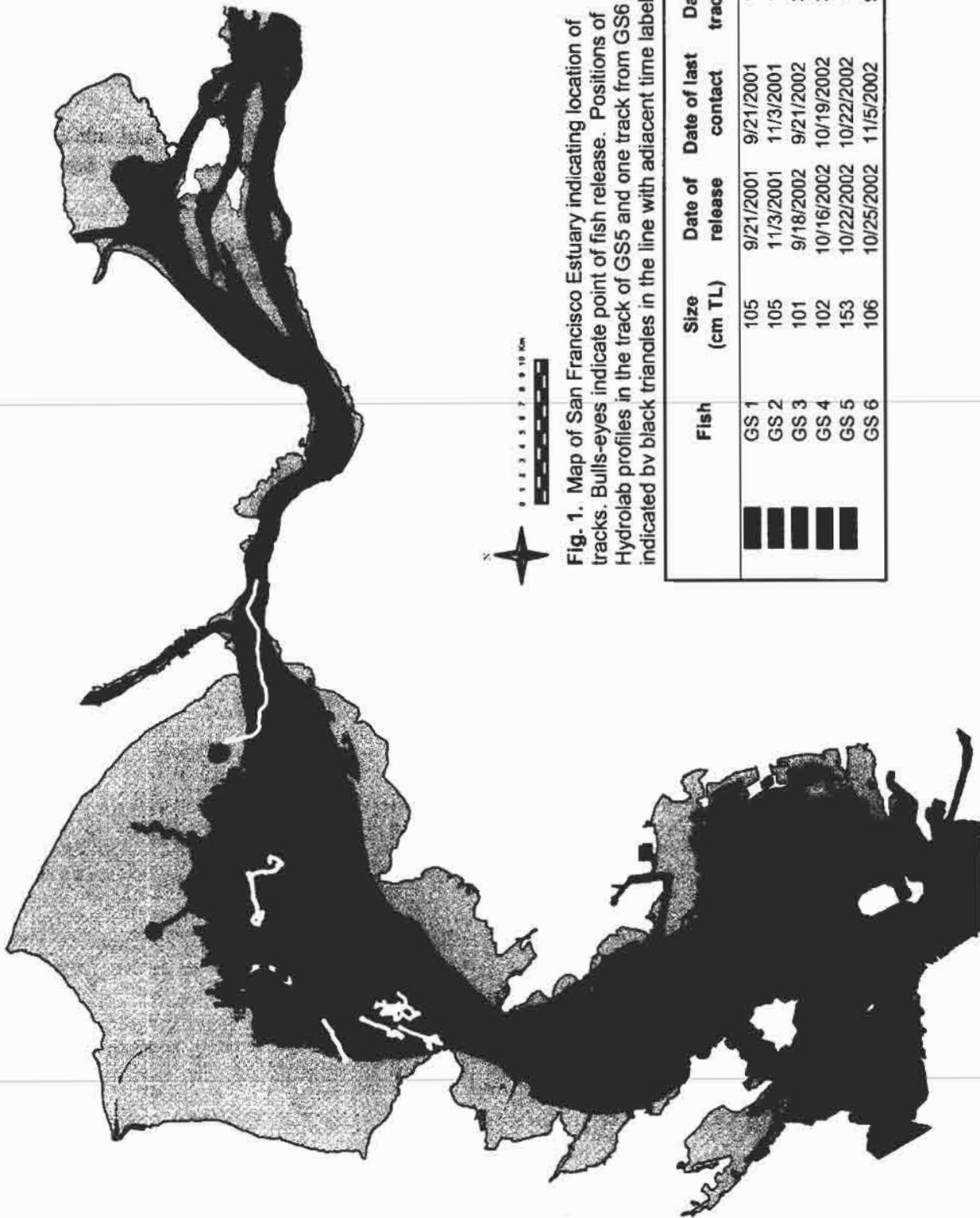
Kelly, J.T., A.P. Klimley, and C.E. Crocker. 2003. Movement of adult and sub-adult green sturgeon (*Acipenser medirostris*) in the San Francisco Estuary. 6th Biennial State of the Estuary Conference, Oakland, CA. Poster, Abstract.

Kelly, J.T., A.P. Klimley, and C.E. Crocker. In prep. Rheotropic orientation and transport of green sturgeon in San Francisco Bay. *Nature*.

Kelly, J.T., A.P. Klimley, and C.E. Crocker. In prep. Movements of green sturgeon in the San Francisco Estuary. *Environmental Biology of Fishes*.

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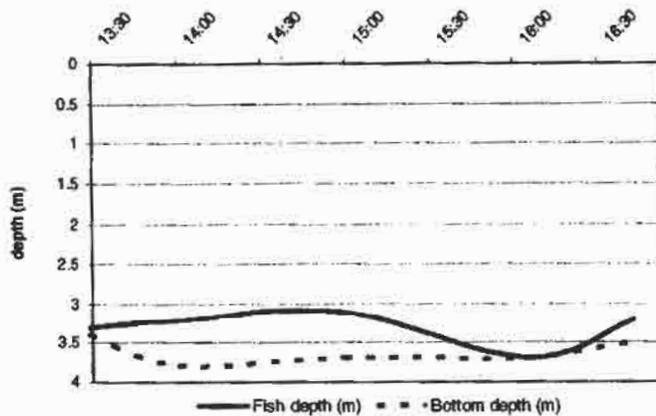
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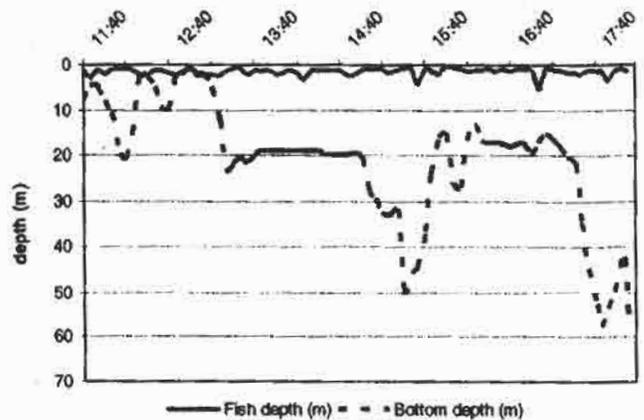
**Fig. 1.** Map of San Francisco Estuary indicating location of tracks. Bulls-eyes indicate point of fish release. Positions of Hydrolab profiles in the track of GS5 and one track from GS6 are indicated by black triangles in the line with adjacent time label.

Fish	Size (cm TL)	Date of release	Date of last contact	Days tracked
GS 1	105	9/21/2001	9/21/2001	1
GS 2	105	11/3/2001	11/3/2001	1
GS 3	101	9/18/2002	9/21/2002	3
GS 4	102	10/16/2002	10/19/2002	3
GS 5	153	10/22/2002	10/22/2002	1
GS 6	106	10/25/2002	11/5/2002	9

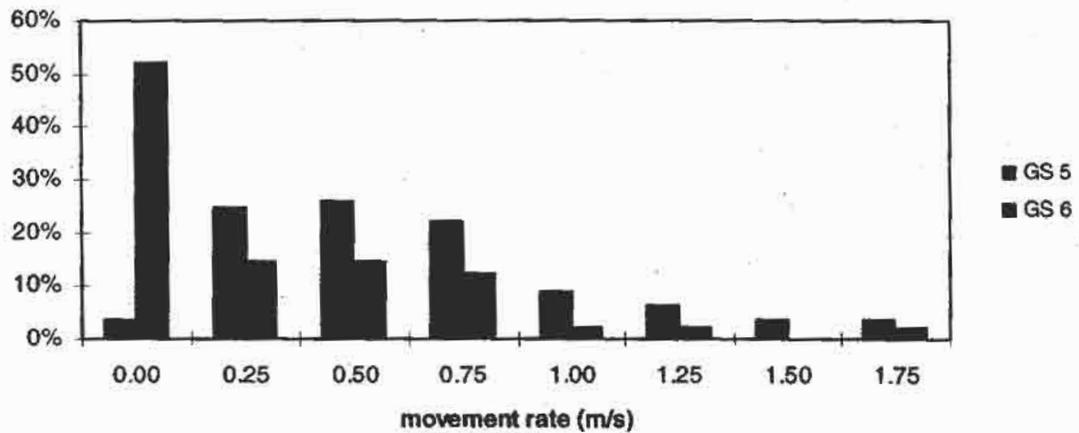
**Fig. 2.** Depth record for GS6 (10/28/02) illustrating the benthic orientation observed during non-directional swimming.



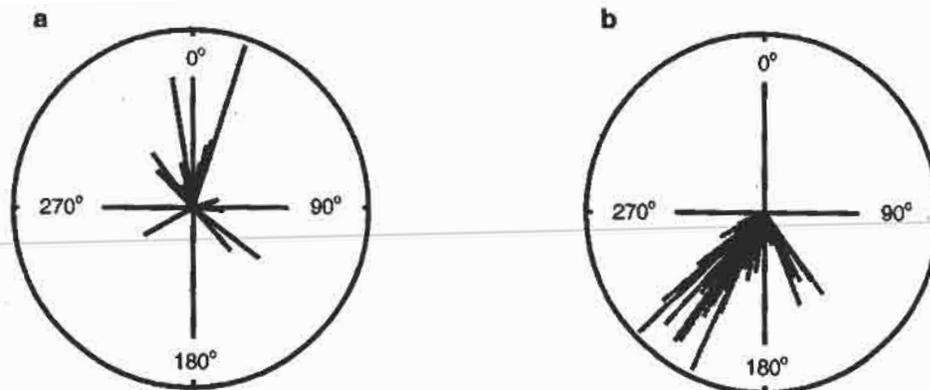
**Fig. 3.** Depth record for GS5 (10/22/02) illustrating surface oriented swimming observed during directed movement.



**Fig. 4.** Comparison of frequency of rates of movement (m/s) exhibited by GS6 (black bars) during a typical non-directional behavior and GS5 (gray bars) during directed movement.



**Fig. 5.** Vector distributions (5 min. intervals) of non-directional (a – GS6) and directional (b – GS5) movements.



## Introduction

The green sturgeon (*Acipenser medirostris*) is a long-lived, iteroparous, anadromous, native fish that occurs in low numbers in the San Francisco Estuary. Adults spawn in freshwater rivers in Oregon and California including the Sacramento River (Moyle et al. 1995). Larvae develop within these freshwater systems, and remain in the estuaries for between one and four years before migrating to the ocean (Beamesderfer & Webb 2002). Adults move into estuaries in the spring, and sexually reproductive individuals continue into natal rivers in late spring/early summer to spawn. Post spawning adults return to the estuary, before migrating back to the ocean in late fall and early winter. Sub-adult fish also are thought to also enter estuaries during summer and fall months.

Little is known about green sturgeon distribution within the San Francisco Estuary and what, if any, physical parameters (e.g. temp, dissolved oxygen (DO), salinity) influence their movements. The goal of this project was to provide a greater understanding of these movements by means of tracking with ultrasonic telemetry.

## Methods

Green sturgeon were captured by trammel net in San Pablo Bay. Fish were surgically implanted with high power, depth sensing ultrasonic transmitters (Vemco V22XP) and released at the point of capture. The tagged fish were tracked using the manual "ground-zero" method described by Nelson (1987). Briefly, this method entails following the fish in a boat using a directional hydrophone to locate the tag signal. The boat is maneuvered as close to the fish as possible, and the location of the boat is recorded at regular intervals via a GPS-equipped laptop PC, which also decodes and stores the depth data telemetered by the tag. Only one fish can be tracked at a time in this method, so tracking efforts focused on that individual until it either could not be relocated or the tag died (expected tag life 10+ days). Track timing and duration were constrained by weather conditions on the bay. Unsafe conditions were common in the afternoon and evening, limiting tracking during these times. At hourly intervals during each track, the salinity, temperature, and DO profiles of the water column were sampled to a maximum depth of 25 m with a Hydrolab Surveyor II.

## Results

Five sub-adult and one adult fish (gender unknown) were tracked in the San Francisco Estuary for 2-16 hours a day over periods ranging from 1-12 days during the fall months of 2001 and 2002 (Fig. 1). Movements can be categorized as either directional or non-directional, with the latter observed more frequently, accounting for 60.8% of observations. Fish moving non-directionally (e.g. Fig. 1:GS6) were observed to remain on or near the bottom (Fig. 2), moving slowly or even not at all (Fig. 4) and making frequent changes in direction (Fig. 5a). Directional movements (e.g. Fig. 1:GS5) were typified by continuous and active swimming (Fig. 4) near the surface (Fig. 3) while holding a steady course for many consecutive hours (Fig.5b). The fish were typically observed to move in the same direction as the prevailing current, though swimming actively, perhaps engaging in selective tidal stream transport. A manuscript detailing orientation with respect to current is forthcoming. Green sturgeon ranged widely across physical parameters, and were found at depths of between 0.7 m and in excess of 15 m,

temperatures between 14.5-20.8 °C, salinities between 8.8-32.1 ppt, and DO contents between 6.5-9.0 mgO<sub>2</sub>/L. Activity appears to be independent of light level with no discernable crepuscular, nocturnal, or diurnal changes in movement. A manuscript describing green sturgeon movements and relationships to physical parameters is in preparation.

### **Discussion**

Green sturgeon make use of much of the San Francisco Estuary, and occasionally make significant large scale movements throughout the region. At the start of the project, it was assumed that these movements would be influenced by physical parameters such as temperature, salinity, and DO with the fish seeking preferred conditions. However, the estuary is vertically well mixed and there are no apparent patterns to sturgeon preferences. When the conditions experienced by the fish at the time of each Hydrolab profile are compared to the mean values of the water column at that time, it is apparent that sturgeon movements are no different than would be expected if they were moving at random. There was no difference between fish and mean column values for temperature, salinity, dissolved oxygen.

Green sturgeon clearly move for some reason, shifting from localized, non-directional "milling" to large scale directional movements. We do not know what the fish were doing while "milling" but foraging is suspected. Reasons for directional movements likely vary with both maturity of the fish and time of year. Movements by sub-adults (e.g. Fig. 1:GS3) and pre and post spawn adults may be related to ranging between foraging sites, while late season movements such as that exhibited by GS5 (Fig. 1) are likely the initiation of migratory behavior.

### **Publications and Presentations**

Movement of green sturgeon in the Greater San Francisco Bay Region. Presentation to Yurok Tribal Council. Arcata, CA, January 2002.

Movement of green sturgeon in the Greater San Francisco Bay Region. Presentation to green sturgeon research planning meeting. Portland, OR, July 2003.

Kelly, J.T., A.P. Klimley, and C.E. Crocker. 2003. Movement of adult and sub-adult green sturgeon (*Acipenser medirostris*) in the San Francisco Estuary. 6th Biennial State of the Estuary Conference, Oakland, CA. Poster, Abstract.

Kelly, J.T., A.P. Klimley, and C.E. Crocker. In prep. Rheotropic orientation and transport of green sturgeon in San Francisco Bay. *Nature*.

Kelly, J.T., A.P. Klimley, and C.E. Crocker. In prep. Movements of green sturgeon in the San Francisco Estuary. *Environmental Biology of Fishes*.

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**Biological Assessment of Green Sturgeon in the Sacramento-San Joaquin  
Watershed, Phases 3-4**

Anadromous Fish Recovery Program, Agreement # 11332-1-G005

**Task 5, Quantitative Green Sturgeon Sampling in San Pablo Bay  
Nina Kogut, Ray Schaffter, Dave Kohlhorst**

The objectives of Task 5 were to assess the distribution and abundance of green sturgeon (GS) in San Pablo Bay, using trammel net samples, and provide GS for Tasks 3 and 4. Sampling took place during late-summer and autumn months. All GS were sampled for fin tissues (via fin clip) for Task 3 (genetic) studies, and some were tagged for Task 4 (tracking) studies. The following table is a summary of the numbers of legal-sized sturgeon caught.

<u>Year</u>	<u>Month</u>	<u># White Sturgeon</u>	<u># Green Sturgeon</u>
2001	August	206	23
	September	227	18
	October	543	5
2002	August	74*	5*
	September	119	3
	October	250	1
2003	(No sampling during 2003)		

\*Sampled only during second half of this month

