

Final Report for Project No. ERP-02-P35 (contract No. 4600002881)

**Selenium Effects on Health and Reproduction of White Sturgeon in the
Sacramento-San Joaquin Estuary**

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November 29, 2004

INTRODUCTION

Selenium (Se) is a potent environmental toxin that threatens higher trophic level species because of its efficient food web transfer (Lemly, 1985; Luoma et al., 1992). The San Francisco Bay-Delta (SFBD) receives Se in agricultural drainage from the Central Valley and in effluents from oil refineries. White sturgeon, *Acipenser transmontanus*, in San Francisco Bay are exposed to high levels of Se through their diet, evidenced by high selenium levels in common prey of white sturgeon (Johns et al, 1988; White et al, 1988; Linville et al., 2002), as well as in sturgeon muscle, liver and eggs (White et al., 1987; White et al., 1988; White et al., 1989; Kroll & Doroshov, 1991; Urquhart and Rigalado, 1991). Risebrough et al. (1977) reported concentrations of 8 to 11 $\mu\text{g/g}$ Se dry weight (dw) in transplanted bivalves in Northern San Francisco Bay. Johns et al. (1988) reported the Se concentration of $6 \pm 3 \mu\text{g/g}$ dw in resident bivalves *Corbicula spp.* sampled near Carquinez Strait. The Selenium Verification Study found Se concentrations of 5.13 – 7.90 $\mu\text{g/g}$ dw in *Corbicula spp.* from the North Bay between 1987 and 1990 (White et al., 1988; White et al., 1989; Urquhart and Rigalado, 1991). The filter feeding exotic bivalve *Potamocorbula amurensis* contains an average Se level of 15 $\mu\text{g/g}$ dw (Linville et al., 2002). This non-native species was introduced into San Francisco Bay in the mid-1980's and has since become the dominant bivalve in the bay (Carlton et al., 1990; Nichols et al., 1990) and a major food source of white sturgeon (Urquhart & Regalado, 1991). The high selenium level and wide distribution of this mollusk is of great concern because its selenium burden significantly exceeds the levels shown to cause toxicity in animals consuming bivalves. Prey items containing greater than 10 $\mu\text{g/g}$ Se have been shown to induce Se toxicity in birds and fish (Heinz et al, 1990; Hamilton et al, 1990; Adams et al, 1998; Lemly, 1998; Skorupa, 1998).

White sturgeon sampled from SFBD between 1986 and 1990 contained Se at concentrations ranging from 9 - 30 $\mu\text{g/g}$ dw in liver (n= 52) and 7 - 15 $\mu\text{g/g}$ dw in muscle (n= 99) (White et al, 1988; Urquhart and Regalado, 1991). Se concentrations in white sturgeon in 1990 reached levels previously linked to adverse effects in other fish (Gillespie & Baumann, 1986; Urquhart & Regalado, 1991; Lemly, 1993). Sturgeon are more prone to Se bioaccumulation, compared to other fish, because of their long lifespan and benthic feeding habits.

The unique reproductive biology of white sturgeon also increases the risk of transferring bioaccumulated Se to offspring. Sturgeon have a prolonged period (at least 2 years, Doroshov et al., 1997) of yolk deposition in their eggs, which is a suspected mechanism of Se maternal transfer to offspring (Kroll & Doroshov, 1991). Transfer and storage of Se in the egg yolk compromises the development and survival of embryos and yolk-sac larvae (Gillespie & Baumann, 1986; Woock et al., 1987; Schultz & Hermanutz 1990; Lemly, 1993) lacking efficient mechanisms and organs (gills, liver and kidney) for Se detoxification and excretion. The Se-enriched yolk causes toxicity, developmental defects and mortalities of embryos and yolk-sac larvae, and therefore may affect sturgeon recruitment.

To mitigate the Se pollution in SFBD, the California San Francisco Bay Regional Water Quality Control Board required oil refineries to reduce the amount of Se in effluents discharged into the Bay, however, refinery effluent continues to be a significant source of Se in this region (Luoma and Presser, 2000). The amount of Se entering the SFBD in the effluents from the San Joaquin Valley is expected to increase in coming years (Luoma and Presser, 2000). Therefore, Se contamination is a high priority for the management of the SFBD ecosystem.

White sturgeon is one of the two endemic sturgeons inhabiting the Pacific Northwest. Both white and green (*A. medirostris*) sturgeon have high zoological value and are important to biodiversity, but white sturgeon has a higher value as a food and game fish. Both species belong to a phylogenetically unique group of ancient ray-finned fish that has survived for over two hundred million years, but has experienced rapid decline over the past century (Birstein, 1993). White sturgeon are more common in SFBD, aggregating in Northern San Francisco Bay (Suisun and San Pablo Bays) and migrating to spawn in the river systems (Kohlhorst, 1980; Kohlhorst et al., 1980).

The goal of this study was to elucidate the effects of Se bioaccumulation on white sturgeon in SFBD. The study pursued two objectives: 1) to determine Se tissue burden in different life stages of wild sturgeon, and 2) to determine the effects of microinjected organic Se on the development and survival of sturgeon embryos and yolk sac larvae. The large size and long ovarian cycle of white sturgeon make it difficult and expensive to study reproductive toxicity in this species under laboratory conditions. Microinjection mimics maternal transfer of environmental contaminants and is used as an important tool in ecotoxicology (Black et al., 1985; Metcalfe et al., 1988). Since microinjection is a slow process which limits the number of eggs treated, we also explored the technique of immersing water-hardening eggs into selenium baths to expose larger quantities of fertilized eggs.

MATERIALS AND METHODS

This project's methodology is divided into two parts, based on the objectives described above. The methodology for egg and larval exposure covers the general methods (culture, staging, selenium exposure and analyses) and detailed experimental procedures.

1a) Wild sturgeon capture in SFBD

Most wild sturgeon were obtained from two sources: California Department of Fish & Game's study of set-line capture efficiency on July 22 and 23, 2003, and a CALFED funded green sturgeon radio tagging study where white sturgeon were caught as a bycatch from April 21 to May 24, 2004. The gill nets used in latter study were 7 ½ inch stretch-bar mesh, 50 fathoms long and 20 feet deep or 9 ¼ inch stretch-bar mesh, 100 fathoms long, and 12 feet deep. The sturgeon were caught primarily between the mouths of the Sonoma Creek and Petaluma River, about 2-2.5 miles off-shore in water 4-10 feet deep. Three other sources were the fish racks at the Tracy Fish Collection Facility (from January 2003 to January 2004; n=6), the fish treadmill project at UC Davis (April 17 and May 7, 2004; n=2), and Fish & Game tagging study in north San Francisco Bay (October

31, 2002; n=3). The capture locations for wild fish are shown in Figure 1 and data on individual fish in Appendix 1.

1b) Tissue sampling and selenium analysis

After capture, fish were euthanized in a water bath with 500 ppm of MS-222 (tricaine methane sulfonate). Morphometric measurements included fork and total length, and live weight. In the larger individuals total gonad weight was also recorded. The pectoral fin ray was removed for aging. Tissue samples were collected from gonad, liver, kidney and muscle, frozen on dry ice and stored at -80°C . A section of each tissue was removed for the determination of moisture content. The samples were homogenized in millipure water using a hand blender and then freeze dried. Selenium measurement was carried out by ICP/AES (Protocol # 8137) at the California Animal Health and Food Safety Laboratory System, School of Veterinary Medicine, University of California Davis (CAHFS). The minimum detection limit was 5 ppb. Quality control measures included standard reference materials, spiked samples, duplication and blanks with each analytical run. All selenium concentrations are expressed for dw.

1c) Age determination

Age was determined from cross sections (0.2-0.6 mm) of base portions of dried pectoral fin rays (Van Eenennaam, et al., 1996). Sections were polished with increasing grades of wet-dry sandpaper and mounted on microscope slides with a cover slip adhesive. Slides were examined under a dissecting microscope with camera lucida and a Nikon Microplan II digital image analyzer with a fine-point light cursor to count the annuli and trace their continuity throughout the section plane. In counting annuli we followed guidelines of Cuerrier (1951), Probst and Cooper (1954), Semakula and Larkin (1968), Sokolov and Akimova (1976), and Brennan and Cailliet (1991).

1d) Statistical analysis

Data analysis was performed using JMP-In v. 4.0 (S.A.S. Institute Inc.). One-way ANOVA model followed by Tukey-Kramer HSD means comparison tests were applied to fork and total length, and live weight. A full factorial three way ANOVA followed by Tukey-Kramer HSD means comparison test was used to determine the effect of age, tissue, and sex on selenium concentration. Total length (cm), fork length (cm), age (yr), sex, capture area, and weight (kg) were used as the regressors for multiple regression analysis of selenium concentration in muscle, liver, kidney and gonad tissues. All analyses were performed on non-transformed data except for liver and muscle selenium for regression analysis, which were log transformed. The accepted significance level was $\alpha = 0.05$.

2a) Egg and larvae culture

All experiments were carried out at the Center for Aquatic Biology and Aquaculture (CABA) at the University of California, Davis. We used indoors flow-through water recirculation systems with biological filter, aeration, YSI thermostat, chiller, and heater. The egg and larvae were held in circular fiberglass tanks (28 cm diameter, 35 cm depth, flow rate 1.0-1.5 l/min). A certified calibrated thermometer (National Institute of Standards and Technology) was used to adjust incubation temperature to $15.7 \pm 0.1^{\circ}\text{C}$.

Natural photoperiod was maintained by artificial light, and approximately 80% of light was blocked by shade-cloth tank covers. Dissolved oxygen and temperature were recorded daily. At the initiation and termination of each trial, water samples were collected from each system to measure pH, ammonia, and, in selected samples, selenium.

2b) Developmental stages

We used key developmental stages to initiate treatments, to collect tissue samples for selenium and to conduct morphological analysis at midpoints and endpoints of each experiment. There were slight variation in stage within the sample due to fast rate of development. Developmental staging followed Dettlaff *et al.* (1993) for Russian sturgeon, with specific characteristics for white sturgeon described by Beer (1981) and Deng (2000) as follows:

Stage 5 (2nd cleavage): the onset of the first cleavage is indicated by the appearance of a furrow in the animal pole of the fertilized egg. Due to the holoblastic cleavage and yolky eggs in sturgeon, the second cleavage furrow appears in the animal pole resulting in four incompletely divided cells sharing yolk and cytoplasm material. White sturgeon eggs reach the stage 5 about five hours post fertilization at 15.7 °C (Deng, 2000).

Stage 19-23 (neurulation): the neural folds start to form around the head region (stage 19), extend to the trunk region (stage 20), and come close together (stage 21-22) to form a neural tube. By stage 23 the neurulation is completed and a suture-line between the two folds is clearly visible.

Stage 29 (S-shaped heart): the heart primordium forms as a straight tube, bends as a C-tube, and later as an S-shaped tube (stage 29). At Stage 29 heart beat commences.

Stage 36 (peak hatch): At this stage, hatched yolk-sac larvae are 10-11 mm in total length, darkly pigmented, and are relatively underdeveloped. They lack eye lenses, mouth, functional gills, and have continuous median fanfold.

Stage 40 (pyloric sphincter formation): at this stage a pyloric sphincter forms between the anterior portion (future stomach) and the posterior portion (future intestine) of the yolk sac. The intestinal portion is void of yolk material, but stomach is not differentiated and filled with yolk.

Stage 45 (Yolk depletion): at this stage all the fins and fin rays have differentiated. The mouth is well developed and equipped with teeth. Four barbells are located in the ventral side of the snout anterior to the mouth. The gill filaments project from the operculum. Stomach, liver, pancreas, and spleen are differentiated, and some fish had extruded the yolk plug and are ready to start exogenous feeding.

2c) Microinjection

We microinjected stage 5 eggs and stage 36 newly hatched larvae. We used a pressure injector (programmable picoinjector IM 300, Narishige Group, Japan), a joystick micromanipulator (MN-151, Narishige group, Japan) mounted on a magnetic stand, and aluminosilicate needles (A150-100-10, Sutter Instruments Co., Novato, CA). Needles were pulled from aluminosilicate tubing (1 mm OD, and 0.67 mm ID) using a programmable Flaming/Brown micropipette puller (P-97, Sutter Instruments Co., Novato, CA) to obtain a 10 µm OD and 100-150 µm long needle tips. Pulled needles were coated (Sigmacote, Sigma, St. Louis, MO), beveled (EG-44 Microgrinder, Narishige Group, Japan), back filled with approximately 5 µl of injection solution followed by an oil plug

of *circa* 10 μ l (paraffin oil, #76235, Fluka Production, Germany) using Eppendorf microloaders. Each needle was individually calibrated using a stereoscope (Wild M38) at 400 magnification, equipped with a pre-calibrated eyepiece micrometer. Needle calibration was done by injecting solution into paraffin oil and measuring the dispensed drop with the eyepiece micrometer. Volume adjustment was done by increasing or decreasing injection time while maintaining the pressure constant at about 20 psi. The target nanoinjection volume throughout all the experiments was 25 ± 2.5 nl (approximately 0.1 % volume of the average size white sturgeon egg). All injections were done under a stereoscope at 160 total magnification (Wild M38), with an X-Y movable stage equipped with a plate holder.

2d) Selenium dosing

Organic selenium (>98% pure seleno-L-methionine, product # S3132, Sigma-Aldrich, St Louis, MO) was used for all microinjections and immersion experiments. Amount of seleno-L-methionine (Se-L-Met) used for the different treatments was calculated based on the target selenium dosing per dry weight basis. The average ovulated white sturgeon egg is 8.25 mg dw, whereas the average newly hatched larvae (Stage 36) is 7.35 ± 0.06 and 7.03 ± 0.09 mg dw when incubated at 14 and 17 °C respectively (Wang, 1984). For calculations of approximate doses of selenium, and preparation of stock solution, we assumed the average white sturgeon hatched larvae to be 7.1 mg dw and the ovulated eggs 8.25 mg dw, for our temperature 15.7 °C.

2e) Egg Immersion

Only freshly fertilized eggs were used for immersion experiments, and the exposure to Se-L-Met was initiated immediately after fertilization. Water hardening results from secretion of hydrophilic colloid and subsequent water uptake into the perivitelline space (Dettlaff et al., 1993). Water uptake begins a few minutes after fertilization, and by 45-50 min post fertilization the perivitelline space reaches maximum volume (Zotin, 1953).

Eggs were placed on Petri dishes (6 - 8 eggs/ml), fertilized with diluted milt (1:100), rinsed in hatchery water, and treated for 2 hr with Se-L-Met dissolved in hatchery water at 15.7 °C inside a temperature controlled laboratory incubator with agitation provided by an orbital shaker (50 oscillations/min). After Se-L-Met exposure, embryos were rinsed four times with hatchery water, with an incubation in fresh hatchery water at 15.7 °C and 50 oscillations/min between the last two rinses. Rinsed eggs were stocked in tanks for incubation, and subsamples were frozen on dry ice and store at -80 °C for selenium analysis.

2f) Selenium analysis

The embryos and larvae were frozen on dry ice and stored at -80°C until preparation for analysis. Each sample was freeze dried and homogenized using a 0.5 mL mortar and pestle. The homogenates were repeatedly freeze-dried and approximately 2 mg of dry material was used for analysis. Total Se concentrations were analyzed by micro-digestion method followed by the fluorescence detection (Fan et al., 1998). The minimum detection limit was 0.2 ppb. Quality control measures included the use of standard reference materials, spiked samples, duplication and blanks with every analytical run. All egg and

larvae selenium concentrations are expressed on dw basis. Selected samples (endogenous selenium in ova and larval selenium in Experiment 2) were processed by ICP/AES method.

2g) Morphological measurements of eggs and larvae.

Egg diameter and total length of newly hatched larvae were measured in 10-15 individuals under a dissecting scope, using a digital image tablet (± 0.01 mm). Larvae at the yolk depletion stage (Stage 45) were measured with a caliper (± 0.5 mm). Ova and larvae were blotted and weighed individually (ww) on a microbalance (± 0.0001 g). Larvae or eggs were placed in tared whirl packs, lyophilized, and weighed to determined dry weights (dw).

Experiment 1: Immersion (pilot study)

There is no published data regarding efficiency of incorporation of test molecules (organic or inorganic) into sturgeon eggs by immersion. We conducted a pilot experiment to determine the relationship between concentration of Se-L-Met in the water and uptake of Se-L-Met by the fertilized egg. The information was used to determine the approximate concentration of Se-L-Met for the target dosing of Se in Experiment 3.

We obtained white sturgeon ova and milt from a commercial white sturgeon farm (Tsar Nicolai Caviar Inc., Wilton, CA), during the spawning of a wild-caught Sacramento River female. The milt was collected by a catheter attached to a 60 ml syringe from the urogenital opening of captive male white sturgeon. The samples of ova (approximately 50 ml) and pooled milt (20 ml) were transported to CABA in a temperature controlled chamber at 15 °C. Upon arrival milt was examined for motility.

We tested wide-spaced three Se-L-Met concentrations (0.0, 1.1, 5.0 and 10.0 mg/ml). Each concentration was run in duplicate dishes. Approximately 75 ova (2.5 ml) were placed on each of eight Petri dishes (60x15 mm), 15 ml of diluted milt (1:100) was added to each dish and fertilization allowed for 3 min. Fertilization solution was decanted, eggs rinsed with hatchery water (15.7 °C) and 7.5 ml of water added to each dish. Se-L-Met stock solution (26.67 mg/ml) was added to dishes to obtain the target concentration of Se-L-Met of 1.1, 5.0, and 10.0 mg/ml, and untreated eggs were used as control. Dishes were placed on an orbital shaker inside an incubator (15.7 °C), and incubation continued for 2 hr. Following incubation, dishes were rinsed 3 times with hatchery water and incubated at 50 oscillation/min at 15.7 °C in 12 ml hatchery water for 10 min before a final rinse. Water was decanted, eggs were placed in a whirl pack, frozen on dry ice, and stored at -80 °C until selenium analysis (ICP/AES, CAHFS).

Experiment 2: Microinjection of yolk-sac larvae (pilot study).

The objective was to test the accuracy of microinjection system and to investigate the post-injection survival of larvae to yolk depletion (Stage 45). Post-neurulated embryos were obtained from spawning of domestic stock (Columbia River origin) at Clear Springs Food Co. (Idaho). Eggs were fertilized on May 19, 2004, and post-neurulated embryos shipped to California in sealed plastic bags with hatchery water (14.5 °C) and oxygen. Upon arrival the embryos (Stage 30-31; Dettlaff et al., 1993) were acclimated to 15.7 °C

for two hours and transferred to crystallizing dishes submerged in tanks. The newly hatch larvae (Stage 36) were used for microinjections.

Newly hatched larvae with normal morphology and behavior (Dettlaff et al., 1993; Beer, 1981) were anesthetized in 150 ppm solution of MS-222 at 15.7 °C for approximately one min. The 100x15 Integrid Petri plate (Falcon #1012) was turned upside down and a water saturated Whatman filter paper (90 x 90 cm) placed on top. Using a transfer plastic pipette, ten larvae were lined up in two columns on the paper top for microinjection. The injection site was the upper mid portion of the yolk sac, between the duct of Cuvier and yolk veins, to avoid puncturing the major blood vessels. On average 10 larvae were injected within 2 min. After injections larvae were allowed to recovered in fresh water at 15.7 °C before stocking in tanks. The control larvae underwent the same procedures (anesthesia and microinjected with autoclaved doubly distilled water, DDH₂O). In addition, we used a second control group of anesthetized but not injected larvae, to account for the physical effect of microinjection.

For calculating target doses of Se-L-Met (MW 196.1) we assumed that yolk-sac larvae at 15.7 °C have 7.1 mg in dw (Wang et al., 1985). Se-L-Met was dissolved in autoclaved vials using autoclaved doubly distilled water (DDH₂O) to 7.0, 14.1, and 28.2 mg/ml. The injection volume was 25 nl per larva, resulting in selenium dosing of 10, 20 and 30 µg/g dw (ppm). Controls consisted of larvae injected with 25 nl of autoclaved DDH₂O and un-injected larvae. A total of 160 larvae were injected in each treatment and control groups. Within each group the 160 larvae were randomly distributed into four tanks; three replicate survival tanks (n=35) and one sampling tank (n=55). All replication were assigned to tanks by using a random number table.

Animals in survival tanks were monitored daily for mortality and morbidity. Sampling tanks were monitored daily for physical (e.g., heart beat, pericardial edema, yolk absorption) and morphological (e.g., cephalic and spinal) abnormalities of larvae. Morphology samples (n=5) were collected and preserved one day post injection and at the end point of the experiment. At sampling, larvae were anesthetized in 150 ppm MS222, overdosed in 500 ppm MS-222, rinsed in DDH₂O, and fixed in 10% buffered formalin containing 4% sucrose. Approximately 45 larvae from each treatment were collected from sampling tank one day post injection for selenium analysis (ICP/AES, CAHFS). Remaining larvae in sampling tanks were used for morphological observations at the end point of the experiment (Stage 45, yolk depletion).

Experiment 3 - 5:

For experiments 3, 4 and 5 (egg immersion, microinjection of embryos, and microinjection of newly hatched larvae, respectively), we obtained white sturgeon ova of three females and milt (pooled from 4 males) from a commercial farm (Stolt Sea Farms, CA). Gametes were handled and transported to CABA (1 hr) as previously described. Egg diameter, wet weight, and dry weight was determined as previously described. A sample of ova from each female (10 g) was frozen on dry ice, and store at -80 °C for selenium analysis (Fluorometry).

Experiment 3: Egg immersion

The eggs of two females (F1 and F2) were used in this experiment. The target selenium concentration were 0 (control), 3 (low), 12 (medium) and 48 (high) $\mu\text{g/g}$ DW. Based on the rate of selenium uptake by white sturgeon eggs (Experiment 1), we exposed eggs to concentrations of 0.00, 0.03, 0.12 and 0.48 mg/ml Se-L-Met for control, low, med, and high respectively. Approximately 450 unfertilized eggs (15 ml) were placed on each of eight plastic dishes (150 mm diameter). Fertilization and immersion procedures followed those described for Experiment 1. Treated and control eggs were counted and stocked in tanks (4 survival tanks and 1 sampling tank per female progeny). Survival tanks were stocked with 120 eggs and sampling tanks with approximately 250 eggs.

Water temperature, dissolved oxygen and mortality were monitored daily, and water samples were collected at the initiation and completion of the experiment to measure ammonia, pH, and selenium. Wet weight and egg diameter was measured in unfertilized ova. The embryos were sampled for morphology and selenium content three times, before two cell stage (pre Stage 4), post-neurulation (Stage 25), and peak hatch (Stage 36). At hatch, we also determined the wet and dry weight, and total length of larvae.

Morphological observations were on live and preserved larvae ($n=10$). For selenium analysis we sampled, at each stage, five whole eggs and five eggs with two outer layers of chorion removed with forceps. Embryos were frozen on dry ice and stored at -80°C for selenium analysis described in section 2f.

Experiment 4: Egg microinjection

Eggs of three females (F1, F2 and F3) were fertilized (1:100 milt dilution), silted with 5 ppt Fuller's earth (Sigma) for 1 hr and incubated in tanks in a flow-through water (15.7°C) re-circulating system. Synchronizing developmental timing of microinjections in three groups of eggs was difficult since the ovulations occurred at short intervals. Microinjections were started at Stages 4-5 (2-4 cell) in F1 and F2, and at Stages 5-6 (4-8 cell) in F3; and were completed at Stages 6-7 (8-16 cell) in F1, F2, and of Stages 7-8 (16-32 cell) in F3, respectively. Approximately 20 ml of 1% agarose (Type I-A, A0169 Sigma, St Louis, MO) was pipetted into Integrid Petri dishes and let to solidify. Twenty-five 3 mm diameter wells were made in the agarose with a stainless steel tube attached to a vacuum line. The embryos exhibiting cleavage were placed in wells and oriented sideways along the animal-vegetal pole axis (approximately 45°) under the stereoscope, so that the injection site was positioned below the egg equator. After injections embryos were gently propped out of the agarose wells and randomly distributed among the tanks.

The target selenium doses were 3, 12, and 48 $\mu\text{g/g}$ dw (low, medium and high treatment groups). The controls were non-injected and sham-injected (DDH_2O) embryos for all three progenies, and 3, 12, and 48 $\mu\text{g/g}$ dw L-methionine (Sigma-Aldrich, St. Louis, MO) for the F3 eggs. Se-L-Met was dissolved in autoclaved vials using autoclaved DDH_2O to 2.46 (low), 9.83 (med), and 39.31 (high) mg/ml and L-Met solution for injection was dissolved in autoclaved DDH_2O in sterile vials. All injections were 25 nl/egg. There were 5 treatment groups in each progeny (no-injected, sham-injected, low, medium, and high doses fo Se-L-Met) and the additional 3 groups for the F3 eggs (low, medium, and high doses of L-Met). Each group had one survival ($n=30$) and one sampling ($n=50$) tank. A

total number of 320 eggs were injected in F1 and F2, and 410 eggs in F3. Before stocking in tanks, non-injected control embryos were manipulated in the same manner and approximately for the same length of time as the injected groups. Treatment groups were randomly assigned to tanks.

Survival and water quality were monitored as described in Experiment 3. Samples for morphology (n=10) and selenium content (n=5) were taken at Stages 9-10 (mid cleavage, 2 hr after injection), at Stage 23-24 (end of neurulation), and at Stage 36 (hatching). For selenium analysis embryos (whole eggs) and larvae were frozen on dry ice and stored at -80°C , and for morphology they were fixed in 10% buffered formalin with 4% sucrose. Observations were made on live and preserved specimens to determine physical conditions (heart beat, yolk absorption) and gross morphological abnormalities.

Experiment 5: Microinjection of yolk-sac larvae

The additional batches of fertilized, eggs of F1 and F3 (see Experiment 4) were incubated at 12.7°C in a separate recirculation system. At start of hatching, temperature was gradually increased to 15.2°C . Microinjection of newly hatched larvae (Stage 36) was performed 90% of larvae had hatched out. Larvae exhibiting normal morphology and behavior were used for experiment (Beer, 1981; Dettlaff et al., 1993).

After the anesthesia (150 ppm MS-222, 15.7°C , ca. 1 min) larvae were placed on a water-saturated filter paper and microinjected as in Experiment 2. On average 10 larvae were injected within 2 min. After injections larvae were allowed to recover in fresh water at 15.7°C before stocking in rearing tanks. Non-injected control larvae were subjected to anesthesia and similar handling procedures.

For dose calculations of Se-L-Met and L-Met, we assumed hatched larvae to have 7.1 mg dw. Se-L-Met was dissolved in sterile vials using autoclaved DDH_2O to 2.11, 6.34, and 19.03 mg/ml. The injection volume was 25 nl per larvae, so the target Se doses were 3, 9 and 27 $\mu\text{g/g}$. Controls were non-injected, sham-injected (25 μl DDH_2O), and L-Met injected larvae. Three concentrations of L-Met (MW 149.2; Sigma-Aldrich, St. Louis, MO) were used for additional control (2.11, 6.34, and 19.03 mg/g).

For each progeny, a total of 445 larvae were used (365 injected and 80 non-injected). Non-injected, sham-injected, and low, medium, and high Se-L-Met groups of 80 larvae were each distributed into one survival (n=30) and one sampling (n=50) tanks. For low, medium, and high L-Met groups, 30 larvae of each group were stocked in a survival tank. All tank assignments were random.

Monitoring survival, abnormalities, and water quality was similar to Experiment 2. Larvae were randomly sampled for morphology (n=10) and selenium content (n=5) at two hours post-injection (Stage 36), at Stage 40 (piloric sphincter formation), and at Stage 45 (yolk depletion). Larvae for selenium analysis were euthanized in MS-222, rinsed in water, frozen on dry ice and stored at -80°C until analysis (section 2f). In addition, each treatment was sampled at the start and end point of the experiment to determine live body weight and total length (n=10).

2i) *Statistical analysis.*

Data analysis was performed using JMP-In v. 4.0.2 (S.A.S. Institute Inc.). One-way ANOVA model followed by Dunnett's and Tukey-Kramer HSD multiple comparison tests were applied to percent survival and hatching after angular transformation, and to untransformed wet weight and total length. In the microinjection experiments, a t-test was used to compare effects of microinjection on survival in non-injected and sham-injected groups prior to ANOVA. When controls were not significantly different, they were pooled for the ANOVA, otherwise the sham-injected group was used. The accepted significance level was $\alpha = 0.05$.

RESULTS

1) Selenium Content in Tissues of Wild Caught White Sturgeon

A total of 46 fish were provided by SFB/DF&G (n=3), Tracy Fish Collection Facility (n=6), Raymond Schaffter/DF&G (n=20), Joseph Cech, Hydrology laboratory, UCD (n=2), and Peter Klimley, tagging study, UCD (n=15) from 10/2002 through 6/2004. We measured fish total length (TL), fork length (FL), and live weight (W), and collected tissue samples for aging (fin ray), and selenium analyses (liver, kidney, gonad, and muscle). Sexing was done by inspection of gonads during tissue collection.

The samples included 19 (41.3%) females and 27 (58.7%) males (Appendix 1). All fish were sub-adult sturgeon with immature gonads, except female #36 in Appendix 1 which had small vitellogenic eggs (just pigmentingst). No ripe males were present in the group. The average age was 9 (range 5-17) and 13 (range 4-18 yr) in females and males, respectively. The average total length of females was 102.5 cm (range 68.0-164.0 cm), and live weight 11.09 kg (range 2.90-27.25 kg). For males, the TL was 127.6 cm (range 61.0-171.0 cm) and W was 13.75 kg (range 2.70-26.10 kg). There was positive correlation and significant relationship between age and TL, FL, and W for females and males (Fig 2 and 3, respectively).

For the tissue selenium burden, seven fish (marked by asterisk in Appendix 1) were excluded from the statistical analysis since they were held in captivity before sampling for a prolonged time (1 to 11 months). We grouped fish (n=39) in three age classes for the ANOVA, age groups 1 (4-8 yr, n=15), 2 (9-13 yr, n=12), and 3 (14-18 yr, n=12). A full factorial three way ANOVA was used to determine the effects of age, tissue, and sex on selenium concentration. There was no significant difference in selenium concentration between females and males (ANOVA, $p > 0.05$). Kidney had significantly higher selenium concentration ($12.36 \pm 3.24 \mu\text{g/g}$, mean \pm SD, n=39) than liver, muscle and gonad (Fig 4). Liver had elevated selenium concentration ($9.75 \pm 5.35 \mu\text{g/g}$) compared to muscle ($6.59 \pm 2.81 \mu\text{g/g}$), but the difference was not significant. Gonad tissue exhibited the lowest selenium burden and very high variation ($4.06 \pm 7.67 \mu\text{g/g}$). Age groups 2 and 3 (9-18 yr) had significantly higher liver and muscle mean selenium concentrations compared to group 1 (4-8 yr, Fig 5). The interactions between age group, sex, and tissue were not significant ($p > 0.05$).

No range!

46-7
N = 39

Since the great majority of fish was captured in only two locations (n=18 in the area 1, and n=21 in the area 2, Fig 1), we compared tissue selenium for the two locations using one-factor (location) and two-factor (location and age) ANOVA. One-factor ANOVA revealed significantly higher selenium concentrations of liver and muscle in the Area 1 (Table 1). However, with the age of fish as an additional factor in ANOVA, there was no significant difference in selenium tissue burden between the fish captured in areas 1 and 2.

In the stepwise multiple regression analysis no significant relationships were found for the gonad and kidney selenium and all regressor variables. The selenium concentrations in muscle and liver were positively correlated with the age and total length of fish. Since these two regressors were highly correlated (see Fig 3), the final models were reduced to a single regressor for muscle (age) and liver (TL) selenium, with all regression coefficients significant at $p < 0.05$ (Fig 6 and 7). Data shows that muscle and liver selenium burdens significantly increase with the age and size of white sturgeon.

2) Microinjection and Immersion Studies

Water temperatures were maintained at 15.7 ± 0.1 °C throughout all the experiments. Dissolved oxygen was above 90% saturation in all tanks. Ammonia in water ranged from <0.003 to 0.014 mg/l NH_3 and pH ranged from 8.2-8.3 (DNRA laboratory, University of California, Davis). Selenium concentration in the water of recirculation systems used for experiments was below 1.3 $\mu\text{g/l}$ at the initiation of experiments, 1.2 $\mu\text{g/l}$ at the end point of experiments (Flame AA on digested samples, SM3114B and SM303E, Sierra Foothill laboratory, Inc., Jackson, California), certified for water selenium analysis at UC Davis facilities.

Experiment 1: Immersion

Wild-caught (Sacramento River) female weigh 45 kg, and 200,000 eggs were collected. On farm the percent hatching was 80% indicating good quality of gametes. The endogenous selenium concentration in unfertilized ova was 10.7 ± 0.1 $\mu\text{g/g}$ (ICP/AES method). The experiment revealed a dose-dependent increase in selenium concentration in the whole fertilized eggs after two hour exposure time to Se-L-Met (Fig. 8). After two hour exposure the concentration of selenium in fertilized white sturgeon eggs with chorion were proportional (1:100) to the Se-L-Met concentrations used.

Experiment 2: microinjection of larvae

Approximately 90% of post neurulated embryos received from Idaho survived to hatching and provided viable yolk-sac larvae for this experiment. Figure 9 and Table 2 show selenium concentrations in larvae one day after microinjection (t_0) and at the experimental end point (t_1 , Stage 45) for controls and treatment groups. Control groups (endogenous Se) exhibited 2.57 ± 0.04 (mean \pm std) of selenium ($\mu\text{g/g}$) at the initiation of the experiment. Concentration of selenium at t_0 in low, med and high treatment group (one sample per group) was 15.8, 21.7 and 46.6 $\mu\text{g/g}$ respectively, approximating target selenium doses of 10, 20 and 40 $\mu\text{g/g}$. At experimental end point selenium concentrations were measured in survivors for each group in three tanks per group. High (8.99 ± 0.49 $\mu\text{g/g}$) and medium (10.70 ± 0.49 $\mu\text{g/g}$) selenium treatment groups had lower

concentration of selenium than at t_0 . Low selenium group had $13.92 \pm 2.06 \mu\text{g/g}$ at experimental end point. Control and sham groups exhibited $5.31 \pm 0.17 \mu\text{g/g}$, twice as much as at t_0 . The discrepancy may relate to two different methods for selenium analysis at t_0 and t_1 (Table 2).

Selenium treatment had a significant effect on mortality, compared to non-injected and sham injected controls (Figure 10). The microinjection technique (larvae manipulation and injection) had no significant effect on the survival of larvae to yolk depletion (stage 45) indicated by practically 100% survival in both non-injected and sham-injected controls. The most prominent abnormality observed in the mortalities in the Se treated larvae were deformed body (bent larvae) and severe edema of pericardial sinus and yolk sac (Figure 11). Many larvae in Se treated groups exhibited bent body and edema, were lethargic, and could not swim normally.

Experiment 3, 4 and 5:

Time from egg collection at the farm to egg fertilization at CABA facilities was less than 2 hours. Table 3 shows the wet weight and diameter of unfertilized ova. Female 1 progeny had significantly larger eggs ($3.59 \pm 0.17 \text{ mm}$ diameter, and $23.95 \pm 1.38 \text{ mg}$ weight) than female 2 progeny ($3.12 \pm 0.15 \text{ mm}$ and $21.88 \pm 0.43 \text{ mg}$). The endogenous egg selenium concentration were 1.97 and $2.62 \mu\text{g/g}$ in Females 1 and 2, respectively (ICP/AES method, CAHFS).

Experiment 3: Egg immersion

All levels of Se-L-Met treatment had a significant effect on the percent mortality compared to control at the end point of the experiment, Stage 36 (Fig. 12). However, the mortality in control (*ca.* 50%) was higher than normally observed in good quality eggs, which was probably effected by gamete transport and handling. There was no significant effect of Se-L-met on wet weight and total length at the end point of the experiment (data not shown). Female 1 and Female 2 progenies significantly differed in weight, Female 1 larvae being heavier than those of Female 2 (Table 4). No difference was found in total length of larvae (Table 4). Both progenies exhibited similar trends in percent cumulative mortality in control and treatments. High Se-L-Met treatment had a significant effect on percent cumulative mortality when compared to control, but no difference was found between control, and low and med Se-L-Met groups (Figure 13). The lowest observed effect (LOEC) was the low Se-L-Met group.

There were no differences in the egg selenium concentration between the treatments (range $6.39 - 8.25 \mu\text{g/g}$, Fluorometry method) and between dechorionated and whole eggs.

Experiment 4: Egg microinjection

ANOVA revealed significant effect of Se-L-Met treatment on the percent mortality at the end point of the experiment (Stage 36). High Se-L-Met treatment had significantly higher mortality compared to other treatment groups and control which did not differ (Figure 14). There was no significant effect of Se-L-met on wet weight and total length at the end point of the experiment (Table 5). All progenies exhibited similar mortality trends over

time for the different control and treatment groups (Figure 15). High Se-L-Met treatment had a significant effect on pooled cumulative mortality (Figure 16).

Selenium concentration between treatment groups was significantly different (ANOVA, $p < 0.05$). Post-injection embryos in the high Se-L-met group had significantly higher selenium content compared to other treatment groups and control (Fig 17). Post neurulated embryos ($t_{1/2}$) in the low and medium Se-L-Met groups exhibited elevated selenium concentration as compared to control and high Se-L-Met group. At the end point of the experiment there was no significant difference in selenium concentration between the treatment and control groups.

Experiment 5: Microinjection of yolk-sac larvae

At the start of the experiment, newly hatched Female 1 larvae had significantly greater weight ($p < 0.001$) but were shorter ($p < 0.05$) than Female 3 larvae (Table 6). Se-L-Met treatment had no effect on body weight and total length by yolk depletion stage (Table 7). Se-L-Met treatment had a significant effect on mortality, relative to non-injected and sham-injected (pooled), and L-Met controls (Figure 18), with significant difference in mortality between the high Se-L-Met treatment group and all other groups. The low and medium Se-L-Met treatment groups did not exhibit significant changes in mortality relative to control groups. Figure 19 shows the percent cumulative mortality for both females progenies which exhibited similar trends, with most mortality occurring 4 to 6 days post-injection. High Se-L-Met treatment had a significant effect on percent cumulative mortality when compared to controls and other treatment groups (Figure 20). The lowest observed effect concentration (LOEC) was the high Se-L-Met group. Percent abnormalities are shown on Figure 21. Almost all larvae treated with Se-L-Met (low – high) exhibited edema and bent bodies (lordosis, kyphosis, and scoliosis).

Selenium concentration in treatment groups were significantly different (ANOVA, $p < 0.05$). At 1-2 hr post injection and at Stage 40 (pyloric sphincter formation) the larvae in the high Se-L-Met group had significantly higher selenium content than the other treatment groups and controls (Fig 22). At the end point of the experiment (Stage 45) there was no significant difference in selenium concentration between the treatment and control groups.

DISCUSSION

The main goal of this study was to elucidate the effect of selenium bioaccumulation on white sturgeon in San Francisco Bay-Delta. White sturgeon is a highly valuable endemic species, important for the ecosystem (controls proliferation of introduced benthic organisms), recreational fishery (some of the most popular game fish on the west coast), and science (ancient ray-finned fish valuable for the study of evolution). As a longlived, late maturing and benthophagous species, white sturgeon has a propensity to accumulate more selenium via food chain compared to other fish of the SFBDD ecosystem. Our study, using mainly sub-adult (i.e., before the first sexual maturity) white sturgeon, revealed

mean selenium concentrations of 12.36 ± 0.52 , 9.75 ± 0.86 , 6.59 ± 0.45 , and 4.06 ± 1.24 $\mu\text{g/g}$ (mean \pm SE) in the kidney, liver, muscle, and immature gonads of white sturgeon.

Selenium in some tissue of white sturgeon (Fig 4) is approaching the levels that have been associated with toxicity and reproductive failure in other fish species (Hilton *et al.* 1980; Hodson *et al.* 1980; Garrett & Inmann 1984; Sorensen & Bauer 1984; Hermanutz *et al.* 1992). Lemly (2002) proposed selenium toxic thresholds for freshwater and anadromous fish at 8 $\mu\text{g/g}$ in muscle, 12 $\mu\text{g/g}$ in liver, and 10 $\mu\text{g/g}$ in ovary or eggs. Mean selenium in white sturgeon muscle and liver from this study are close to these thresholds, and several individual fish in the upper range of distributions greatly exceed the proposed thresholds. Although mean value of selenium in gonad of fish sampled in this study did not exceed the toxicity threshold, one female had ovaries containing 46.7 $\mu\text{g/g}$ selenium and three others had ovaries with 7.3 – 9.7 $\mu\text{g/g}$ selenium, which approaches the proposed threshold of 10 $\mu\text{g/g}$. Out of six sturgeon females sampled in the Sacramento River in the 1990's, four had eggs ranging from 8 – 12 ppm selenium and one had eggs with 29 ppm (Kroll & Doroshov, 1991). Selenium was found to be highest in the kidney, an organ that has a major role in selenium elimination during chronic exposures (Diplock 1976; Hilton & Hodson 1983; Sorensen 1986). Decreased weight, kidney lesions, and mortality in rainbow trout have been associated with kidney selenium levels similar to those found in white sturgeon from this study (Hicks *et al.* 1984).

None of the studies cited above used white sturgeon as a test species, however recent unpublished studies have shown that excess selenium leads to toxicity in white sturgeon. Liver selenium levels of 21.9 ± 10.6 $\mu\text{g/g}$ were associated with liver damage in juvenile white sturgeon (*ca.* 575g) after six months of dietary organo-selenium exposure at 20 $\mu\text{g/g}$ (Linville *et al.* In progress-a). Dietary exposure of 40 $\mu\text{g/g}$ seleno-L-methionine led to impaired growth in juvenile white sturgeon (25 – 30g) after two weeks (Tashjian *et al.* In progress). In the same study, the activity level of juvenile white sturgeon decreased with increasing selenium exposure. A study investigating the effects of selenium exposure on salinity tolerance in juvenile white sturgeon indicates a strong interactive effect of dietary selenium exposure and elevated salinity on survival (Tashjian & Hung In progress). Sturgeon mortality and time to death were both positively correlated with levels of selenium in the diet.

Recently, female white sturgeon have been shown to transfer selenium to their young, leading to severe edema and deformities. Adult females with liver and gonad selenium concentrations as low as 11.0 $\mu\text{g/g}$, produced larvae with significant defects (Linville *et al.* In progress-c). The adults had been exposed to 33 $\mu\text{g/g}$ dietary organo-selenium for approximately 6 months. The predominate route of maternal transfer was, most likely, via the yolk protein precursor, vitellogenin (Kroll & Doroshov 1991; Linville *et al.* In progress-b). The effects of selenium in white sturgeon have been the focus of several recent studies at the University of California at Davis, some of which were not discussed above (Tashjian & Hung In press; Tashjian *et al.* Submitted). Evidently, white sturgeon are susceptible to selenium toxicity at environmentally relevant levels.

We found a high variation in selenium levels of sturgeon sampled in this study. This variation was observed in other studies on white sturgeon and appears to be linked to different foraging locations within San Francisco Bay (Stewart *et al.* 2004a; Stewart *et al.* 2004b). Moreover, we observed a significant positive relationship between selenium in muscle and the age of fish (Fig 6) and between liver selenium and the fish length (Fig 7). Selenium levels in 4-8 yr old white sturgeon were lower than the levels in older fish. Selenium has not typically been shown to accumulate with age, probably because it does not accumulate in fat or form irreversible complexes. The elevation of tissue burden seen after 8 years of age could be associated with a change in foraging patterns at this stage of sturgeon development. Sturgeon exhibit a delayed sexual maturity, with males typically maturing at age 10 – 12 years and females at 15 – 32 years (PSMFC, 1992). It is plausible that dietary changes accompany sexual maturity. Another possible scenario for dietary change with age is that increased size and strength could lead to increased ability to compete for prime feeding locations. Stewart and colleagues (Stewart *et al.* 2004b) have shown that food web pathway and foraging location determine the extent of selenium exposures for the top predators in San Francisco Bay.

White sturgeon collected in this study contained somewhat lower selenium levels than those reported by Stewart *et al.* (2004b). These authors reported a mean liver selenium of 24 $\mu\text{g/g}$ in 15 white sturgeon (age 14 – 20 yr) collected from northern San Francisco Bay in the year 2000. Age and, possibly, feeding patterns may partially explain this difference since all of the sturgeon collected in Stewart *et al.* (2004) study were older than 9 years. We found that sturgeon younger than 9 years appeared to contain less selenium in their tissues, possibly due to changes in foraging patterns. However after excluding the younger age group (4 – 8 yrs) from our data set, the mean liver concentration remains significantly lower ($11.4 \pm 6.1 \mu\text{g/g}$) compared to Stewart *et al.* (2004) data. Another possible explanation for this discrepancy is the effect of seasonality. Selenium levels in *Potamocorbula amurensis*, the primary source of food selenium for white sturgeon from this area, are strongly influenced by seasonal variability (Luoma & Presser 2000; Linville *et al.* 2002). Selenium is at highest level in these bivalves after the periods of low river inflow, possibly due to differences in hydraulic residence times. Stewart *et al.* (2004b) sampled white sturgeon after a prolonged period of low river inflow (January). Conversely, 35 of the 39 sturgeon sampled in this study were collected following a higher flow regime in March – August. Regardless of the impacts of seasonality on sturgeon bioaccumulation, Stewart *et al.* (2004a and 2004b) have shown that selenium levels in white sturgeon from San Francisco Bay exhibit a high degree of variation. All but three liver samples in our study fell within the range of previous data (Stewart *et al.*, 2004b). Gathering additional data and comparing with existing data sets may lead to a more distinct pattern of selenium bioaccumulation in white sturgeon from San Francisco Bay.

One of the most likely effects of selenium bioaccumulation on white sturgeon is a maternal transfer of organic selenium to the embryos and yolk-sac larva stages. In this study we attempted to mimic transfer by the immersion of freshly fertilized eggs and microinjections of eggs and larvae, using Se-L-Met. Exposure of white sturgeon embryos to selenium by immersion was only successful using very high levels (1-10 ppt) of Se-L-Met (Experiment 1, Fig 8.). A previous study successfully used immersion of the

Sacramento splittail embryos in sodium selenite to show selenium-induced deformities (Teh *et al.* 2002). Immersion Se-L-Met concentrations used in this study to target environmentally relevant levels of selenium were not entirely successful (Experiment 3). We did not detect significant difference between selenium levels in treated embryos, however we did observe greater mortality in treated embryos compared to controls (Figs 12 and 13). A possible reason for a low selenium assimilation is the three-layer chorion in sturgeon embryos, which includes an outer gelatinous coat which could trap dissolved Se-L-Met (Dettlaff *et al.* 1993); however, intact and dechorionated (but not denuded, since the inner chorion remained) eggs had similar concentrations of selenium at all doses. It may be useful to further investigate the immersion exposure range necessary to achieve environmentally relevant selenium levels in sturgeon embryos.

Microinjection of white sturgeon embryos did result in selenium exposures at environmentally relevant levels (Experiment 4, Fig 17). Selenium exposures to embryos in the high treatment injections were statistically different from all other treatment injections. Selenium exposures were not statistically different between control, low and medium injections. This was most likely due to using too narrow of an injection range. Embryos injected with the high selenium contained a mean of 20.8 µg/g selenium at 1–2 hours post injection, and eventually experienced 61.11% control-corrected mortality (Figs 14, 15, 16, 17). The selenium level in the high treatment significantly exceeded Lemly's proposed toxicity threshold of 10 µg/g selenium in fish eggs (Lemly 2002). Control mortalities were high (37.8% in DI water sham and 29.1% in L-methionine sham), indicating that white sturgeon eggs are sensitive to handling and injections at early stage of development.

Microinjection of yolk-sac larvae proved to be the most reliable method to study the effects of excess selenium on the development of this species (Experiments 2 and 5). In the first trial (Experiment 2) we successfully targeted the exposure levels of 10, 20 and 40 µg/g selenium in larvae (Table 2; Fig 9). The observed increase of selenium in control larvae between the start and end of the experiment was most likely due to different sample sizes (n=60 and 10) and different analytical methods were employed for these two analyses (ICP/AES at start, Fluorometry at end). In the second trial (Experiment 5), the actual Se concentrations in microinjected larvae differed from, but were proportional to target exposure levels of 3, 9 and 27 µg/g selenium (Fig 22). In both the embryo and larval injections, we were unable to accurately obtain target exposures levels that were less than 10 µg/g apart. Because of water solubility of Se-L-Met, small amounts of the compound may have diffused out of the embryo or larvae directly following injection. Overall, microinjection of white sturgeon larvae resulted in environmentally relevant levels of selenium exposures. Actual exposure levels of selenium in larvae ranged from ca. 8 – 47 µg/g (Fig 9 and 22). The loss of selenium between the start and end of the experiment could have been due to metabolism, although limited previous work indicates that selenium accumulated in egg yolk is retained during the early larval stages (Linville *et al.* In progress-c).

The observed effects of selenium on larval development included edema, spinal deformities and mortality. Mortality was low in sham injected larvae (less than 2%), and

in larvae exposed to ca. 8.5 µg/g selenium (4% and 11% mortality, Experiment 5), but high in larvae exposed to ca. 16 – 22 µg/g selenium (40-48 %, Experiment 2) and in larvae exposed to either 16.5 (Experiment 5) or 47 (Experiment 2) µg/g selenium (70%, Figs 10, 18, 19). In Experiment 5, most mortalities took place between days 4 and 5 post-hatch. The observed mortalities showed a steep increase in 16 µg/g selenium exposure. We observed defects in only one sham-injected larvae group (less than 3% with spinal deformities). Low and variable levels of edema and spinal deformities (including lordosis, kyphosis and scoliosis) were observed in larvae exposed to 8.5 µg/g selenium (4 and 12%, Fig 21). However very high percent edema and spinal deformities were observed in larvae exposed to 16 – 22 µg/g (30-45%), 16.4 µg/g (68%), and 47 µg/g selenium (68%, Figs 11 and 21). As with mortalities, we observed a sharp rise in abnormalities at higher than 16 µg/g selenium. The variations in mortality and abnormality rates could be due to variation in selenium dose received by individual larvae. These results indicate that white sturgeon larvae experience selenium-induced toxicity at environmentally relevant levels. The very low control mortality and abnormality rates indicate that white sturgeon larvae tolerate microinjection well, and that microinjection of yolk-sac larvae is a good model for investigating selenium-induced toxicity in early life stages.

Developmental toxicity of endogenous (maternal) selenium was shown in recent studies (Linville *et al.*, In progress) where in two groups of 8 hatchery-raised white sturgeon females were fed diets with normal (1 µg/g; control) and elevated (33 µg/g; treatment) organic selenium for approximately 6 months, during the late phase of vitellogenesis. Upon full egg maturation (*ca.*, six months of dietary exposures) sturgeon were artificially induced to spawn. Selenium levels in treatment fish were significantly higher than in control females (8.6, 8.7, and 11.2 µg/g in liver, ovary and eggs, respectively, compared to 1.5, 1.4, and 2.2 µg/g in control). Three control and 3 treatment fish produced fertile eggs. The progeny were allowed to develop in a clean environment to Stage 45 (yolk absorption). Developing larvae showed a statistically significant relationship between selenium concentration and the abnormality rate (edema and spinal deformities; $R^2 = 0.57$, $p < 0.05$). In 3 treatment progenies larvae selenium concentrations ranged 7.5-11.8 µg/g, and the abnormality rates were up to 23%. The highest level of selenium concentration and larval defects in control female was 2.88 µg/g and 1.1% respectively.

There are certainly differences between the microinjection and maternal exposure studies due to effects of injection stress, form of selenium used (free amino acids in microinjection versus protein bound in maternal exposure), and the route of selenium exposure (injected selenium versus selenium incorporated into yolk proteins). However, both studies indicate hazardous level of selenium at concentrations exceeding ca. 15 µg/g in eggs and larvae.

The observed effects on larvae agree with those described in several field and laboratory experiments. The most common types of developmental defects produced by selenium toxicity are severe edema and various forms of terata, including spinal curvatures (Gillespie & Baumann 1986; Woock *et al.* 1987; Lemly 1993, 1997). Holm *et al.* (2003) found 41.85 and 31.85 µg/g selenium (converted from wet weight using 80% moisture) in

the eggs of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) collected from seleniferous areas in Canada. These eggs produced larvae with edema and classic deformities known to be associated with selenium toxicity. Gillespie and Baumann (1986) spawned female bluegills (*Lepomis macrochirus*) from a high selenium reservoir with egg selenium concentrations of 12 – 55 µg/g. The resulting larvae exhibited edema, deformities and complete mortality. Other field and laboratory studies have shown similar results in bluegill (Wooock *et al.* 1987; Hermanutz *et al.* 1992; Coyle *et al.* 1993). Schultz and Hermanutz (1990) experimentally demonstrated that fathead minnow (*Pimephales promelas*) embryos containing *ca.* 19.5 µg/g selenium (converted from wet weight using 80% moisture) resulted in larvae with edema and spinal deformities. Hamilton and colleagues (2002) found that eggs from wild razorback suckers (*Xyrauchen texanus*) containing greater than 20 µg/g selenium represented a high level of hazard to this endangered species.

Several possible mechanisms of selenium toxicity have been described. Palace *et al.* (2004) recently determined that selenomethionine exposure can lead to oxidative stress in fish larvae, which is known to cause edema and spinal curvatures. They linked superoxide production to methioninase enzyme activity and the ensuing metabolism. Superoxide activity affects the embryo until significant production of superoxide dismutase provides a detoxification pathway. Individual variation in the expression of either of these enzymes could lead to different timing and frequencies of effects. The selenium can be incorporated into biological molecules in place of sulfur because the two elements have similar chemical properties (Stadtman 1974; Diplock 1976). However, selenium and sulfur bonds may have different strength and function in biological molecules (Reddy & Massaro 1983), thus some selenium-substituted compounds may become unstable or dysfunctional (Stadtman 1974; Lemly 1998). Selenium can also inhibit protein synthesis (Vernie & Van Leewenhoekhuis 1987) and react with sulfhydryl groups of proteins or other molecules (Martin 1973), causing disruptions of histogenesis and deformities, which are a common effect of selenium toxicity (Lemly 1998).

In summary, white sturgeon from San Francisco Bay-Delta have been shown to contain potentially hazardous levels of selenium in this and other studies (Stewart *et al.* 2004b). Some white sturgeon adults collected from San Francisco Bay contained liver selenium levels close to those associated with reproductive toxicity in selenium dietary experiments in white sturgeon (Linville *et al.* In progress-b; Linville *et al.* In progress-c). The microinjection of selenium in white sturgeon larvae demonstrated significant increases of mortality and abnormality rates (including edema and spinal deformities) in larvae containing above 15 µg/g selenium. The larval microinjection experiments used environmentally relevant selenium levels and revealed defect and mortality rates similar to those observed in field and laboratory studies of selenium toxicity in other fish. We suggest that white sturgeon of San Francisco Bay-Delta should be carefully monitored for tissue selenium burden, particularly in reproductive phase.

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Table 1. Selenium concentration (mean \pm SE, $\mu\text{g/g}$) in the different tissues from fish capture in Area 1 (n = 18) and Area 2 (n= 21). Area 1 (San Pablo Bay) include fish capture sites 1 and 2, and Area 2 (above Suisun Bay) include fish capture sites 3, 4, and 5 (Fig. 1). Concentrations in liver and muscle are significantly different in one way ANOVA (capture area) but do not differ when the age of fish is used as a covariate.

	Area 1	Area 2
Gonad	4.36 \pm 0.92	3.82 \pm 2.15
Kidney	12.41 \pm 0.66	12.32 \pm 0.79
Liver	12.20 \pm 1.34	7.65 \pm 0.89 (*)
Muscle	8.20 \pm 0.66	5.22 \pm 0.44 (*)

Table 2.

Target tissue selenium concentration, and whole larvae selenium content ($\mu\text{g/g}$ dry weight) one day post-injection (t_0 , ICP/AES method) and at the experimental end point (t_1 , Stage 45, Fluorometry). Experiment 2.

	Target Selenium	Selenium at t_0	Selenium at t_1
Non-injected	0.0	2.60	5.43 \pm 0.28
Sham-injected	0.0	2.54	5.19 \pm 0.24
Low	10	15.8	13.92 \pm 2.06
Med	20	21.7	10.71 \pm 0.49
High	40	46.6	8.99 \pm 0.49

Table 3. Initial egg diameter and weight (mean \pm SD) of unfertilized eggs from Females 1 and 2 used in Experiments #3.

	Egg diameter (mm)	Egg weight (mg)
Female 1	3.59 \pm 0.17	23.95 \pm 1.38
Female 2	3.12 \pm 0.15	21.88 \pm 0.43

Table 4. Effect of Se-L-Met on weigh and length of sturgeon larvae (mean \pm SD, n=10) at hatching (Stage 36, Experiment 3). Asterisk denote significant difference between the females ($p < 0.05$). No significant differences were detected between control and treatments.

	Weight (mg)		Length (mm)	
	F1	F2	F1	F2
Control	26.19 \pm 0.77	22.59 \pm 1.42 *	12.91 \pm 0.28	12.84 \pm 0.48
Low	25.26 \pm 0.93	23.04 \pm 1.02 *	12.46 \pm 0.23	13.28 \pm 0.35 *
Med	26.24 \pm 2.88	23.07 \pm 1.36 *	12.82 \pm 0.38	13.09 \pm 0.46
High	25.95 \pm 1.26	22.40 \pm 1.06 *	12.96 \pm 0.34	13.14 \pm 0.71

Table 5. Effect of Sel-L-Met treatment on weight and length of sturgeon larvae (mean \pm SE) in survival tanks (n=3) at hatching (Stage 36, Experiment 4). No significance difference was found between the controls and treatment groups.

	Weight (mg)	Length (mm)
Control	23.07 \pm 1.44	12.93 \pm 0.16
Low	22.21 \pm 0.98	12.62 \pm 0.07
Med	22.24 \pm 0.84	12.68 \pm 0.27

Table 6. Initial length and weight (mean \pm SD, n=15) of larvae from females (#1 and #3) used for microinjection in Experiment 5.

	Weight (mg)	Length (mm)
Female 1	24.23 \pm 1.82	12.48 \pm 0.33
Female 3	17.66 \pm 2.22	12.85 \pm 0.35

Table 7. Weight and length (mean \pm SE) of larvae at Stage 45 (yolk depletion) from treatment and control groups in Experiment 5. Sample size was 2 progenies except for High Se-L-Met (no larvae survived for F1) and Low L-Met.

	Weight (mg)	Length (mm)
Non-injected	42.55 \pm 2.96	18.45 \pm 0.39
Sham-injected	42.28 \pm 1.74	18.60 \pm 0.50
L-Met Low (n=1)	45.04	18.57
L-Met Med	41.76 \pm 2.51	18.33 \pm 0.23
L-Met High	41.74 \pm 1.00	18.09 \pm 0.01
Se-L-Met Low	42.49 \pm 2.52	18.50 \pm 0.27
Se-L-Met Med	42.44 \pm 2.45	18.67 \pm 0.30
Se-L-Met High (n=1)	37.87	17.56

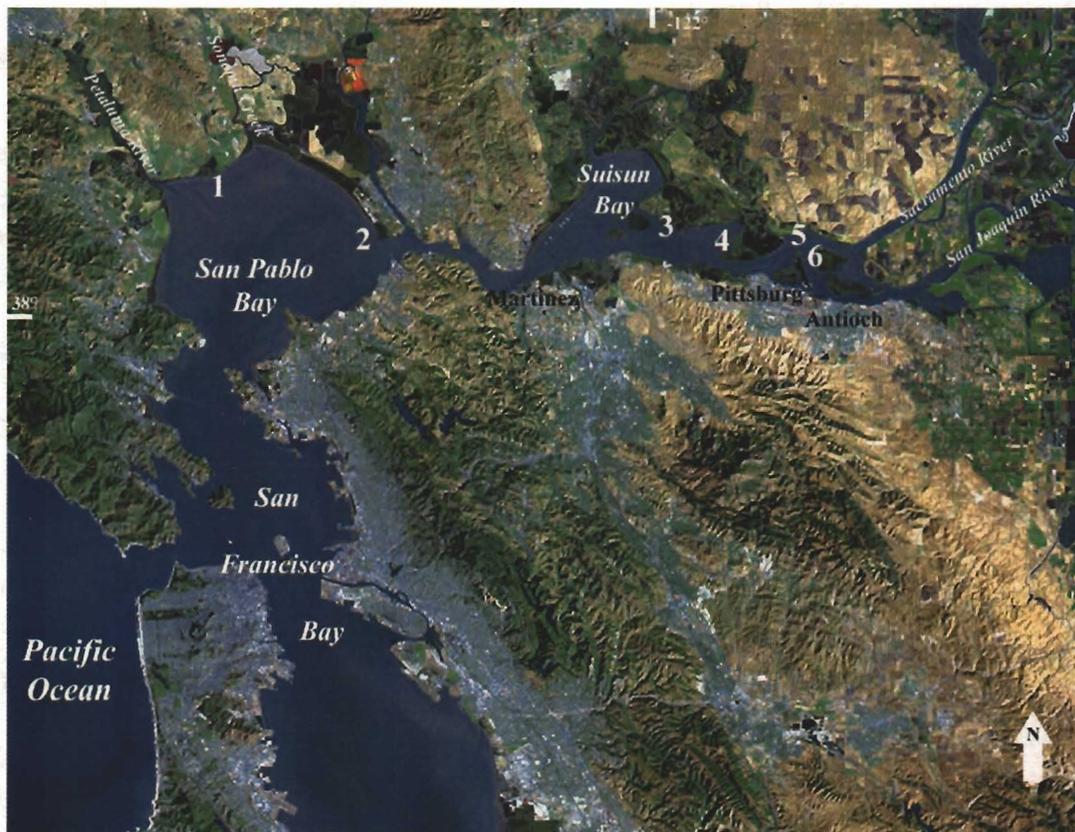


Figure 1. Locations of white sturgeon captures for the selenium study. 1. Between Petaluma River and Sonoma Creek, 2-2.5 mi off-shore and in 4-10" deep water (n=15); 2. North San Pablo bay at 4-8" depth (n=3). 3. Suisun Bay Cut off (n=1); 4, 5, and 6. Honker Bay, Channel Island, and Broad Slough North respectively (n=20).

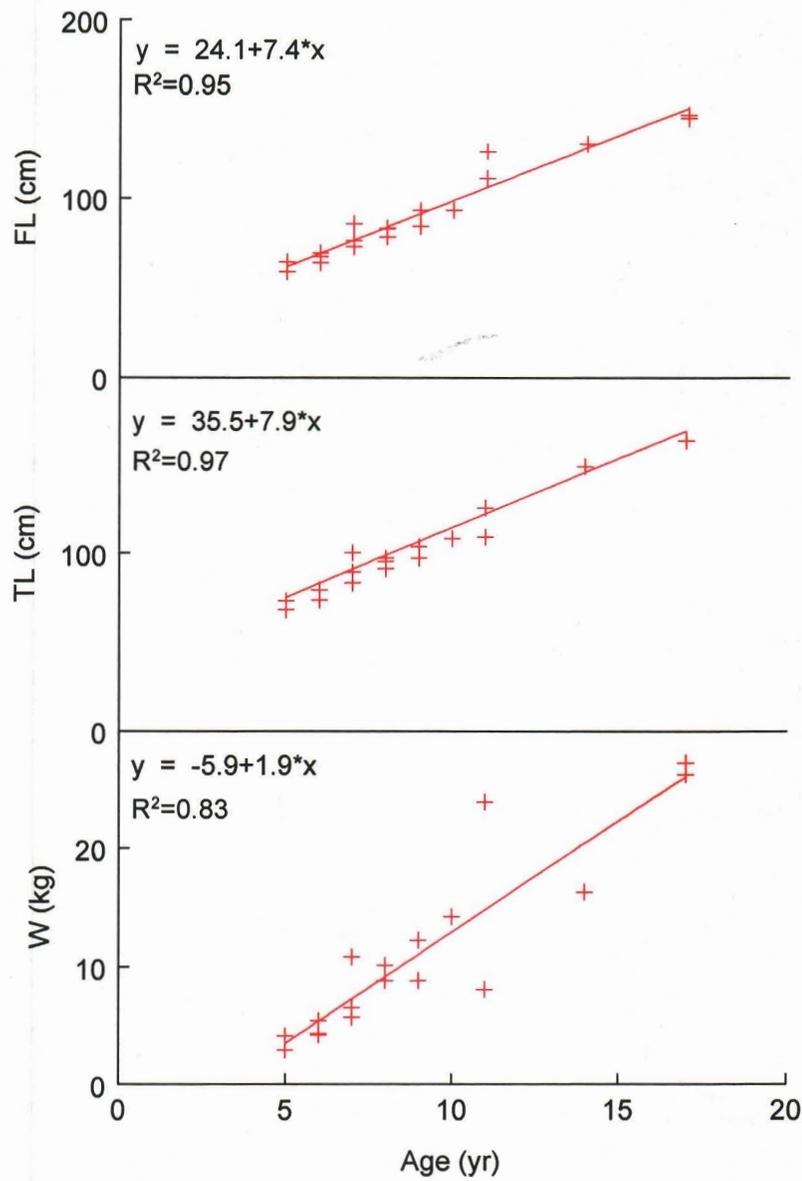


Figure 2. Scatter plots and linear regressions of the female length (fork and total) and live weight on age (n=19).

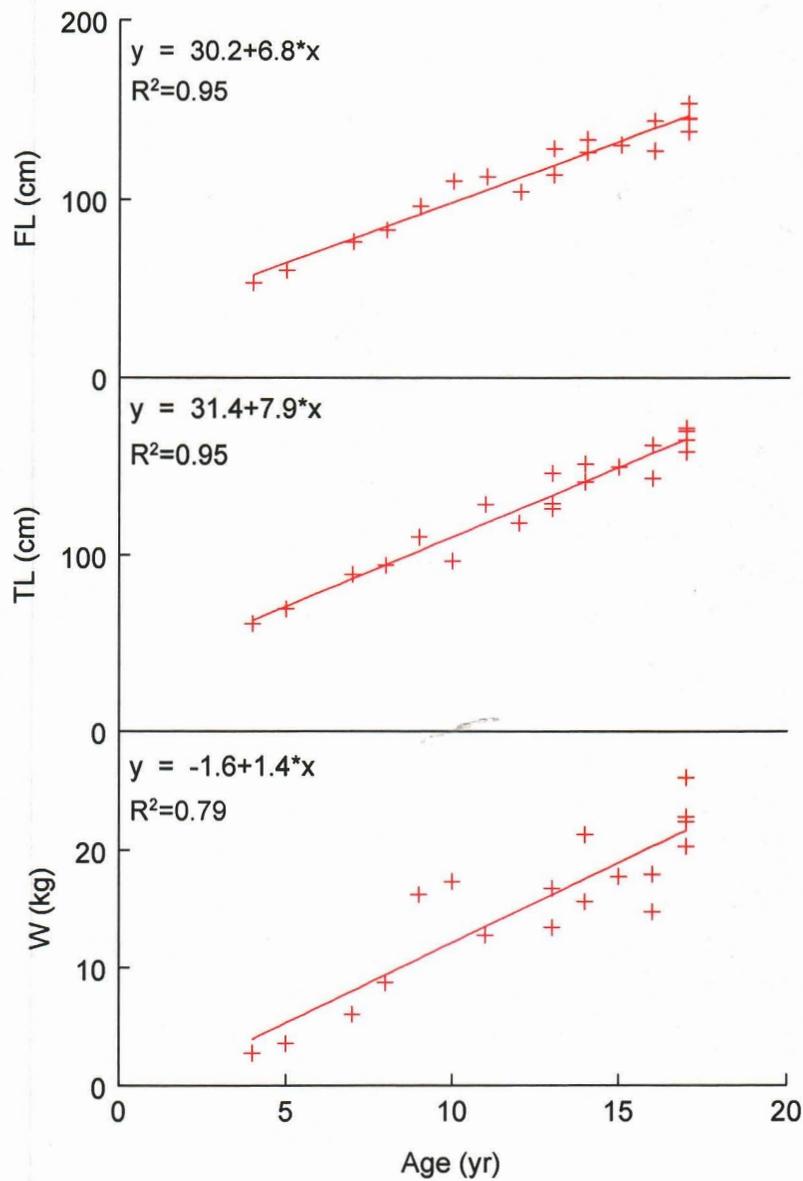


Figure 3. Scatter plots and linear regressions of the male length (fork and total) and weight on age (n=27).

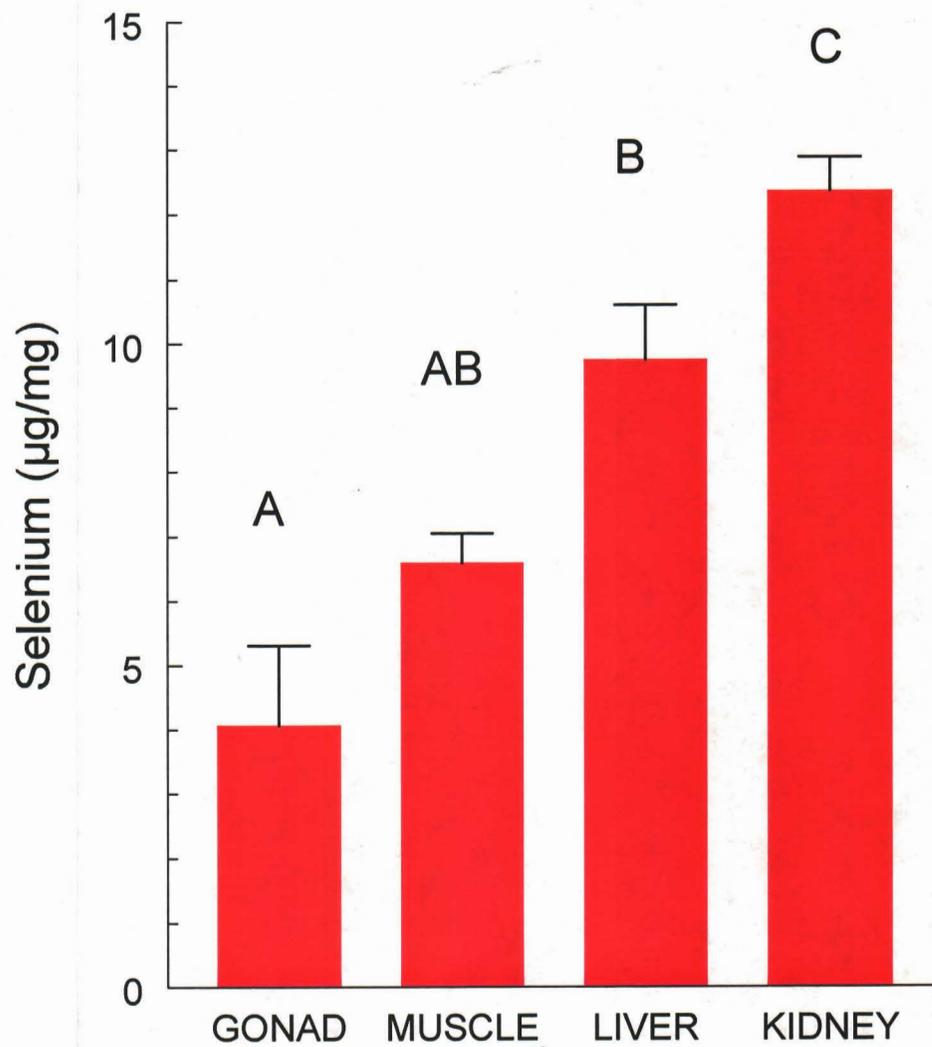


Figure 4. Selenium concentration (mean \pm SE) in different tissues of sturgeon (n = 39). Different letters indicate significantly different means ($p < 0.05$).

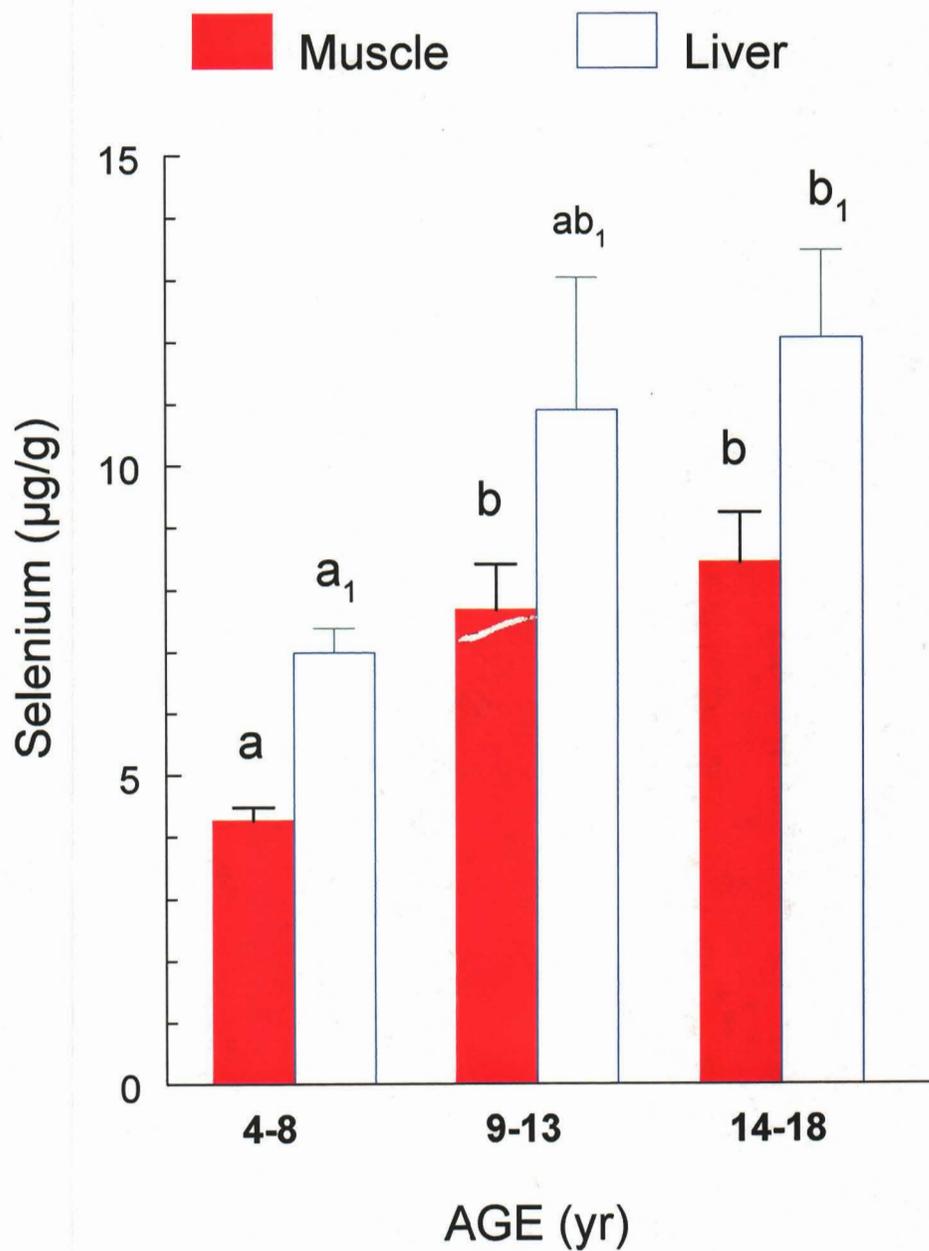


Figure 5. Selenium concentration in liver and muscle (mean \pm SE) for different age groups (n = 15, 12, and 12 fish for age groups 4-8, 9-13 and 14-18, respectively). Different letters indicate significantly different means for liver and muscle ($p < 0.05$)

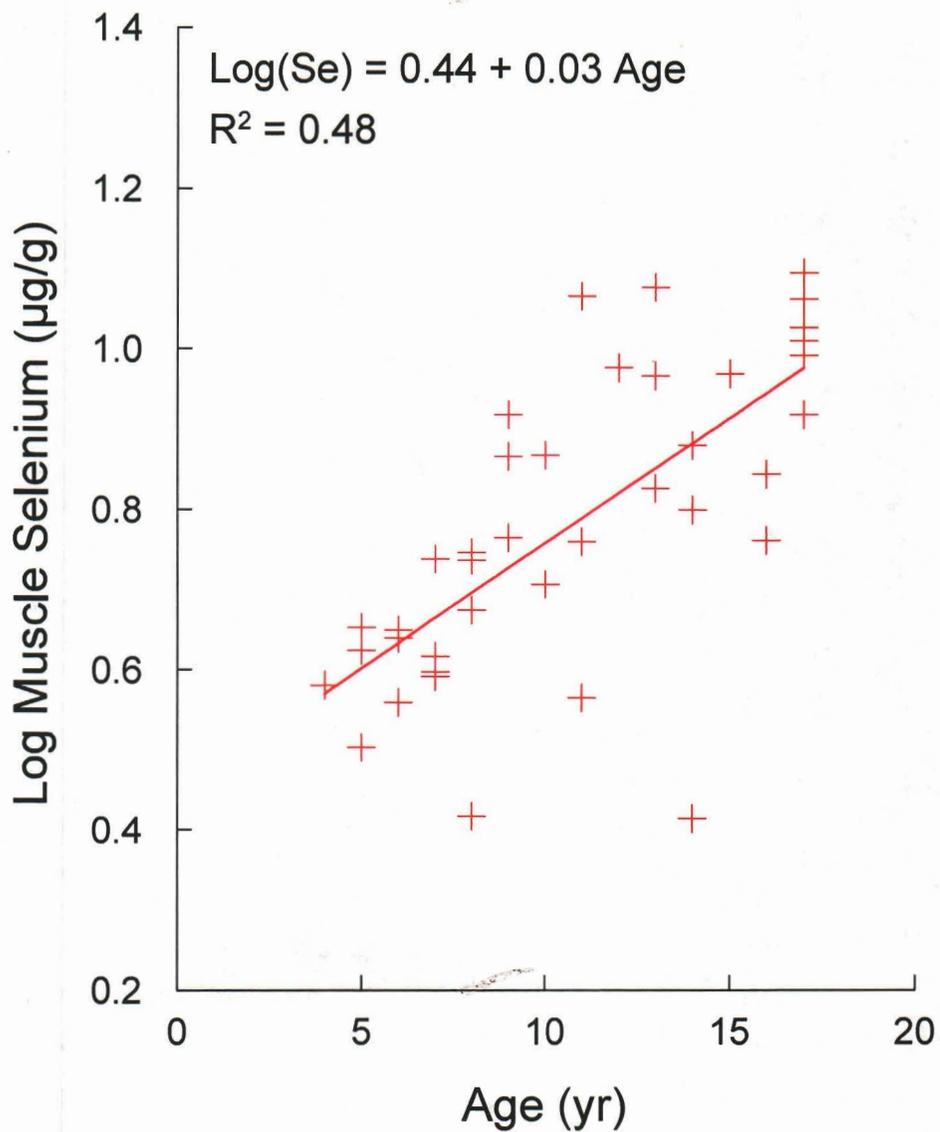


Figure 6. Scatter plot, and regression of muscle selenium concentrations on age of fish. Both regression coefficients were significant, $p < 0.001$. ($n=39$).

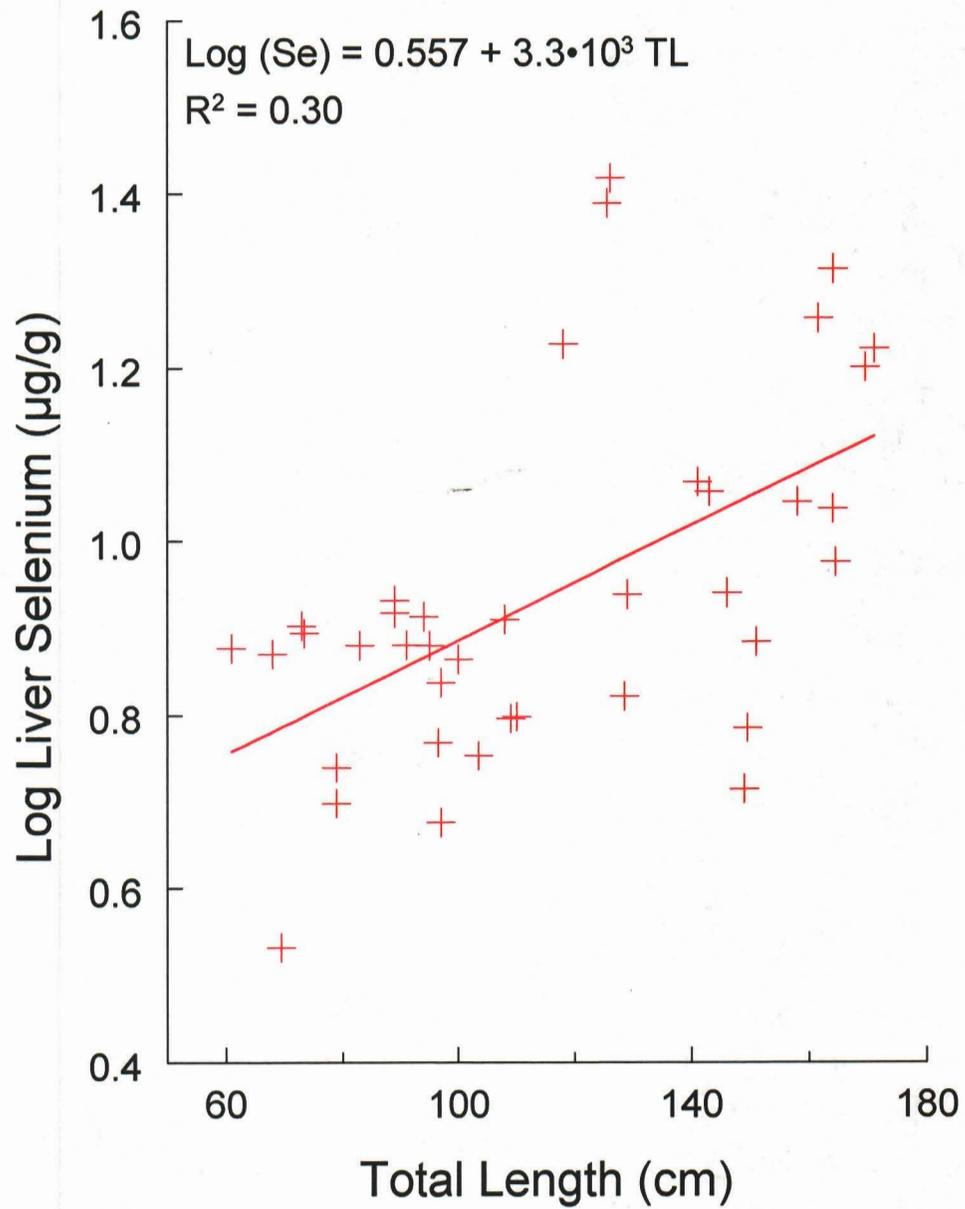


Figure 7. Scatter plot and regression line of liver selenium concentration on total length. Both regression coefficients were significant, $p < 0.001$. ($n=39$).

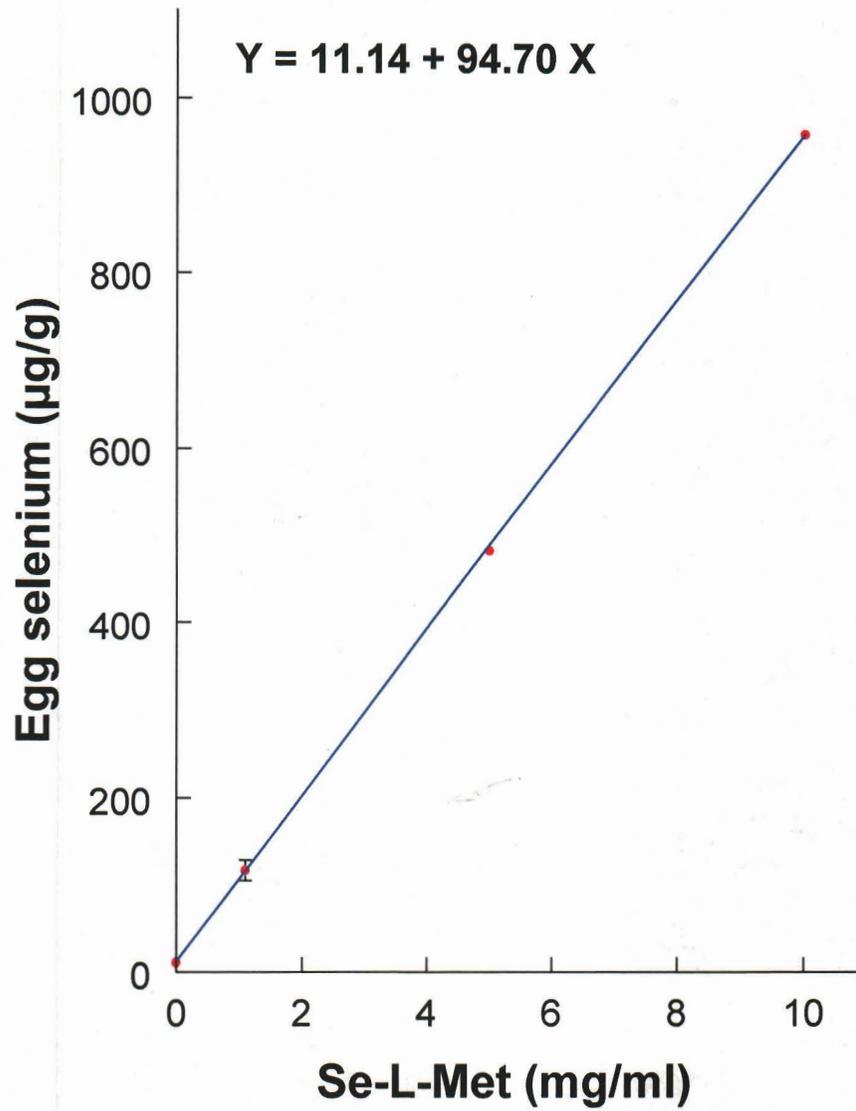


Figure 8. Selenium content ($\mu\text{g/g}$ dry weight) in the whole (with chorion) eggs of white sturgeon eggs after 2 hr water immersion exposure to Sel-L-Met in Experiment 1.

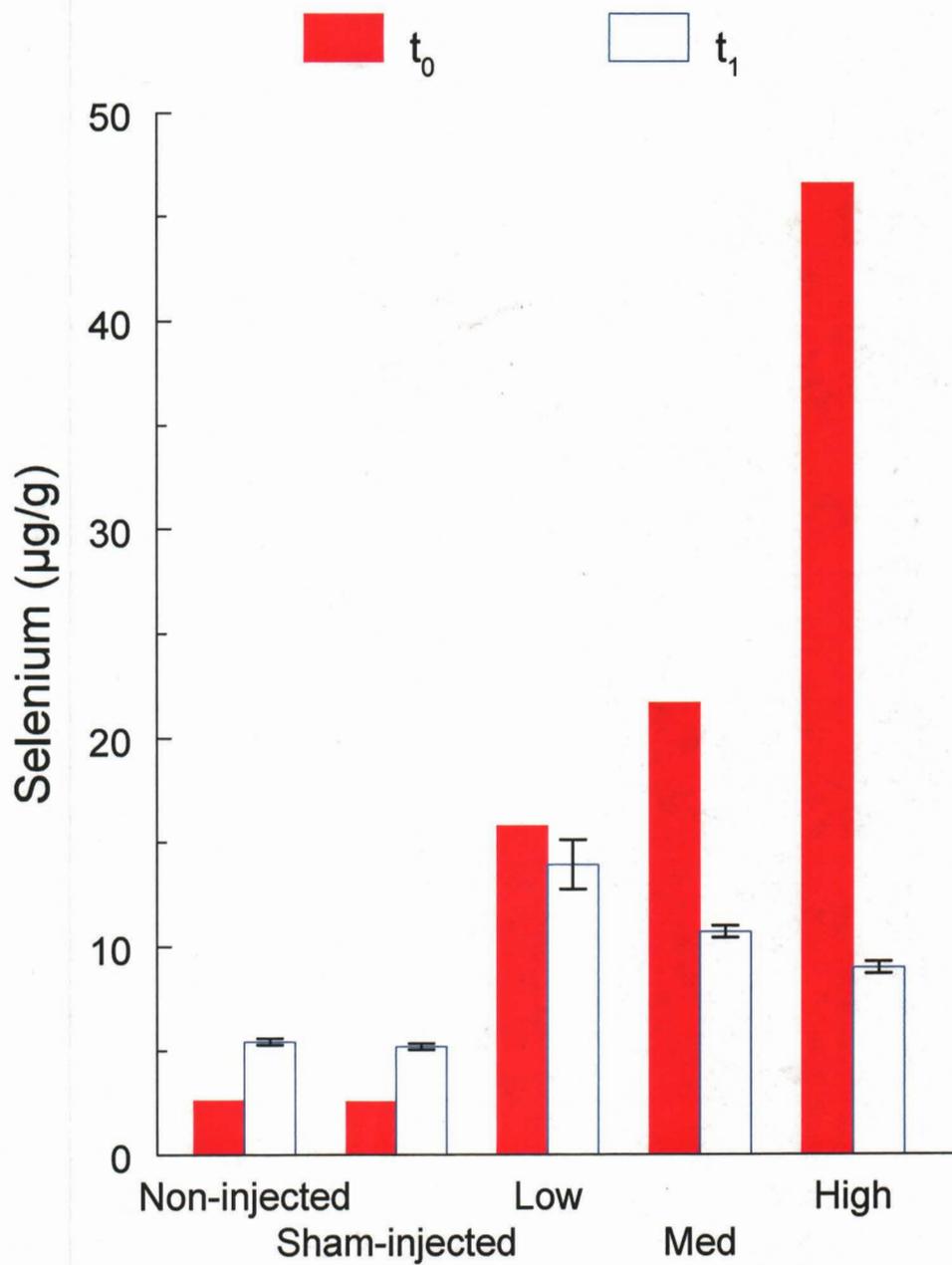


Figure 9. Mean \pm SE selenium concentration in larvae after microinjection in Experiment 2 (t_0 , $n=1$) and at the end point of the experiment (t_1 , Stage 45; $n=3$).

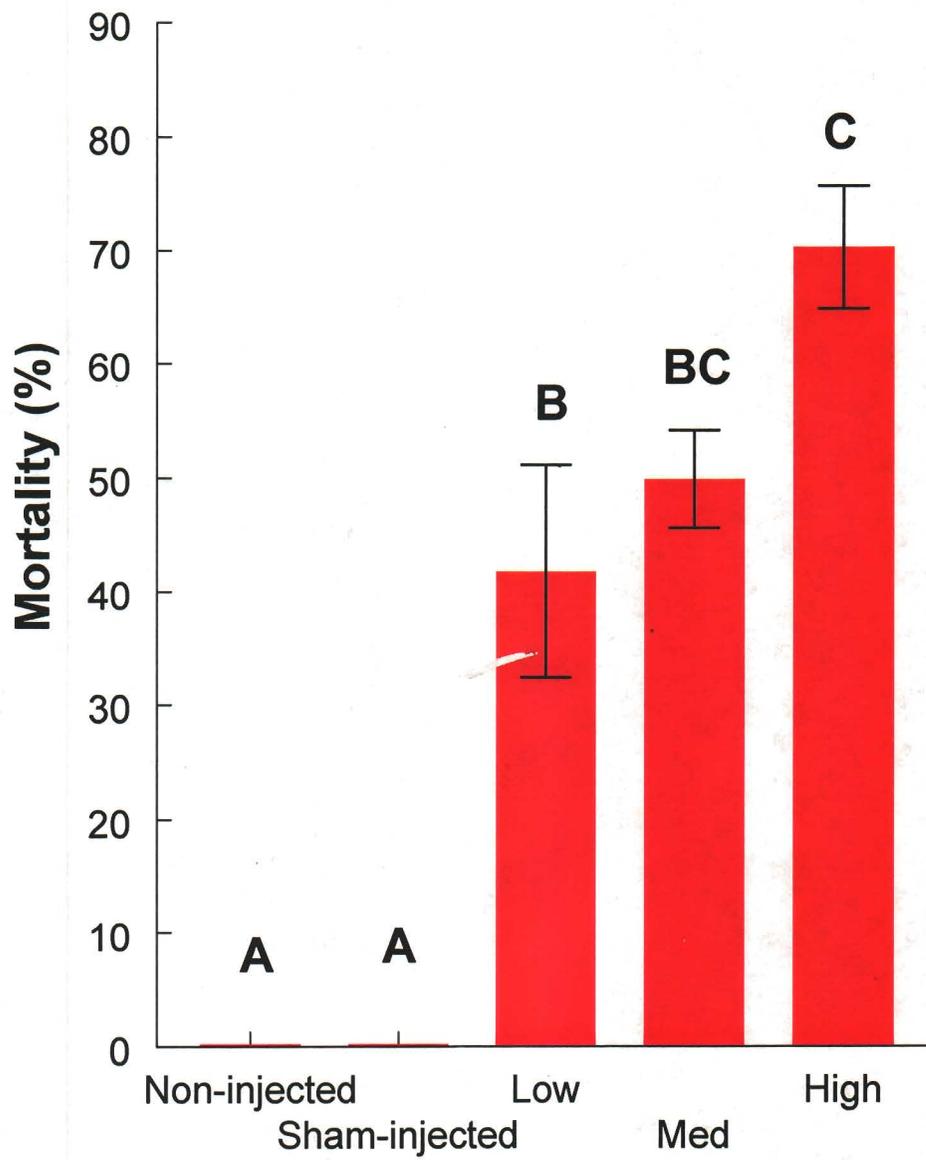


Figure 10. Effect of Se-L-Met treatment on mortality (mean \pm SE, n=3) at Stage 45 (Experiment 2). Letters above the bars show significant difference between the group means.

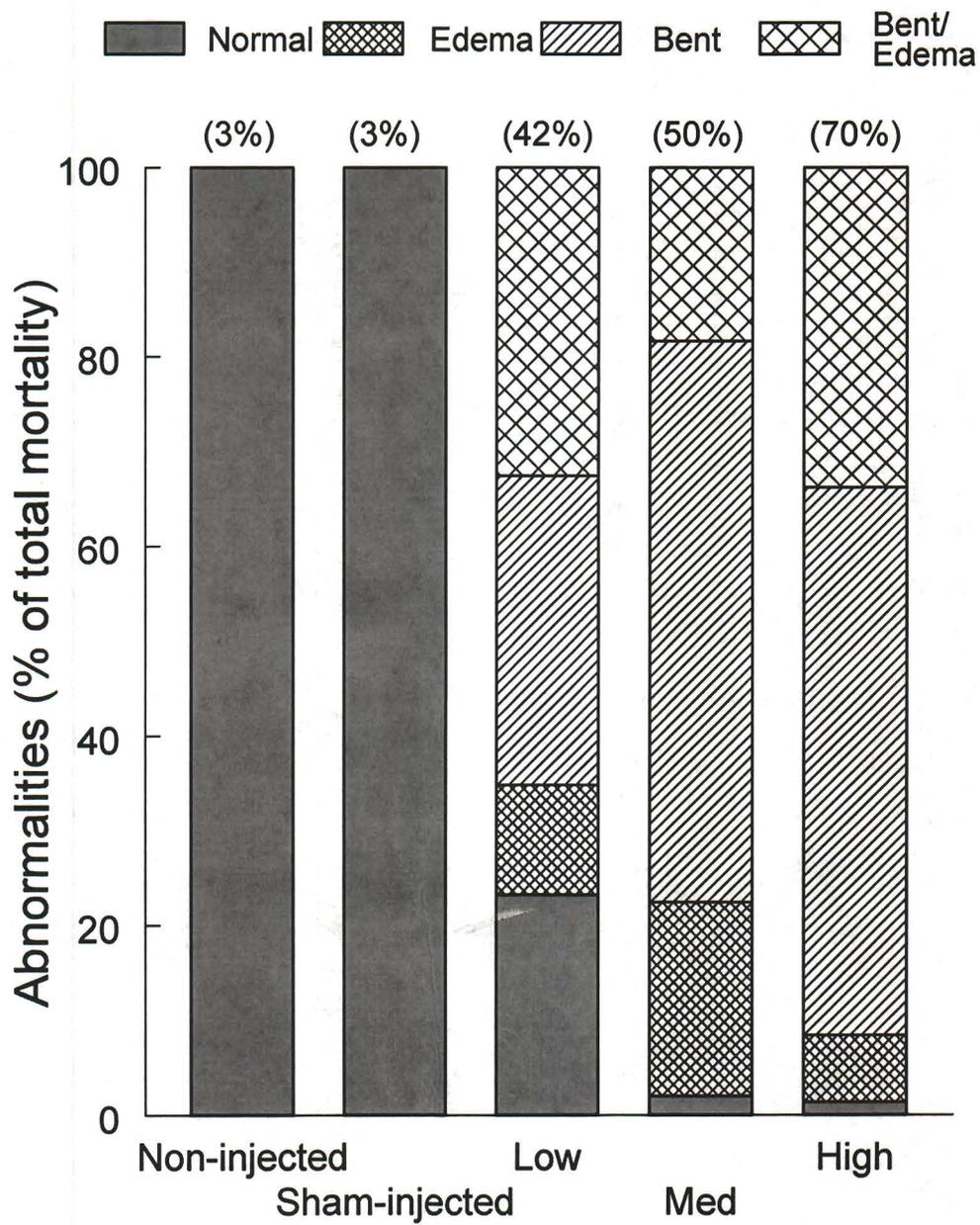


Figure 11. Experiment 2. Proportion of normal or abnormal larvae within mortalities (mean, n=3) in survival tanks at Stage 45. Percent of total mortalities is above the bars.

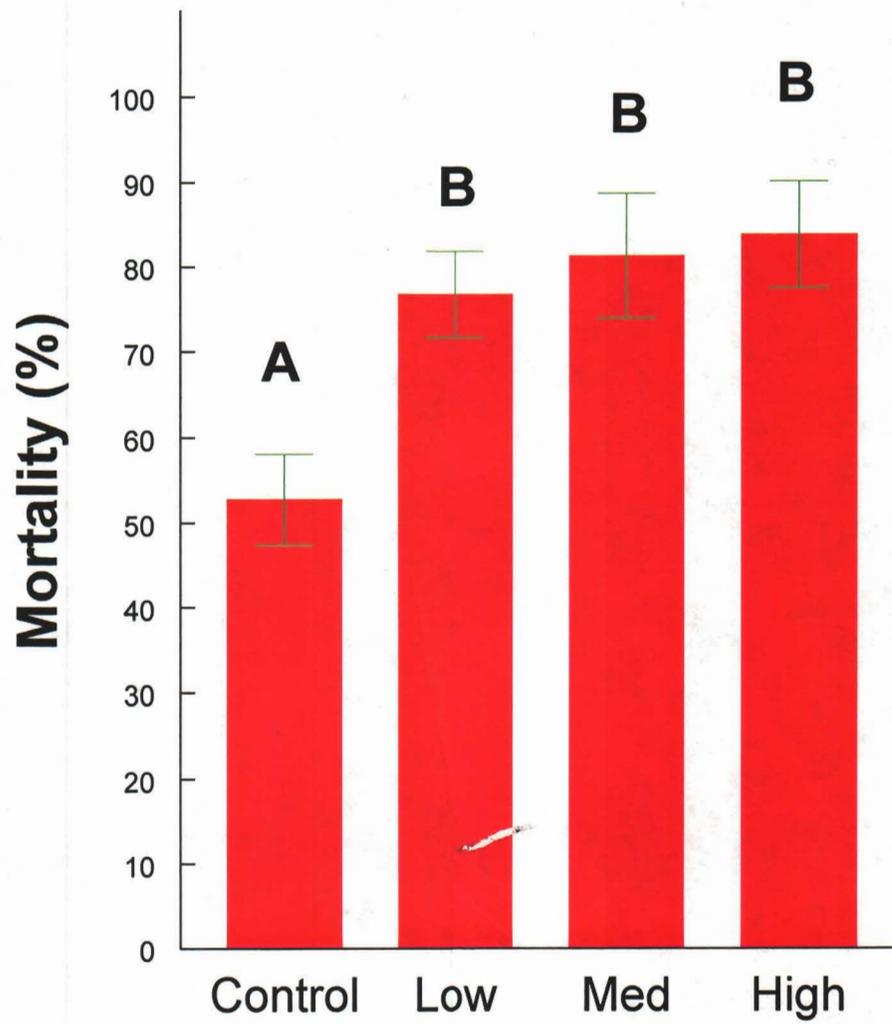


Figure 12. Effect of Se-L-Met treatment on mortality in survival tanks (mean \pm SE, n=2) at Stage 36 (Experiment 3). Letters above the bars show significant difference between group means.

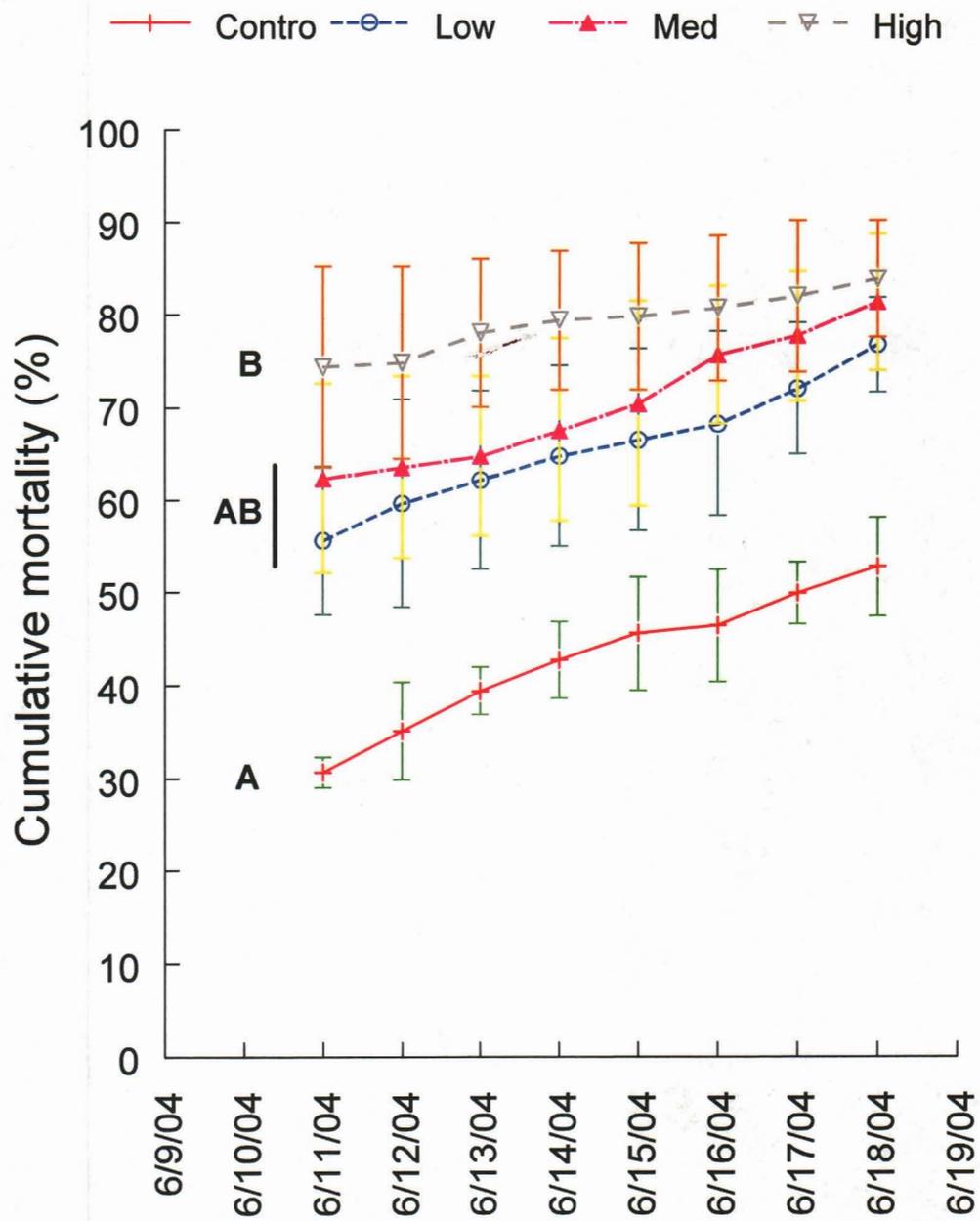


Figure 13. Experiment 3. Average cumulative mortality (mean \pm SE.; $n=2$) in treatment groups. Letters show significant difference between control and treatment groups.

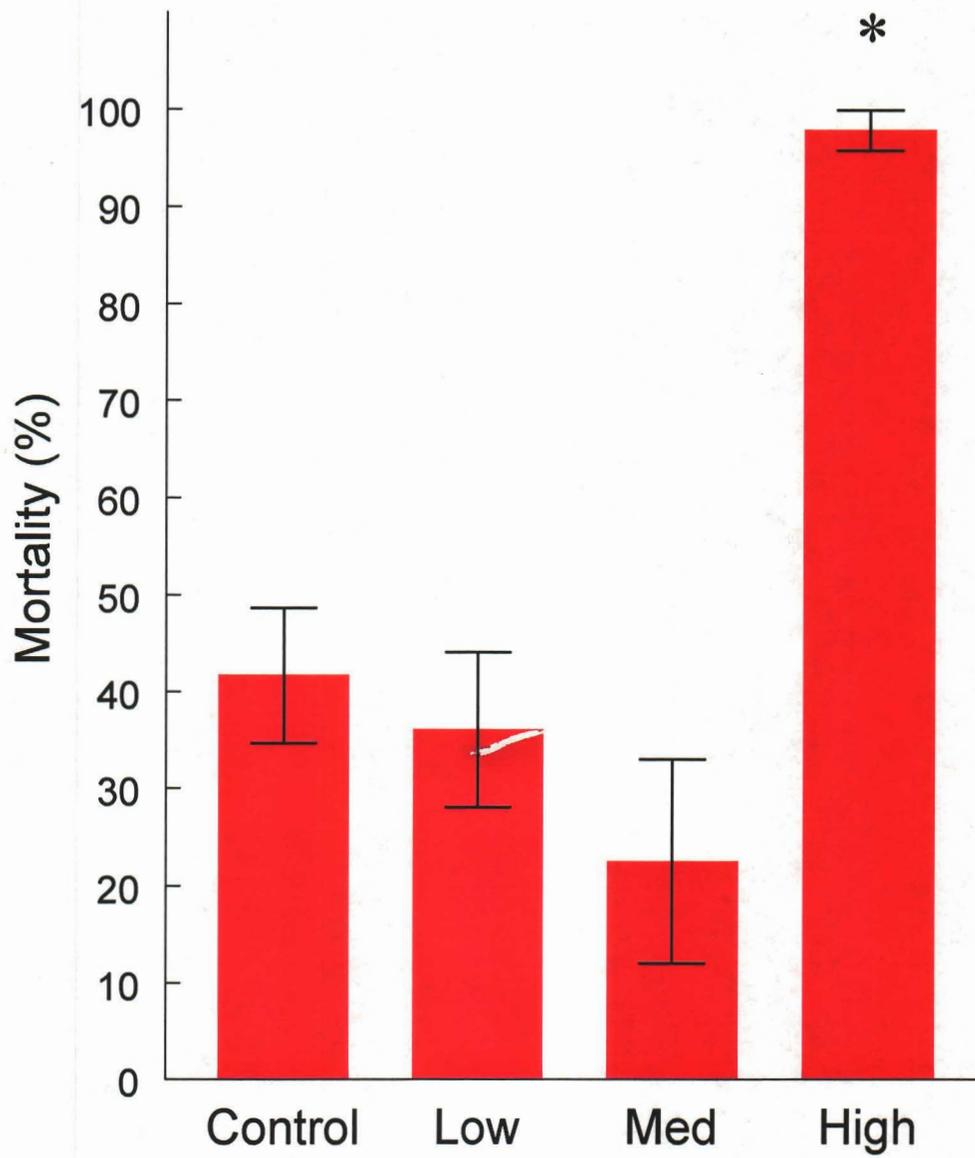


Figure 14. Experiment 4. Effect of Se-L-Met treatment on mortality (mean \pm SE, n=3) Stage 36. Asterisk above the bars indicates significantly different mean.

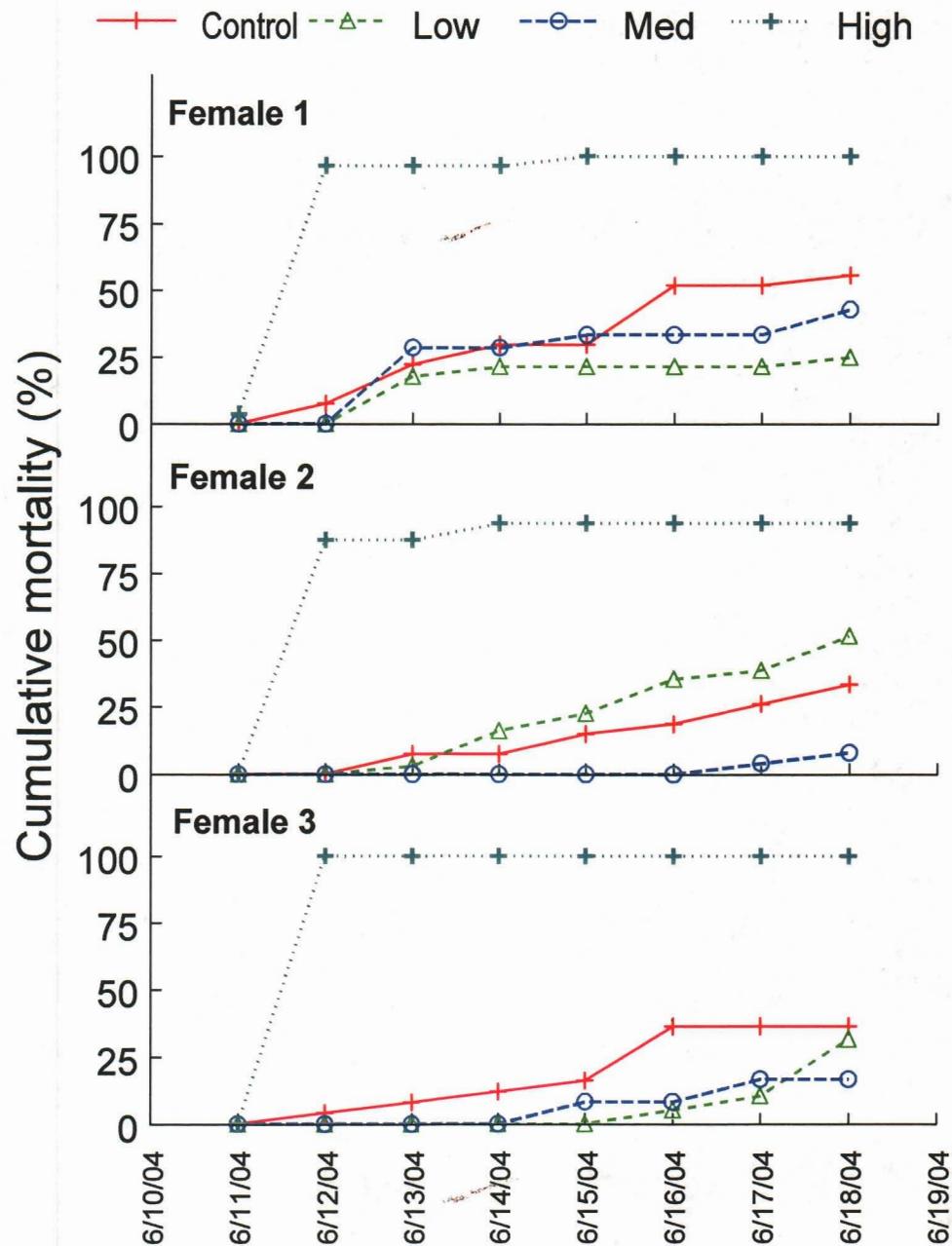


Figure 15. Cumulative mortality profiles in different treatments for the progenies of three females (Experiment 4).

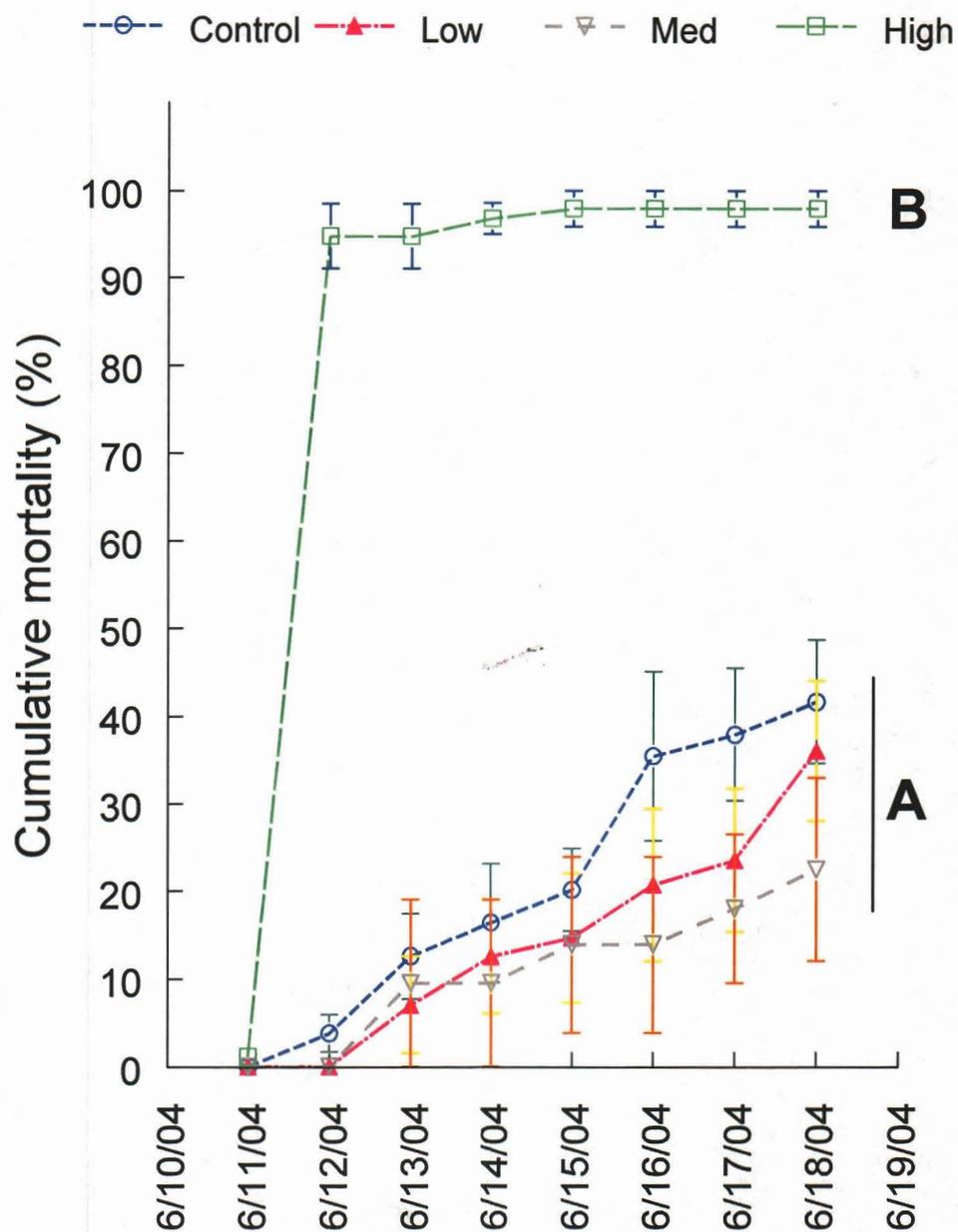


Figure 16. Cumulative mortality (mean \pm SE, $n=3$) in different treatment groups of Experiment 4. Letters show significant different treatments.

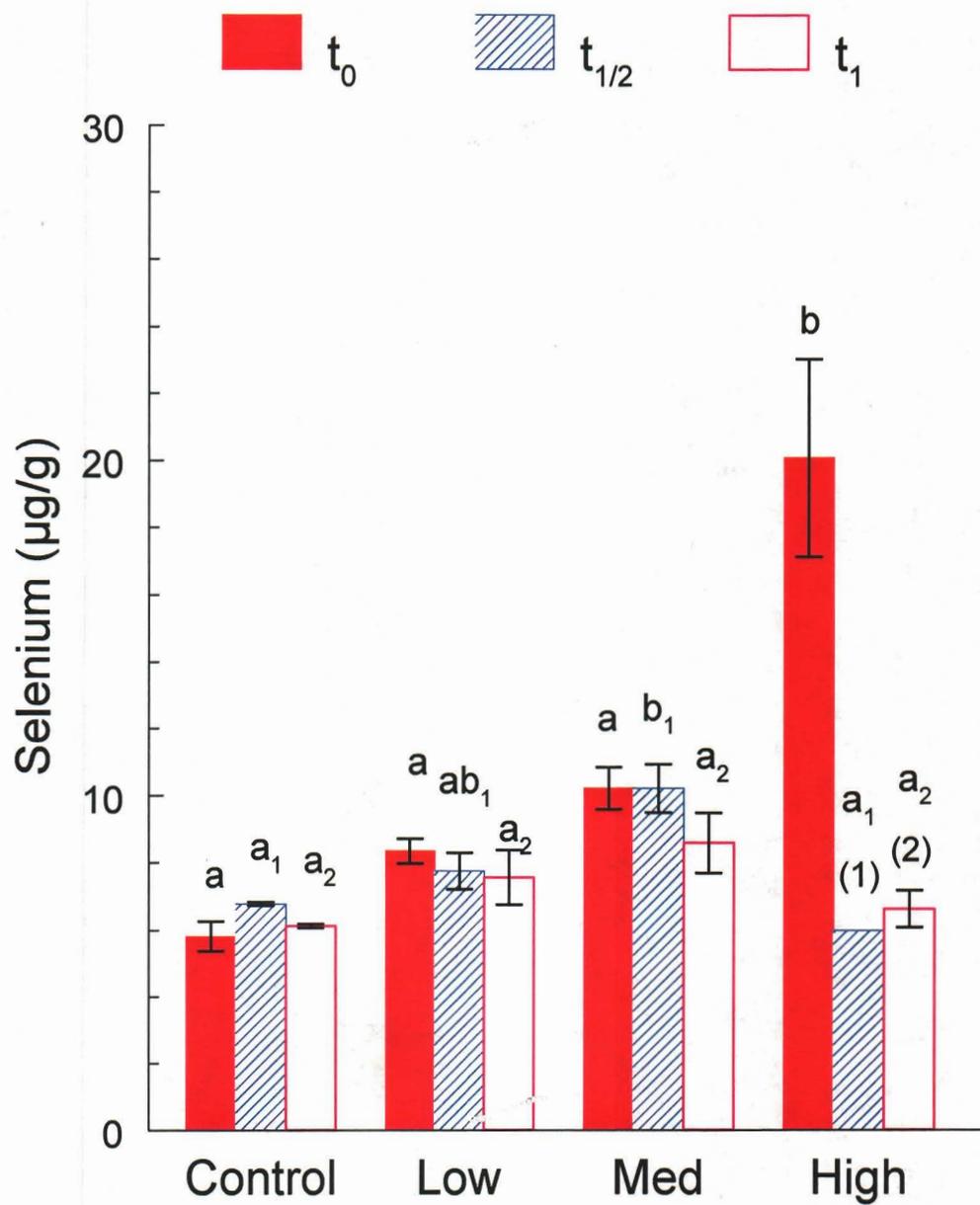


Figure 17. Selenium concentration (mean \pm SE, n=3 unless specified in parentheses) sturgeon embryos at Stages 9-10 (t₀, cleavage), 23-24 (t_{1/2}, post-neurulation), and 36 (t_{end}, hatching) injected Se-L-Met (Experiment 4). Target doses were 0 ppm (Control), 3 ppm (Low), 12 ppm (Med) and 48 (high). Letters show significantly different means at similar stage among treatment groups (p<0.05).

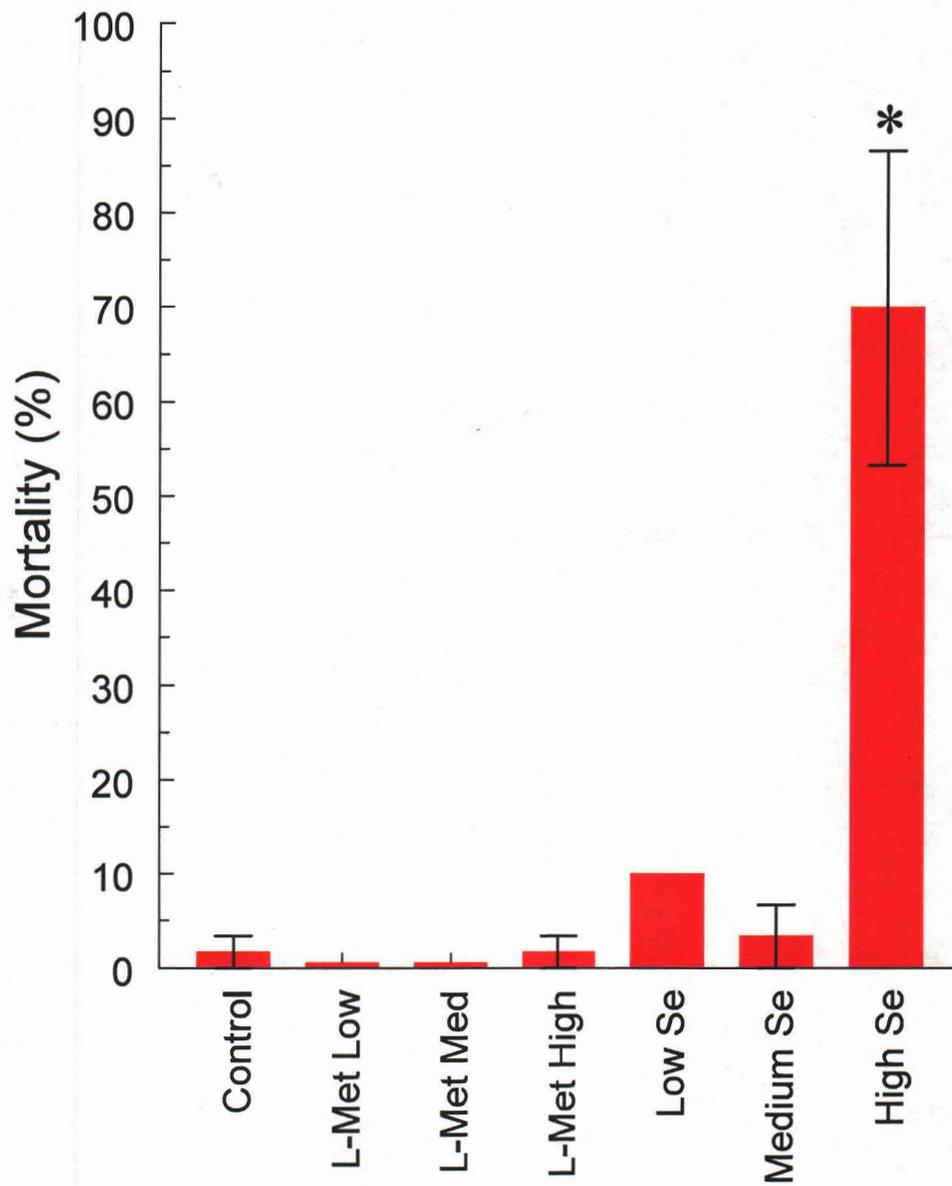


Figure 18. Effect of Se-L-Met treatment on mortality (mean \pm SE, n=2) at Stage 45 in Experiment 4. Asterisk above the bar indicates significant difference between treatment groups. Non-injected and sham-injected larvae are pooled in control.

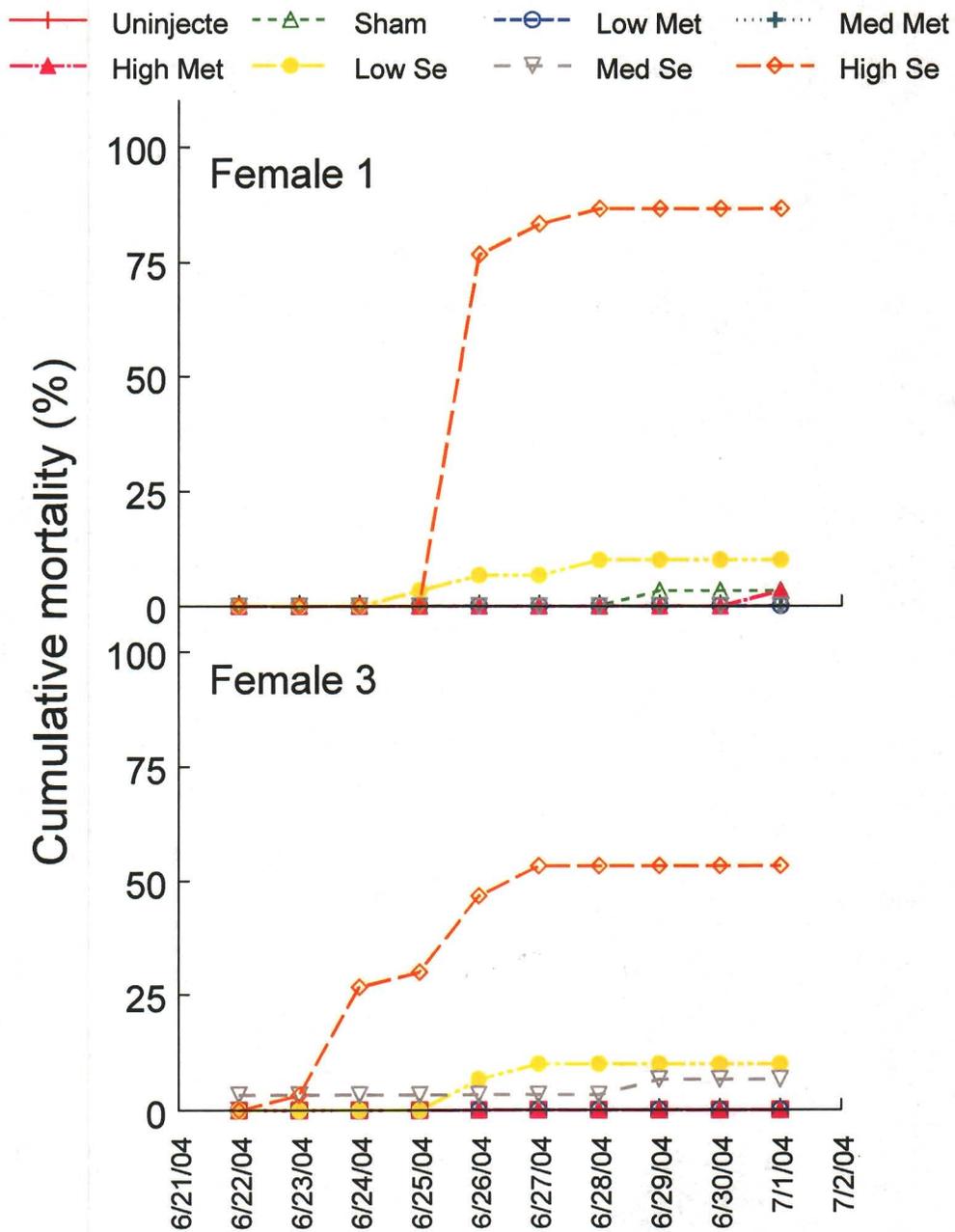


Figure 19. Cumulative mortalities in the progenies of two female, Experiment 5.

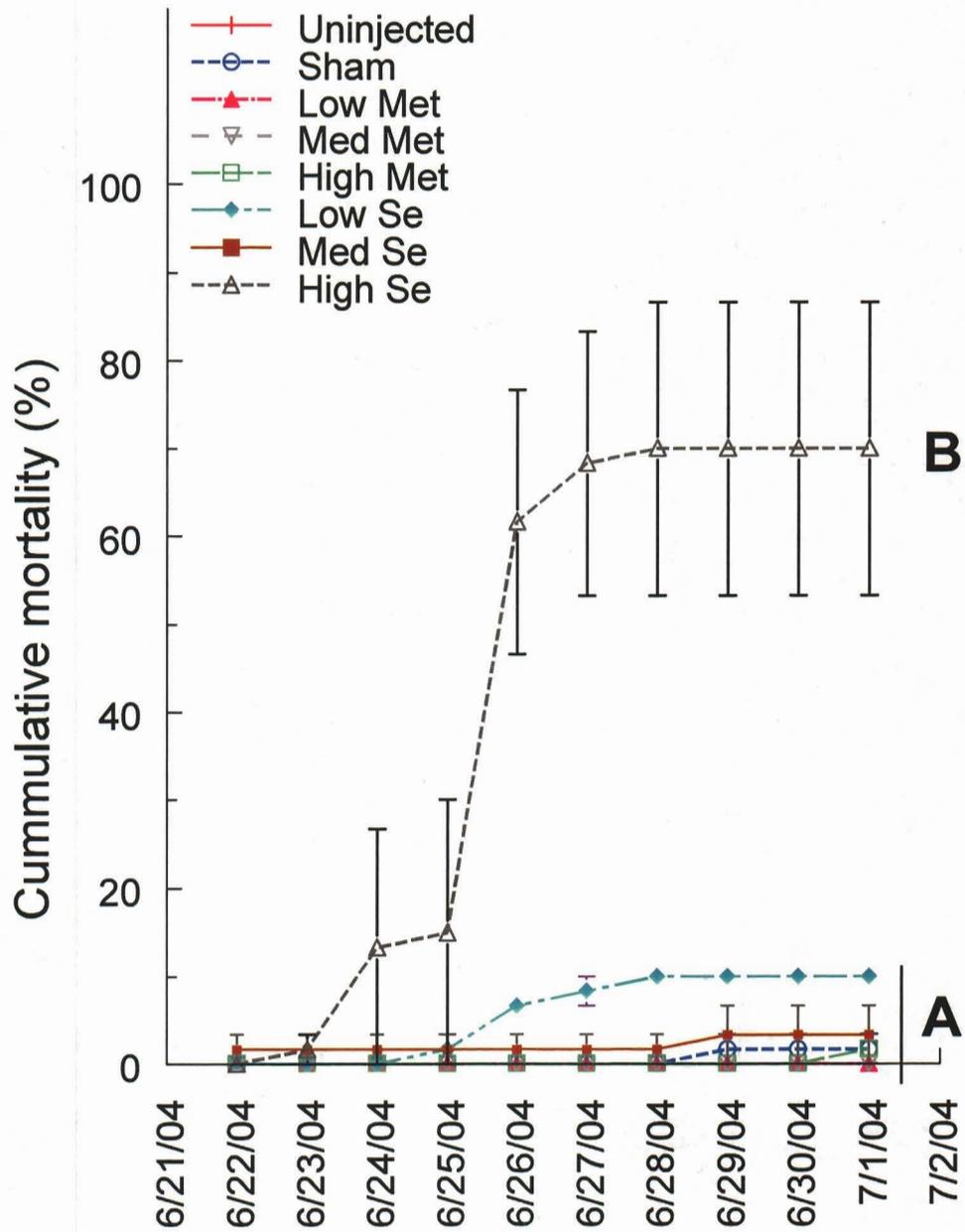


Figure 20. Cumulative mortality (mean \pm SE; $n=2$) in different treatments, Experiment 5. Letters show significantly different treatments.

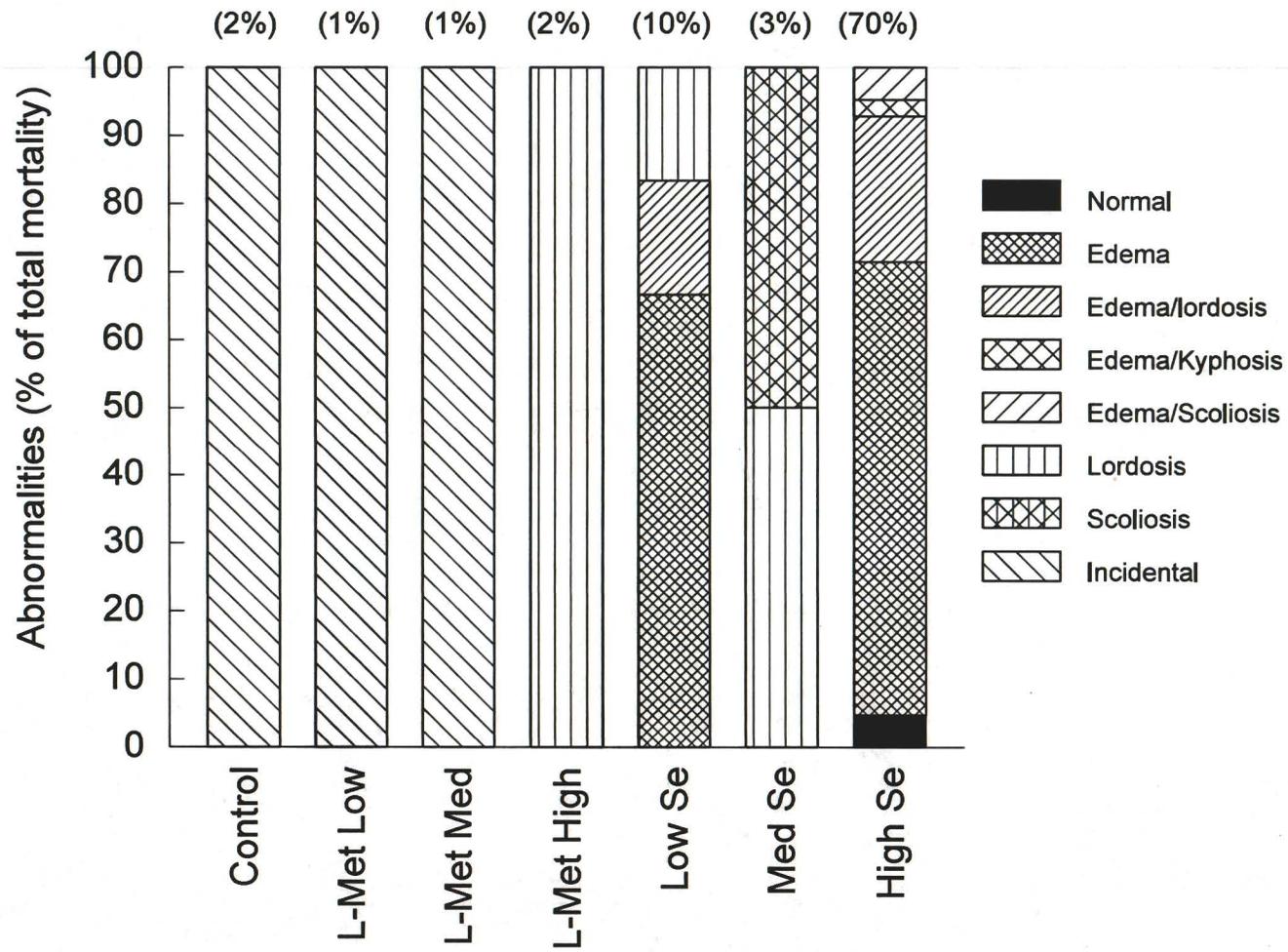


Figure 21. Abnormalities amongst total mortalities (Stage 45, yolk depletion) in survival tanks (mean, n=2). Percent of total mortalities is above the bars.

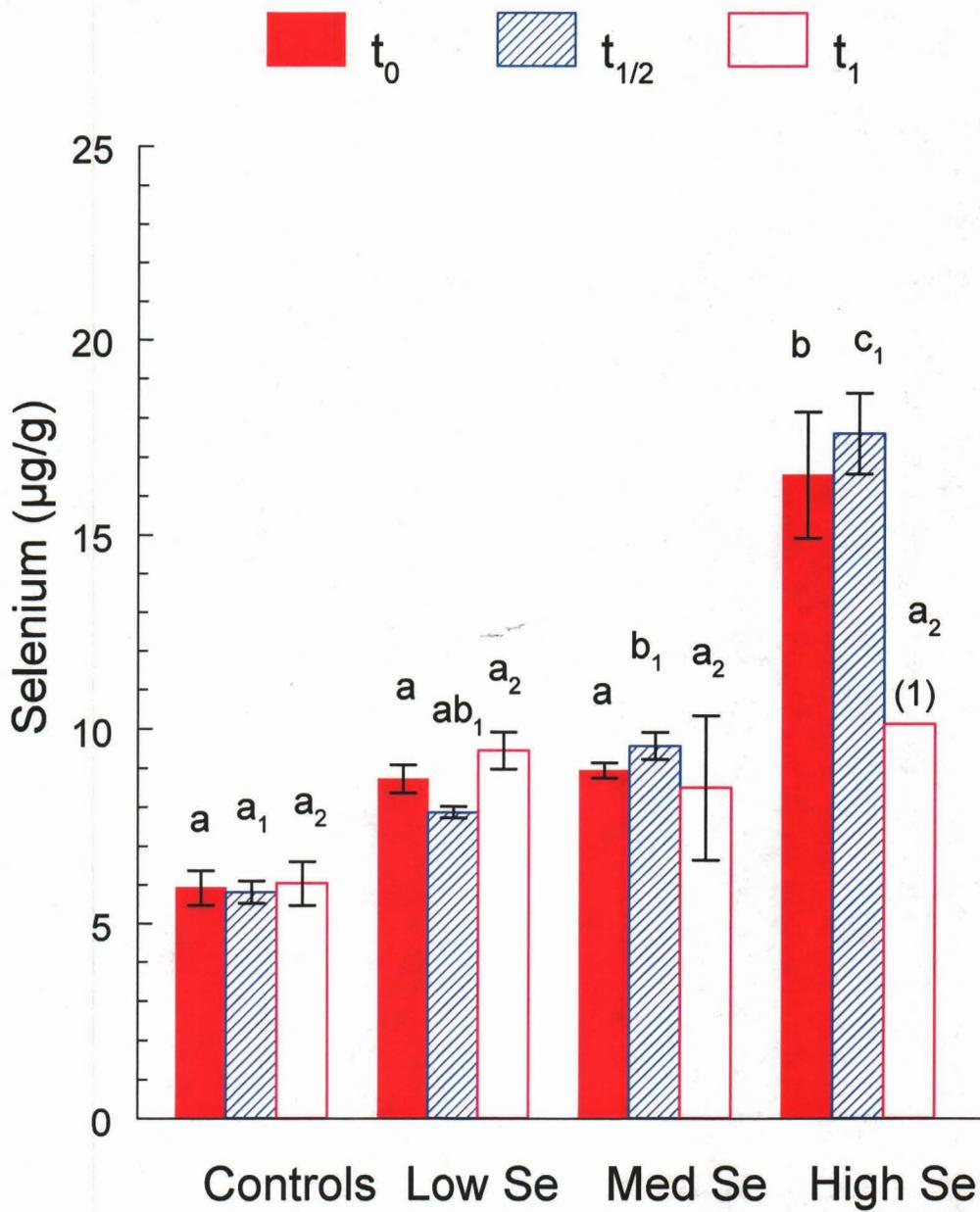


Figure 22. Larval selenium concentrations (mean \pm SE, $n=2$ unless specified in parenthesis) at Stages 36 (t_0), 40 ($t_{1/2}$), and 45 (t_1) in Experiment 5. Target doses were 0 ppm (Control), 3 ppm (Low), 9 ppm (Med) and 27 (high). Letters denote significant different means at similar stage among treatment groups ($p < 0.05$).

Appendix 1. Body size, age, capture location and selenium levels in tissues sampled, from wild-caught white sturgeon. All selenium values are in dry weight. Fish excluded from statistical analysis are marked by asterisk.

FISH #	Fork Length (cm)	Total Length (cm)	Live Weight (Kg)	Gonad Weight (g)	GSI (%)	Liver Se (µg/g)	Muscle Se (µg/g)	Kidney Se (µg/g)	Gonad Se (µg/g)	Sex	Age	Capture Location	Date of Sampling
1	na	126.0	na	na	na	26.2	11.9	16.2	2.53	M	13	Lat 38° 4.64' N Lon 122° 18.31" W	10/31/2002
2	104.0	118.0	na	na	na	16.9	9.46	8.96	Na	M	12	Lat 38° 4.84' N Lon 122° 18.59" W	10/31/2002
3	78.0	91.0	na	na	na	7.61	5.44	15	7.8	F	8	Lat 38° 4.84' N Lon 122° 18.59" W	10/31/2002
4 *	84.0	93.0	4.7	na	na	3.83	2.96	15.8	1.71	M	8	Tracy Fish Retention Facility	3/10/2003
5 *	120.0	132.0	12.85	na	na	13.9	2.86	10.6	2.57	M	13	Tracy Fish Retention Facility	3/10/2003
6 *	120.0	134.0	9.45	na	na	5.59	6.18	15.7	5.43	M	14	Tracy Fish Retention Facility	3/10/2003
7	93.0	108.0	14.2	na	na	8.14	5.07	9.4	1.69	F	10	Channel Island and Broad Slough North**	7/22/2003
8	93.0	103.5	12.2	na	na	5.67	8.27	8.77	1.12	F	9	Channel Island and Broad Slough North**	7/22/2003
9	85.5	100.0	10.8	na	na	7.33	3.9	12.8	1.23	F	7	Channel Island and Broad Slough North**	7/22/2003
10	96.0	110.0	16.2	na	na	6.3	7.33	13.7	0.77	M	9	Channel Island and Broad Slough North**	7/22/2003
11	76.0	89.0	6.5	na	na	8.28	3.95	14.1	1.42	F	7	Channel Island and Broad Slough North**	7/22/2003

Appendix 1 (continued). Body size, age, capture location and selenium levels in tissues sampled, from wild-caught white sturgeon. All selenium values are in dry weight. Fish excluded from statistical analysis are marked by asterisk.

FISH #	Fork Length (cm)	Total Length (cm)	Live Weight (Kg)	Gonad Weight (g)	GSI (%)	Liver Se ($\mu\text{g/g}$)	Muscle Se ($\mu\text{g/g}$)	Kidney Se ($\mu\text{g/g}$)	Gonad Se ($\mu\text{g/g}$)	Sex	Age	Capture Location	Date of Sampling
12	82.5	95.0	8.8	na	na	7.6	4.71	11.6	1.5	F	8	Channel Island and Broad Slough North**	7/22/2003
13	67.0	79.0	4.3	na	na	4.99	4.45	10.6	1.31	F	6	Channel Island and Broad Slough North**	7/22/2003
14	64.0	73.0	4.1	na	na	8.0	4.2	9.92	1.98	F	5	Channel Island and Broad Slough North**	7/22/2003
15	60.0	69.5	3.5	na	na	3.41	3.18	7.86	0.97	M	5	Channel Island and Broad Slough North**	7/22/2003
16	58.5	68.0	2.9	na	na	7.43	4.48	9.97	4.33	F	5	Channel Island and Broad Slough North**	7/22/2003
17	53.0	61	2.7	na	na	7.54	3.8	12.1	0.71	M	4	Channel Island, Broad Slough North and Honker Bay**	7/24/2003
18	63.5	73.5	4.2	na	na	7.85	4.35	16.8	2.65	F	6	Channel Island, Broad Slough North and Honker Bay**	7/24/2003
19	72.5	83	5.7	na	na	7.6	5.46	12.0	1.8	F	7	Channel Island, Broad Slough North and Honker Bay**	7/24/2003
20	69.0	79	5.4	na	na	5.49	3.62	10.5	1.28	F	6	Channel Island, Broad Slough North and Honker Bay**	7/24/2003

Appendix 1 (continued). Body size, age, capture location and selenium levels in tissues sampled, from wild-caught white sturgeon. All selenium values are in dry weight. Fish excluded from statistical analysis are marked by asterisk.

FISH #	Fork Length (cm)	Total Length (cm)	Live Weight (Kg)	Gonad Weight (g)	GSI (%)	Liver Se ($\mu\text{g/g}$)	Muscle Se ($\mu\text{g/g}$)	Kidney Se ($\mu\text{g/g}$)	Gonad Se ($\mu\text{g/g}$)	Sex	Age	Capture Location	Date of Sampling
21	82.5	94	8.7	na	na	8.2	5.56	14.6	1.03	M	8	Channel Island, Broad Slough North and Honker Bay**	7/24/2003
22	83.0	97	10.1	na	na	4.74	2.61	8.12	1.42	F	8	Channel Island, Broad Slough North and Honker Bay**	7/24/2003
23	76.0	89	6	na	na	8.55	4.13	14.4	1.82	M	7	Channel Island, Broad Slough North and Honker Bay**	7/24/2003
24	84.0	97	8.8	na	na	6.89	5.8	12.7	2.04	F	9	Channel Island, Broad Slough North and Honker Bay**	7/24/2003
25	110.0	96.5	17.3	na	na	5.87	7.36	13.1	0.8	M	10	Channel Island, Broad Slough North and Honker Bay**	7/24/2003
26	126.0	109	23.9	na	na	6.26	5.74	11.1	3.72	F	11	Channel Island, Broad Slough North and Honker Bay**	7/24/2003
27 *	82.0	95	4.3	na	na	20.3	4.41	9.47	7.06	M	9	Tracy Fish Retention Facility	2/26/2004
28 *	100.0	114	8.85	na	na	5.21	4.97	11.5	3.42	M	13	Tracy Fish Retention Facility	2/26/2004
29 *	128.0	147.0	12.5	na	na	16.5	5.28	12.0	5.31	M	16	Tracy Fish Retention Facility	2/26/2004

Appendix 1 (continued). Body size, age, capture location and selenium levels in tissues sampled, from wild-caught white sturgeon. All selenium values are in dry weight. Fish excluded from statistical analysis are marked by asterisk.

FISH #	Fork Length (cm)	Total Length (cm)	Live Weight (Kg)	Gonad Weight (g)	GSI (%)	Liver Se ($\mu\text{g/g}$)	Muscle Se ($\mu\text{g/g}$)	Kidney Se ($\mu\text{g/g}$)	Gonad Se ($\mu\text{g/g}$)	Sex	Age	Capture Location	Date of Sampling
30	111.0	125.5	8.05	na	na	24.5	11.6	24.6	46.7	F	11	Lat 38° 4.67' N Lon 121° 58.81" W	3/18/2004
31	143.5	161.5	17.95	82.1	0.46	18.1	6.97	14.6	15.1	M	16	Lat 38.04891 N Lon 122.21718 W	4/21/2004
32	112.5	128.5	12.75	116.75	0.92	6.65	3.67	8.63	1.73	M	11	Lat 38.04891 N Lon 122.21718 W	4/27/2004
33	144.5	169.5	22.8	132.65	0.58	15.9	11.5	14.8	4.15	M	17	Lat 38.04891 N Lon 122.21718 W	4/27/2004
34	126.0	141.0	15.6	180	1.15	11.7	6.29	10.7	1.62	M	14	Lat 38.06010 N Lon 122.21050 W	4/30/2004
35	113.5	129.0	13.4	122.95	0.92	8.69	9.24	9.41	1.33	M	13	Lat 38.06010 N Lon 122.21050 W	4/30/2004
36	145.0	164.0	27.25	2000	7.34	20.6	10.2	13.6	7.31	F	17	Lat 38.05134 N Lon 122.19477 W	5/7/2004
37	147.0	164.0	26.25	243.7	0.93	10.9	12.4	12.2	9.75	F	17	Lat 38.04524 N Lon 122.19853 W	5/9/2004
38	127.0	143.0	14.75	167.2	1.13	11.4	5.76	12.6	5.61	M	16	Lat 38.05116 N Lon 122.19489 W	5/9/2004
39	153.0	171.0	22.4	600	2.68	16.7	8.27	13.0	5.19	M	17	Lat 38.05429 N Lon 122.20550 W	5/12/2004
40	128.0	146.0	16.75	196.6	1.17	8.73	6.69	11.9	1.54	M	13	Lat 38.05570 N Lon 122.20570 W	5/15/2004
41	130.5	149.0	16.25	182.2	1.12	5.19	2.59	6.42	2.18	F	14	Lat 38.05042 N Lon 122.21807 W	5/17/2004
42	133.0	151.0	21.3	80.62	0.38	7.68	7.57	13.5	1.8	M	14	Lat 38.05619 N Lon 122.21658 W	5/19/2004

Appendix 1 (continued). Body size, age, capture location and selenium levels in tissues sampled, from wild-caught white sturgeon. All selenium values are in dry weight. Fish excluded from statistical analysis are marked by asterisk.

FISH #	Fork Length (cm)	Total Length (cm)	Live Weight (Kg)	Gonad Weight (g)	GSI (%)	Liver Se (µg/g)	Muscle Se (µg/g)	Kidney Se (µg/g)	Gonad Se (µg/g)	Sex	Age	Capture Location	Date of Sampling
43	145.0	164.5	26.1	1100	4.21	9.46	10.6	17.5	2.71	M	17	Lat 38.05619 N Lon 122.21658 W	5/19/2004
44	130.0	149.5	17.75	153.9	0.87	6.11	9.3	12.4	1.79	M	15	Lat 38.01162 N Lon 122.02272 W	5/20/2004
45	137.5	158.0	20.3	54.5	0.27	11.1	9.79	12.0	1.94	M	17	Lat 38.04419 N Lon 122.19648 W	5/24/2004
46 *	148.0	152.5	14.95	84.5	0.57	14.3	1.34	6.89	2.32	M	18	Sacramento R. at Colusa	6/15/04