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Reproductive Status of Western Mosquitofish Inhabiting Selenium-Contaminated Waters in the Grassland Water District, Merced County, California

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Abstract. This study was implemented to determine if western mosquitofish (*Gambusia affinis*) populations in the Grassland Water District suffer from impaired reproduction because of seleniferous inflows of agricultural drainwater from the Grassland Bypass Project. During June to July 2001, laboratory trials with pregnant female fish collected from two seleniferous treatment sites exposed to selenium-laden drainwater and two nonseleniferous reference sites yielded fry that averaged >96% survival at birth. In addition, none of the newborn fry exhibited evidence of teratogenesis, a typical consequence of selenium toxicity. Chemical analysis of postpartum female fish and their newborn fry indicated that mosquitofish from seleniferous sites accumulated relatively high body burdens of selenium (3.96 to 17.5 μg selenium/g in postpartum female fish and 5.35 to 29.2 μg selenium/g in their fry), whereas those from nonseleniferous sites contained lower body burdens (0.40 to 2.72 μg selenium/g in postpartum female fish and 0.61 to 4.68 μg selenium/g in their fry). Collectively, these results strongly suggest that mosquitofish inhabiting selenium-contaminated waters are not experiencing adverse reproductive effects at current levels of selenium exposure.

The Grassland Bypass Project diverts brackish, selenium-contaminated agricultural drainwater from approximately 44,000 ha of irrigated croplands on the west side of the San Joaquin Valley into the San Luis Drain (SLD) for disposal in North Mud Slough and the San Joaquin River (Quinn *et al.* 1998). Field surveys indicate that the Grassland Bypass Project is influencing selenium concentrations in fish. Whole-body measurements of selenium in green sunfish (*Lepomis cyanellus*) and bluegill (*L. macrochirus*) vary from 12 to 23 μg/g (dry weight; hereafter, unless indicated otherwise, selenium concentrations in fish will be expressed on a dry-weight basis) in the SLD, 2.4 to 11 μg/g in North Mud Slough upstream from the SLD, and 7.6 to 18 μg/g in North Mud Slough downstream from the SLD (Saiki *et al.* 2001). Recent data on selenium body burdens in western mosquitofish (*Gambusia affinis*) are not available for the SLD. However, mosquitofish from North Mud Slough average approximately 3 μg selenium/g above the SLD and 10 μg selenium/g below the SLD (Beckon *et al.* 1998). In contrast, published guidelines for selenium toxicity in fish suggest that whole-body concentrations as low as 4 μg selenium/g are associated with mortality of juvenile fish and reproductive failure (Lemly 1993, 2002).

In this study, we attempted to determine if western mosquitofish populations inhabiting selenium-contaminated surface waters in the Grasslands suffered from impaired reproductive ability. Mosquitofish were targeted for investigation because this species occurs nearly everywhere on the San Joaquin Valley floor and, because of its live-bearing mode of reproduction, there is no need to artificially fertilize and incubate its eggs. Moreover, female mosquitofish from the SLD exhibited a high incidence of stillbirths and abnormal young during 1984 to 1985 when dissolved concentrations of selenium exceeded 300 μg/L or approximately 10 times higher than at present (Saiki and Ogle 1995).

Specific objectives of this study were as follows: (1) determine if reproductive success (percent survival of newborn fry) varies among adult female western mosquitofish collected from seleniferous versus nonseleniferous habitats; (2) compare brood size (numbers of fry) and lengths and weights of newborn fry produced by adult female fish; and (3) determine if selenium concentrations in newborn fry are associated with body burdens of this element in postpartum female fish.

Materials and Methods

Western mosquitofish were captured with dip nets, minnow seines, minnow traps (baited with bread), and lift nets from the SLD at Gun Club Road (a selenium-contaminated site), from North Mud Slough at Gun Club Road (MSN1; a reference site located 4.6 km upstream from the mouth of the SLD that has not received seleniferous drainwater since the Grassland Bypass Project became operational); from North Mud Slough at State Highway 140 (MSN2; a selenium-contaminated

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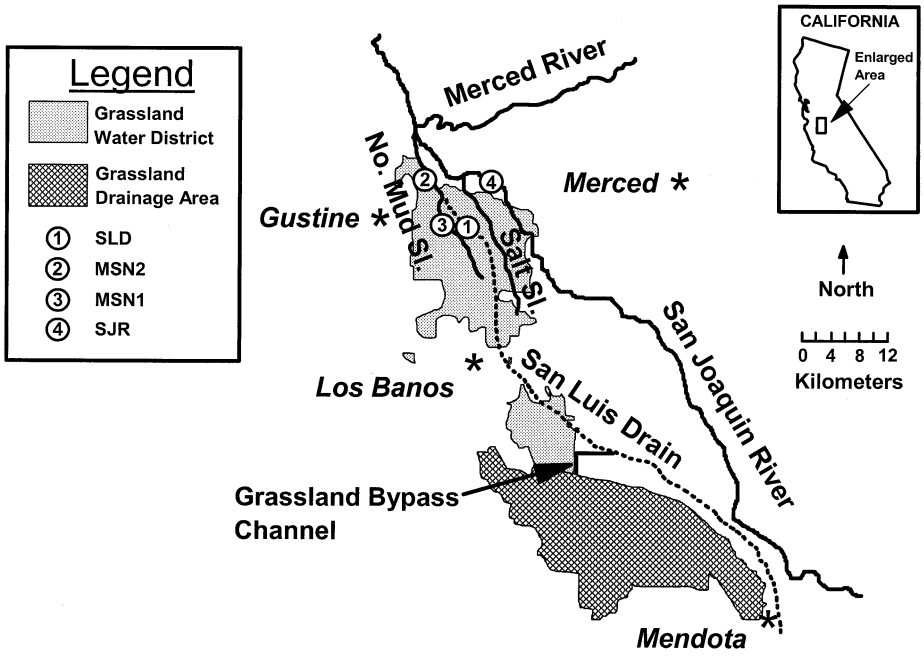

Fig. 1. Map of the study area

site located 6.2 km downstream from the mouth of the SLD); and from the San Joaquin River at Lander Avenue (SJR; a reference site with no history of receiving seleniferous drainwater; Figure 1). Mosquitofish for the first of two trials were collected on June 18, 2001. However, after female fish from SJR experienced high mortality during and immediately after transport to Dixon because of failure of the aeration system in the holding tank, new fish from this site were collected on June 27. Mosquitofish for the second trial were collected on July 16. In all instances, mosquitofish were held overnight in aerated holding tanks and used in experiments only if mortalities in each tank were negligible (<5%).

With 1 exception, 20 pregnant female fish (identified by prominent dark-colored spots on each side of the abdomen) from each sample site were held for as long as 2 weeks in individual 2-L glass beakers to quantify live and dead births and to make other measurements. Western mosquitofish from SLD were exceptional because only 16 female fish were captured in June, and just 1 female fish was captured in July. Within each beaker, the female fish was placed into a small-mesh livebearer enclosure to decrease the likelihood of cannibalism after the birth of the young. Water in each beaker was gently aerated with an air stone and, at 48-h intervals, approximately two thirds of the volume was replaced with fresh site water from where the female fish had been captured. Water temperature in the beakers, which averaged 21.1°C to 22.1°C, was regulated with a thermostatically controlled central air conditioner. Solid waste was siphoned off during water changes. Fish were fed daily to satiation with a commercial diet (TetraMin Tropical Flakes; Tetra Sales [distributors], Blacksburg, Virginia). Other details of the experimental procedure were previously reported by Saiki and Ogle (1995).

When female fish gave birth, both the female fish and their newborn fry were removed from glass beakers and placed into clean glass petri dishes. Live and dead fry were counted, and then the female fish and live fry were killed by overdosing with MS-222 (Finquel brand of tricaine methanesulfonate; Argent Chemical, Redmond, Washington). All female fish and fry were measured for total length and weight, and the live and dead fry were visually examined under low magnification with a binocular microscope for gross evidence of external abnormalities (teratogenic symptoms such as spinal curvature; missing or deformed fins, eyes, and mouths; and edema; Lemly 1997). Every third female fish and her fry were saved for selenium determinations (the female fish and as many as three five-fry composites were packaged in sterile polyethylene cryovials and immediately frozen at −10°C in a freezer); for gut analysis for cannibalized fry (only female fish were preserved in 10% buffered formalin); or archived by initially fixing the female fish and her fry in Davidson's solution, then transferring the specimens after 2 days to 99% isopropyl alcohol. Excess fry from broods targeted for selenium determinations and all fry from female fish designated for gut analysis were also archived.

In October 2001, a total of 37 postpartum female fish, 90 composite samples of newborn fry, and subsamples of crystalline MS-222 destined for selenium determinations were shipped on dry ice by overnight carrier to the Columbia Environmental Research Center (CERC) in Columbia, Missouri. In addition, subsamples of TetraMin Tropical Flakes were shipped unfrozen to CERC. At CERC, the fish samples and MS-222 were transferred into small 1.5-ml high-density polyethylene vials provided by the University of Missouri Research Reactor (MURR) in preparation for lyophi-

Table 1. Reproductive success (percent live births) of female western mosquitofish from various sites and trials

Site or *F*	Trial No.	Mean Live Births (%)
MSN1	1	99.2
MSN1	2	98.8
MSN2	1	96.6
MSN2	2	>99.9
SJR	1	99.2
SJR	2	99.2
SLD	1	99.6
SLD	2	—[a]
$F_{6,106}$		0.99 (NS)

[a] No data. Only one female fish was captured from the SLD during trial 2, and she did not give birth.
NS: Not significant, MSN: North Mud Slough, SJR: San Joaquin River, SLD: San Luis Drain.

lization and selenium measurement by neutron activation analysis. Two certified reference tissues [Institute for Reference Materials and Measurements Certified Reference Material 422 cod muscle ($N = 3$) and National Research Council Canada DORM-2 dogfish muscle ($N = 3$)] were included for CERC internal quality control. Lyophilization at CERC was accomplished with a Virtis Genesis 35 EL lyophilizer (SP Industries, Gardiner, New York). All dried samples were then transported to MURR for determination of selenium. At MURR, samples of National Institute Standard Reference Material (SRM) 1577 bovine liver were included for internal quality control. Each standard or sample was placed in a shuttle rabbit and irradiated via a fast pneumatic tube system (Morris *et al.* 1981) using an irradiation position that exhibited thermal and epithermal neutron flux densities of 8×10^{13} n/cm^2/s and 2×10^{12} n/cm^2/s. The shuttle rabbit was returned to the laboratory where the sample vial was removed and transferred to the appropriate counting geometry. All samples were analyzed after a 5-s irradiation using a 15-s decay and 25-s real-time count with a high-resolution gamma-ray spectrometer. The gamma-ray spectrometer quantified selenium concentrations by measuring decay of selenium-77m with the standard comparator method (Baskett *et al.* 1993, 2001).

Accuracy and precision of the neutron activation technique were assessed from reference materials submitted by CERC with the samples of western mosquitofish and MS-222 and from MURR's internal quality-control samples. Recovery of selenium from reference materials was excellent, with results approaching or falling within certified range values. Method precision as determined from replicate tissue analyses varied from 1.1% to 5.3% relative standard deviation (%RSD). Variance in selenium concentrations measured in composite samples of mosquitofish fry from the same parental female fish was generally ≤22%. All quality-control results were considered to fall within acceptable limits as specified by CERC.

The subsamples of TetraMin Tropical Flakes were analyzed by CERC for moisture content by lyophilization and for selenium by a dry-ash procedure with hydride generation flow injection atomic absorption spectroscopy (Brumbaugh and Walther 1989; May *et al.* 2001). A dogfish muscle certified reference tissue (National Research Council Canada DORM-2) analyzed with the fish food yielded a selenium recovery of 106%. When fish food was spiked with organic selenium, recovery was 108%, whereas when spiked with inorganic selenium, recovery was 110%. Variance in selenium concentrations measured in subsamples of the TetraMin Tropical Flakes was 0.8% RSD.

The MS-222 contained undetectable (<0.010 μg/g) concentrations of selenium. In contrast, the TetraMin Tropical Flakes contained 1.02 to 1.03 μg selenium/g, which is similar to concentrations in net plankton, chironomid larvae, and amphipods from reaches of the San Joaquin River and its tributaries that do not receive seleniferous inflows of agricultural drainwater (Saiki *et al.* 1993).

Raw data were tabulated as Microsoft Excel files, then imported into SAS software (version 8.1; SASI, Cary, North Carolina) for statistical comparisons. All data were angular-transformed (if percentages) or logarithmically transformed before computing means and performing statistical tests of significance (e.g., Pearson product-moment correlation; analysis of variance [ANOVA]; and analysis of covariance [ANCOVA]). Unless indicated otherwise, the probability of type 1 error for all statistical tests was specified as $p = 0.05$. When F values for ANOVA were significant, we used Tukey's Studentized range test to compare geometric means for statistical differences. Before conducting ANCOVA, we verified that relations between covariates were parallel (Kleinbaum and Kupper 1978). When F values for ANCOVA were significant, we compared least-squares means by using individual Student t tests with p values adjusted for the number of simultaneous comparisons (a Bonferroni-type approach).

Table 2. One-way ANCOVA for mean brood size of adult female western mosquitofish from various sample sites and trials: The covariate is the total length of the adult female[a]

Sample Site or F	Trial No.	Mean Brood Size[b]
MSN1	1	20.1 B
MSN1	2	13.3 A
MSN2	1	20.9 B
MSN2	2	18.9 B
SJR	1	21.8 B
SJR	2	23.9 B
SLD	1	23.0 B
SLD	2	—[c]
$F_{6,112}$		3.63*

[a] Before ANCOVA was conducted, all regression lines under comparison were determined to be parallel ($F_{6,112} = 0.62$, $p = 0.7143$).
[b] Least-squares means followed by the same capital letter are not significantly different according to individual Student t tests where p values are adjusted for the number of simultaneous comparisons.
[c] No data. Only one female fish was captured from the SLD during trial 2, and she did not give birth.
* $p = 0.0026$.
MSN: North Mud Slough, SJR: San Joaquin River, SLD: San Luis Drain.

Results and Discussion

Survival of Newborn Fry

A total of 113 adult female western mosquitofish gave birth to 2715 fry during the 2 trials. On average, the percentage of live births was high at both seleniferous sites (96.6% to 99.9%) and nonseleniferous sites (98.8% to 99.2%; Table 1). These results contrast with those reported by Saiki and Ogle (1995) when selenium concentrations in adult female fish from the SLD averaged at least 5 times higher than at present. In the earlier study, fry survival averaged 70% to 77% in female fish from the SLD and 97% to 99% in female fish from the Volta Wildlife Area, a nonseleniferous reference site (Saiki and Ogle 1995).

Except for a low frequency of aborted larvae (fry containing prominent yolk sacs) and stillborn fry, no obvious anomalies (e.g., missing or deformed eyes, deformities in fins or other external appendages, edema) were observed during the present study. In contrast, many dead fry born to selenium-contaminated female fish from the SLD from 1984 to 1985 exhibited anomalies such as arrested development (resemblance to eyed embryos), spinal curvature, and missing fins even though live fry from the same broods were fully developed (Saiki and Ogle 1995; M.K. Saiki, unpublished observations made on various occasions during July 26 and August 24, 1984, and during July 9 and July 30, 1985).

Brood Size and Other Fry Characteristics

Examination of gut contents and oviducts of 34 female western mosquitofish from the present study yielded an average of 0.49 cannibalized fry/female (95% confidence interval [CI] 0.21-0.84 cannibalized fry/female fish) and 0.54 unborn fry/female (95% CI 0.13–1.09 unborn fry/female fish). Thus, counts of fry

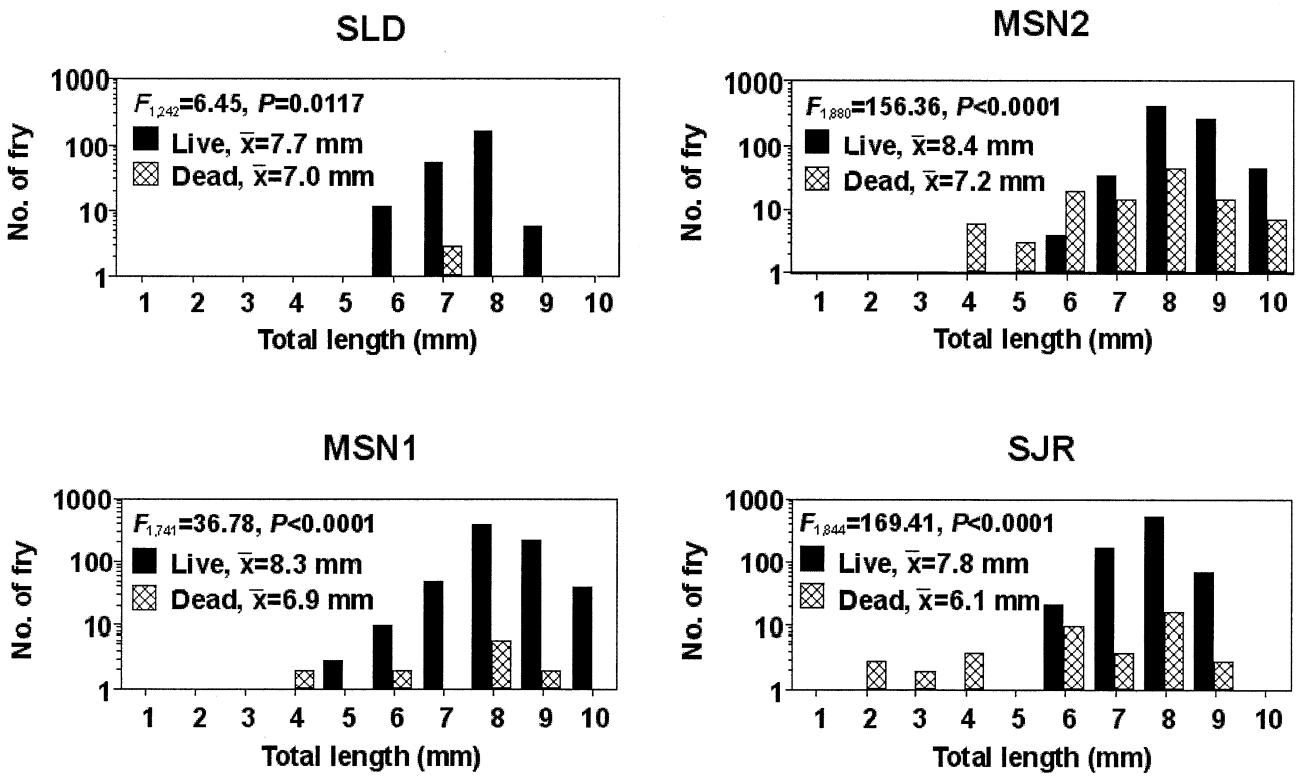

Fig. 2. Comparison of total lengths among live and dead fry born to adult female western mosquitofish

Table 3. Pearson product-moment correlations between female size and selected variables ($N = 113$)

Female	Brood Size	Fry Survival (%)	Mean Fry Total Length (mm)	Mean Fry Weight (g)
Total length (mm)	0.73*	−0.23	−0.17	0.20
Weight (g)	0.68*	−0.24	−0.18	−0.21

* Significant according to adjusted Bonferroni $p = 0.00625$.

recovered from test chambers were reasonable estimates of brood size. According to one-way ANCOVA, mean brood size (adjusted for total length of female fish) varied significantly among sample sites and trials ($F_{6,112} = 3.63$, $p = 0.0026$), with exceptionally small broods produced by female fish from MSN1 during the second but not the first trial (Table 2). Although brood size in mosquitofish can be influenced by day length, temperature, physiologic senility (old age), and perhaps other factors (Constantz 1989), we are not aware of any major environmental perturbations occurring before fish collections for the second trial that would easily account for an exceptionally small brood size at MSN1.

At both seleniferous and nonseleniferous sites, stillborn fry were consistently smaller (shorter) than live fry (Figure 2). A similar pattern was reported by Saiki and Ogle (1995), who attributed the phenomenon to the presence of aborted embryos and premature larvae among the "stillborn fry."

As judged by a Bonferroni $p = 0.00625$ (adjusted for the number of simultaneous comparisons), total length and weight of adult female western mosquitofish were significantly correlated with brood size (numbers of fry born to each female) but not with percent fry survival, mean fry length, and mean fry weight (Table 3). Similar relations were reported by Saiki and Ogle (1995).

Selenium Body Burdens

Four postpartum female western mosquitofish from the SLD contained 13.0 to 17.5 μg selenium/g, whereas their broods of newborn fry averaged 17.8 to 29.2 μg selenium/g. In contrast, 12 female fish from MSN2 contained 3.96 to 13.3 μg selenium/g, whereas their broods averaged 5.35 to 29.1 μg selenium/g. Again in contrast, 10 female fish from MSN1 contained 1.72 to 2.77 μg selenium/g, whereas their broods averaged 2.26 to 4.68 μg selenium/g. Finally, 11 female fish from the SJR contained 0.40 to 1.29 μg selenium/g, whereas their broods averaged 0.61 to 2.49 μg selenium/g. Overall, the mean selenium concentrations in broods were directly correlated with selenium concentrations in female fish (Figure 3).

According to two-way ANOVA, the "sample site" × "life stage (females or fry)" interaction for selenium concentrations was not significant ($F_{3,66} = 0.34$, $p = 0.7954$). However, the main effects were significant for "sample site" ($F_{3,66} = 423.99$, $p < 0.0001$) and "life stage" ($F_{1,66} = 54.59$, $p < 0.0001$). On average, highest concentrations of selenium occurred in western mosquitofish from the SLD, followed by MSN2, MSN1,

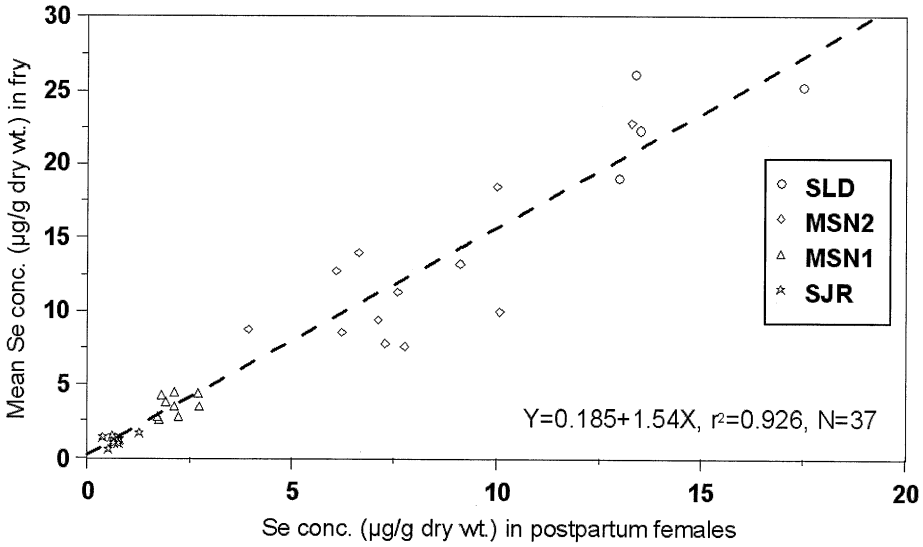

Fig. 3. Relation between selenium concentrations in whole-body samples of adult postpartum female western mosquitofish and their newborn fry. Selenium concentrations for fry are geometric means from as many as three five-fish composite samples

and SJR (Table 4; also see Figure 3). In addition, selenium concentrations were higher in newborn fry than in postpartum female fish (Table 5; also see Figure 3). The spatial variations in selenium body burdens of mosquitofish were similar to patterns reported by Saiki *et al.* (2001) for bluegill and green sunfish sampled in 1997, which was shortly after the Grassland Bypass Project became operational. To our knowledge, the higher concentrations of selenium measured in newborn fry than in postpartum female fish have not been previously documented in the scientific literature. However, several investigators have reported that selenium concentrations are generally higher in ovaries or eggs than in skeletal muscle tissue of several egg-laying fishes (e.g., Cumbie and Van Horn 1979; Lemly 1985, 2002; Gillespie and Baumann 1986; Coyle *et al.* 1993; Hamilton and Waddell 1994; Kennedy *et al.* 2000). Such findings suggest that the selenium content in newly hatched offspring results from parental transfer through the eggs (e.g., Gillespie and Baumann 1986; Schultz and Hermanutz 1990; Coyle *et al.* 1993; Lemly 2002).

Table 4. Selenium concentrations in whole-body samples of western mosquitofish from various sample sites

Sample Site or F	N	Selenium (µg/g dry weight)[a]
SLD	8	18.1 A
MSN2	24	9.31 B
MSN1	20	2.72 C
SJR	22	0.907 D
$F_{3,66}$		423.99*

[a] Geometric means followed by the same capital letter are not significantly different ($p > 0.05$) according to Tukey's Studentized range test.

* $p < 0.0001$, MSN: North Mud Slough, SJR: San Joaquin River, SLD: San Luis Drain.

Discussion

Lemly (1993, 2002) proposed that selenium concentrations as low as 4 µg/g in whole fish or 10 µg/g in ovaries and eggs were sufficient to cause reproductive failure, at least in sensitive species such as bluegill, largemouth bass (*Micropterus salmoides*), and other centrarchids. Hamilton (2002, 2003) and others also recommended that selenium concentrations not exceed approximately 4 µg/g in whole fish to avoid toxic effects on growth, reproduction, or survival. However, still other investigators have argued that these thresholds are too conservative and have proposed thresholds of 6 to 9 µg/g for whole fish and 17 µg/g for ovaries (e.g., DeForest *et al.* 1999; Brix *et al.* 2000). In contrast, selenium concentrations in postpartum female western mosquitofish from the SLD and MSN2 varied from 3.96 to 17.5 µg/g whereas their newborn fry contained 5.35 to 29.2 µg/g without exhibiting overt evidence of reproductive failure.

Mosquitofish (*Gambusia* spp.) may be among a handful of fish species that can tolerate extraordinarily high concentrations of selenium. Cherry *et al.* (1976) noted that selenium concentrations averaged 9 µg/g (wet weight basis; approximately 36 µg/g dry weight assuming 75% moisture) in western mosquitofish exposed to seleniferous ash basin effluent from a coal-fired power plant. Although other fishes were absent from the drainage, there was no indication that this concentration of selenium had adversely affected the mosquitofish (Cherry *et al.* 1976). In selenium-contaminated Belews Lake in North Carolina, a population of the closely related eastern mosquitofish (*G. holbrooki*) accumulated 9.91 µg selenium/g wet weight (approximately 40 µg selenium/g dry weight) in white skeletal muscle (whole-body concentrations were not given) and continued to persist long after other fishes had disappeared (Lemly 1985). Saiki (1986) reported that western mosquitofish sampled from the SLD in 1983 contained as much as 370 µg selenium/g, whereas other cohabiting fish species contained much lower concentrations. In September 1983, a massive fish kill of undetermined cause eliminated most or all fishes except for mosquitofish (Saiki and Lowe 1987). In the months immediately after the fish kill, only mosquitofish were collected from

Table 5. Selenium concentrations in whole-body samples of postpartum female western mosquitofish and their newborn fry

Life Stage or F	N	Selenium (μg/g dry weight)[a]
Fry	37	4.58 A
Adult	37	2.82 B
$F_{1,66}$		54.59*

[a] Geometric means followed by the same capital letter are not significantly different ($p > 0.05$) according to Tukey's Studentized range test.
* $p < 0.0001$.

the SLD despite several attempts to capture fish with gill nets and seines. However, as noted by Saiki and Ogle (1995), when examined in 1984 to 1985, mosquitofish containing > 100 μg selenium/g suffered from a relatively high incidence of stillbirths that included deformities even though they also produced fully developed live fry.

In conclusion, results from this study strongly suggest that western mosquitofish inhabiting selenium-contaminated waters in the Grassland Water District are not experiencing adverse reproductive effects at current levels of selenium exposure. After failing to detect adverse reproductive or teratogenic effects in wild cutthroat trout (*Oncorhynchus clarki lewisi*) inhabiting a selenium-contaminated reach of the Elk River in southeastern British Columbia, Kennedy *et al.* (2000) speculated that this population may have developed an evolved tolerance to high tissue concentrations of selenium. Although beyond the scope of our study, the seemingly high tolerance of mosquitofish to increased body burdens of selenium could have a genetic basis. Other investigators have reported that offspring were more tolerant than parental stock among eastern mosquitofish exposed to high concentrations of mercury (Mulvey *et al.* 1995) and uranium (Keklak *et al.* 1994). According to Hamilton and Palace (2001), short-lived, rapidly reproducing species such as mosquitofish are more likely to develop evolved tolerances to selenium and other trace elements than species with longer reproductive cycles.

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