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Article

Lifetime Chronicles of Selenium Exposure Linked to Deformities in an Imperiled Migratory Fish

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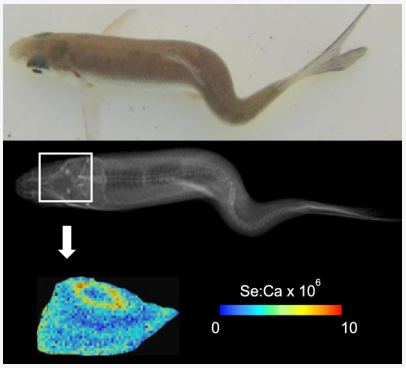
ABSTRACT: Aquatic ecosystems worldwide face growing threats from elevated levels of contaminants from human activities. Toxic levels of selenium (Se) shown to cause deformities in birds, fish, and mammals can transfer from parents to progeny during embryonic development or accumulate through Se-enriched diets. For migratory species that move across landscapes, tracking exposure to elevated Se is vital to mitigating vulnerabilities. Yet, traditional toxicological investigations resolve only recent Se exposure. Here, we use a novel combination of X-ray fluorescence microscopy and depositional chronology in a biomineral to reveal for the first time provenance, life stage, and duration of toxic Se exposure over the lifetime of an organism. Spinal deformities observed in wild Sacramento Splittail (*Pogonichthys macrolepidotus*), an imperiled migratory minnow, were attributed to elevated Se acquired through maternal transfer and juvenile feeding on contaminated prey. This novel approach paves the way for diagnosing sources, pathways, and potential for a cumulative exposure of Se relevant for conservation.



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■ INTRODUCTION

Chemical contaminants from industry, agriculture, and urban runoff seep into aquatic environments and disrupt biological systems at levels ranging from molecules to ecosystems, with sublethal levels having delayed and profound population-level impacts where examined.[1,2] Human activities, including coal combustion, mining, and agricultural practices, may concentrate selenium (Se) resulting in bioaccumulation that can affect an organism's physiology, health, and fitness as well as the community of organisms in a food web.[3] Selenium is found naturally in soils and minerals and is an essential nutrient required for oxidative and enzymatic processes.[4] However, the differences in nutritionally optimal versus toxic levels are typically narrow. Elevated dietary exposure exceeding 3 μg/g can disrupt protein synthesis by substituting Se for sulfur in ionic disulfide bonds, induce oxidative stress, and modulate expression of key genes involved in bone formation resulting in striking deformities in developing offspring of fish, birds, and mammals.[5−7]

The ecological and conservation importance of revealing the sources and pathways of spine-deforming Se toxicity in nature is substantial, yet remains a significant challenge in toxicological studies.[8] Investigations are often limited to detecting recent exposures because contaminants in muscles or soft-tissues change over time due to depuration, metabolic transformation, and tissue recompartmentalization, making measurements difficult to interpret.[9−12] Further, discovering fish with spinal deformities is rare in the wild due to their increased mortality risk. For migratory species, such as Sacramento Splittail (*Pogonichthys macrolepidotus*), listed as a species of special concern by California,[13] the recent observation of deformed individuals with symptoms of Se toxicity presented an opportunity to apply new analytical tools to diagnose sources and pathways of exposure in nature.

Here, we use a novel combination of microchemical tracers and deposition chronology in a biomineral (aragonitic otoliths, part of the fish's hearing/balance system) to reveal when and where during development individuals with spinal deformities were exposed to elevated Se. Specifically, we were able to map selenium concentrations using synchrotron-based two-dimensional X-ray fluorescence microscopy (SXRF) and strontium isotopes (^{87}Sr:^{86}Sr) in incrementally deposited otolith layers corresponding to different life stages and habitats. This approach allowed us to deduce whether individuals collected in the wild obtained Se through maternal transfer in the estuary (embryo and yolk portion of otolith; Figure 1A), versus direct ingestion on Se-enriched prey in the freshwater (10 days post-hatch, exogenous feeding; Figure 1B), or from both sources (estuary and freshwater) and pathways (maternal and direct feeding).

Otoliths are metabolically stable and provide a permanent chronology of Se exposure over the lifetime of fish.[14−16]

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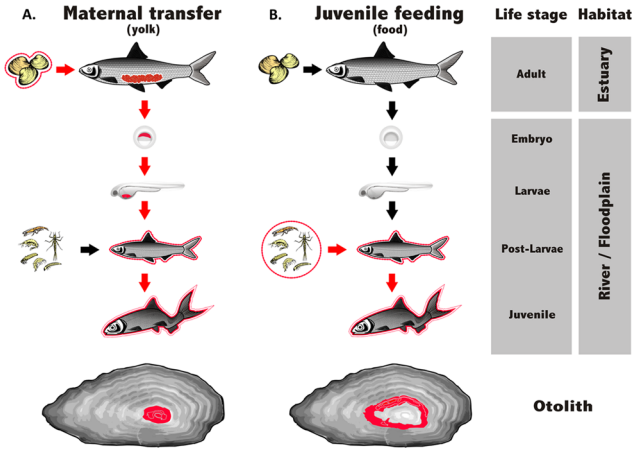

Figure 1. Conceptual model of selenium (Se) diet pathways and otolith incorporation. (A) Reproductive females feeding on elevated Se in prey in the estuary sequester Se in eggs during vitellogenesis; elevated Se is transferred to embryos and larvae, via yolk and causes juvenile vertebral malformation. The maternal Se pathway is documented through incorporation in the embryo and larval portions of juvenile otoliths. (B) Juveniles can directly ingest prey with elevated Se after hatching and yolk-feeding thereby impacting vertebrae formation. The juvenile feeding pathway is documented in the post-larvae and juvenile life stages in the otolith deposited 10 days after hatching. Diet pathways of elevated Se and ultimate otolith incorporation are highlighted in red.

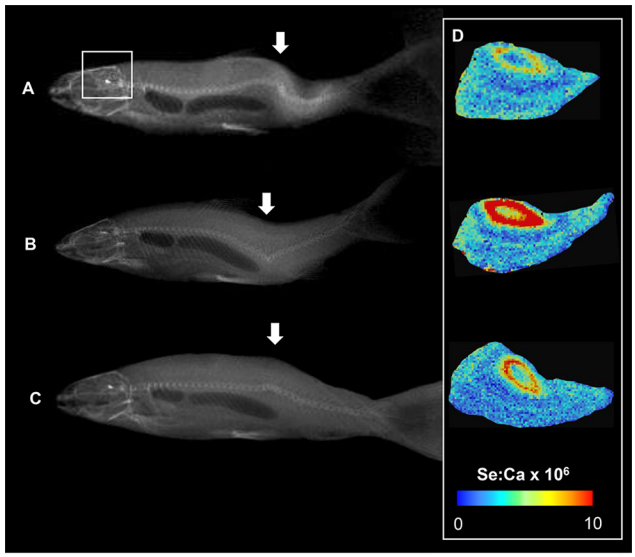

Figure 2. Juvenile splittail spinal deformities and selenium chronology. Radiographs showing splittail with three categories of spinal deformities and affected vertebrae (white arrow): scoliosis (A), lordosis (B), and kyphosis (C) and location of otolith in the fish cranium (A; white box). Selenium to calcium (Se/Ca) distribution in the otoliths from the same fish (D) shows a halo of elevated selenium outside of the core (maternal influence) suggestive of an elevated selenium diet while in freshwater.

Otoliths are formed of daily concentric layers of $CaCO_3$ and protein with the older layers occupying the central core region and younger layers sequentially formed on the top. This process continues throughout the lifetime of the fish resulting in a daily record of a variety of elemental constituents from a fish's local environment.[17] Some trace elements, such as strontium, are benign to fish, and their isotopic ratios can also be used to track movements as they migrate among chemically different waterways, such as estuaries and rivers.[18,19] When chemistry data are linked to the daily growth bands in fish otoliths, detailed time series of individual fish exposures to particular contaminants at different ages can be constructed.[20]

The effluent from oil refineries within the estuary and legacy agricultural practices in the upstream watershed are the two leading, geographically distinct point sources of anthropogenic Se in the upper San Francisco Estuary (estuary), California.[21] Sacramento Splittail (splittail), a cyprinid benthivore endemic to the San Francisco estuary and watershed, feed, migrate, and reproduce between these two potentially elevated Se environments at different life stages. Extensive work has been done to establish Se thresholds and reduce the exposure of wildlife to elevated Se in the estuary and San Joaquin River over several decades, yet juvenile splittail with visible morphological (Figure S1) and spinal deformities (Figure S2) was recently observed. These gross deformities and morphological distortions are consistent with Se toxicity, which include scoliosis (lateral curvature of the spine), kyphosis (outward curvature of the spine), lordosis (concave curvature of the lumbar and caudal regions of the spine; Figure 2A−C), as well as deformities of fins, skull, jaws, and bulging eyes. The extent to which splittail are threatened by point sources of Se resulting in spinal deformities in the estuary and/or freshwater has a direct bearing on which water bodies may remain impaired for wildlife.

There are two primary pathways for Se toxicity to result in observed spinal deformities in organisms. Elevated Se can be transferred from females to their progeny, altering embryonic development (Figure 1A); or an individual can be exposed directly to toxic levels in food, modifying the spine subsequent to endogenous feeding (Figure 1B). The majority of toxicological research on Se exposure across taxa suggests that spinal deformities in young are primarily transgenerational.[22,23] Deformed progeny resulted when parents were fed elevated Se diets or exposed prior to spawning.[22,24] Because of this, there is concern that adult splittail are at heightened risk to Se toxicity in the estuary because their diet includes the invasive Asian clam, *Potamocorbula amurensis*,[25] which is known to bioaccumulate Se.[26] Selenium levels in the clam (2−20 μg/g dry weight) have exceeded dietary concentrations known to cause reproductive toxicity in the wildlife making vulnerable other clam-eating native fishes like white sturgeon, *Acipenser transmontanus*, as well as diving ducks.[27,28] Therefore, females with high Se body burdens from foraging in the estuary could produce progeny with the observed deformities through maternal transfer of Se in yolk during development (Figure 1A).

There is also evidence that juvenile fish exposed to Se-enriched diets develop the same deformities. For example, Se-enriched feeding experiments with 7-month-old juvenile splittail have induced spinal deformities.[29] Flowing into the estuary, the San Joaquin River is known to have historical and ongoing elevated Se from agricultural irrigation practices shown to impact fish and wildlife.[30−32] One of the best-documented cases of Se toxicity occurred in the 1980s when laboratory and field studies confirmed that Se in agricultural irrigation drainage water in the San Joaquin River caused extensive deformities in wild populations of aquatic birds. This included disfiguring impacts with birds missing eyes, beaks, wings, legs, and feet, and reproductive failures.[33,34] When

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Table 1. Diagnosis of Spinal Deformities, Se Exposure, and Natal Assignments for Juvenile Splittail[a]

		exposure Se/Ca > 5 × 10^{-6}			juvenile natal assignment			
Fish_ID	diagnosis	embryo	yolk (days)	exogenous (days)	^{87}Sr:^{86}Sr (N)	SD	assigned	posterior probability
14001C	lordosis	no	0	22	0.70729 (14)	0.00011	San Joaquin	0.999
14002C	scoliosis	no	0	27	0.70737 (12)	0.00012	San Joaquin	1.000
14003C	kyphosis	yes	10	63	0.70735 (15)	0.00019	San Joaquin	1.000
14004C	lordosis	yes	10	64	0.70731 (14)	0.00015	San Joaquin	0.999
14005C	lordosis	no	0	10				
14007C	kyphosis	no	5	26	0.70741 (12)	0.00021	San Joaquin	1.000
14008C	kyphosis	no	0	3	0.70738 (14)	0.00014	San Joaquin	1.000
14010C	lordosis	no	0	15	0.70735 (13)	0.00015	San Joaquin	0.999
14016N	lordosis	yes	0	32	0.70731 (12)	0.00015	San Joaquin	0.999
14017N	kyphosis	no	0	28	0.70732 (15)	0.0001	San Joaquin	0.999
14018N	undiagnosed	no	0	28	0.70733 (16)	0.00014	San Joaquin	0.999
14020N	undiagnosed	yes	10	61	0.70744 (12)	0.00017	San Joaquin	1.000
14023N	undiagnosed	no	0	16	0.70741 (15)	0.0002	San Joaquin	1.000
14024N	kyphosis	yes	3	43	0.70735 (13)	0.0001	San Joaquin	1.000
14025N	lordosis	no	0	7	0.70748 (13)	0.00022	San Joaquin	1.000
14028N	undiagnosed	no	0	4	0.70739 (10)	0.00016	San Joaquin	1.000

[a]Juvenile splittail were radiographed at the UC Davis Veterinary Sciences Radiology Laboratory and diagnosed for spinal deformities (scoliosis, lordosis, kyphosis, and undiagnosed) prior to elemental mapping of otoliths. The duration of elevated Se exposure (Se/Ca > 5 × 10^{-6}) was quantified as presence/absence in the embryo portion of the otolith and days of exposure in yolk and exogenous life stages. Average strontium isotope values and standard deviation (SD) for spot measurements (N) in otoliths representing natal habitat values for individual juvenile spilttail 10 days post-hatch. Natal strontium values were evaluated using known strontium isotope habitat values for the Sacramento and San Joaquin Rivers (Data file S1) and fish were assigned to their river-of-origin. The strength of assignment (posterior probabilities) suggests that all juveniles were born and reared in the San Joaquin River prior to capture. Note: Fish 14005C does not have strontium isotope measurements as the sample was damaged during the analysis.

splittail are young and first begin to feed on plankton and insects in the freshwater floodplains, they can be exposed to elevated levels of Se directly in their diets (Figure 1B).

The study presented here evaluated the potential pathways, habitat sources, and duration of Se exposure linked to spinal deformities in nature. The potential pathways and habitat sources include (i) transfer of estuary-sourced Se from parents to eggs and yolk influencing embryonic development; (ii) direct accumulation of Se-enriched diet sources in freshwater rearing habitats after exogenous feeding; and (iii) multiple exposures across life stages and habitats. Unlike measurements of Se in tissues that only provide information on recent exposure, combining chemistry and biomineral analysis allowed a retrospective estimate of the number of days individuals were vulnerable to elevated Se across multiple aquatic ecosystems during their lifetimes.

■ MATERIALS AND METHODS

Study Organism. Evidence of declining trends in splittail population numbers[35] resulted in the federal listing of splittail as threatened under the US Endangered Species Act.[36] Although the federal listing status was remanded in 2003,[37] splittail retain classification as a species of Special Concern by the California Department of Fish and Wildlife and is of conservation importance.[13] Reproducing splittail migrate from the estuary to spawn in freshwater channels and floodplains of the Sacramento and San Joaquin rivers and recruit as sub-yearlings into the estuary where they feed, grow, and live the majority of their lives (5−7 years of age).[38]

Experimental Design. Young-of-the year splittail (30−90 days of age) were collected in the San Francisco Delta on the San Joaquin River at the Fish Salvage Collection facility, Byron, California on May 24, 2011 and transported to the University of California, Davis' Center for Aquatic Biology and Aquaculture Facility as part of ongoing genetic and physiology studies on the species and held captive for ∼4 years prior to sacrifice. All individuals were handled in accordance with approved protocols as part of the University of California's Institutional Animal Care and Use Committee standards. The majority (>80% of 1000 fish) were observed to exhibit spinal deformities as young-of-the-year. A total of 16 fish that represented individuals ranging in severity of morphological deformities including some that appeared normal were sacrificed on October 1, 2014 for radiographic and otolith analyses. Two additional fish (young-of-the-year 2013) that spent their entire lives in captivity were used as controls (control-culture). They were the progeny of adult splittail collected in the wild and spawned in the aquaculture facility and fed a standard diet (Rangen Inc., soft-moist 1/32 in., Buhl, Idaho). Two adults caught in the estuary in 2010 were also used as controls to place the chemical chronologies of nondeformed fish into context (control-wild). Individuals (excluding controls) were externally examined, photographed (Figure S1), and assessed visually as either having normal or deformed morphology. Individuals were then radiographed at the University of California's Veterinary Medical Clinic (Figure S2). Radiographs were read, and individuals were diagnosed as having scoliosis, kyphosis, lordosis, or undiagnosed spine morphology (Table 1). Previous research documenting spinal deformities used higher resolution radiographs allowing for the detection of smaller-scale aberrations such as inconsistent distances among vertebrae.[39] Therefore, individuals that appeared to have normal radiographs were considered "undiagnosed", since the detection of potential aberrations could go unobserved using our instrumentation. It remains unclear the extent to which these undiagnosed individuals are impacted physiologically or behaviorally.

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Individuals were placed into four groups for further statistical analyses: (1) "deformed" individuals with phenotypic and/or radiographic evidence of spinal deformity, (2) undiagnosed individuals with inconclusive evidence for spinal deformity based on limited resolution of radiographs but were from the same cohort as the deformed individuals, (3) "control-culture" individuals who were hatched and reared in captivity, and (4) "control-wild" individuals who were collected as adults in the San Francisco Estuary and did not exhibit evidence for spinal deformities.

Otolith Preparation and Daily Ages. Lapilli are the largest otoliths in splittail and daily ring deposition has been validated.[40,41] One lapilli otolith per fish was embedded in West Systems 105 epoxy resin before being sectioned in the frontal plane using a low-speed diamond saw. The core and natal portions were further revealed using 1500 grit sandpapers and 3 μm lapping films. Finished preparations were cleaned by sonicating in deionized water and surface-wiped with ethanol prior to elemental mapping. All otolith microstructure imaging and age and growth measurements were performed in Image-Pro Premier (Media Cybernetics Inc., Rockville, MD) at 200× magnification. The first 60−100 daily increments were counted along the primary growth axis on the rostral side depending on the quality of the preparation starting with the first increment after the hatch check (Figure S3 and Data file S4). This transect and increment data were later used to link the chemical maps with daily ages to create a time series of Se exposure.

Elemental Mapping in Wild Splittail Otoliths. Selenium, strontium (Sr), and calcium (Ca) concentrations were analyzed in splittail otoliths at Cornell's High Energy Synchrotron Source (Cornell University, Ithaca, NY) using scanning X-ray fluorescence microscopy on the F3 beamline per established techniques.[15,42] This instrument allows for spatial mapping of elemental concentrations using a non-destructive technique with minimal interferences among Se and other elements. Briefly, a multilayer monochromator (0.6% bandwidth) produced an X-ray with 16 energy keV focused on the otolith with a single glass capillary necessary to achieve 20 μm spot resolution over the entire otolith. The photon flux was about 0.5×10^{11} counts per second, and a fluorescence spectrum was integrated for 1 s. To increase the sensitivity of Se and reduce the potential for overwhelming Ca fluorescence, an aluminum attenuator was applied to the quad Vortex silicon drift detector. Fluorescence spectra were calibrated using an in-house otolith pellet previously described.[14] Distributions of absolute elemental concentrations were analyzed with PyMCA.[43] Commercial GIS software (Esri, Redlands, California) was used to analyze spatial patterns of elemental concentration data in otoliths. Because otoliths are 96% calcium carbonate, Se is normalized to Ca in otoliths by convention to account for imperfections in sample preparation and instrument performance.[14,15,44]

Strontium Isotope Measurements and Habitat Source Assignment. Sr isotopes in otoliths of migratory fish can trace broad-scale movements of individuals from marine to inland water as well as smaller-scale habitat-use within freshwater.[18,19,38,45] The ^{87}Sr:^{86}Sr in the embryo and yolk portion of the otolith reflects water ^{87}Sr:^{86}Sr experienced by females during maturation,[46,47] while the exogenous feeding portion reflects the ^{87}Sr:^{86}Sr in the juvenile rearing habitat.[18] We used a laser-ablation multicollector inductively coupled plasma mass spectrometer at the University of California Davis, (MC-LA-ICPMS; Nu plasma HR interfaced with a New Wave Research Nd:YAG 213 nm laser) to measure ^{87}Sr:^{86}Sr from the core to the edge of the splittail otoliths to reconstruct the portion of the juvenile otolith under maternal influence and associate the portion of the otoliths exhibiting elevated Se/Ca with the location in the watershed the juvenile was rearing at the time. The transect consisted of consecutive spots that were 40 μm in diameter. At each spot, the laser pulsed at 10 Hz for 25 s and varied between 3 and 8 J/cm^2 depending on sample strontium concentrations (Data file S3). Spot analyses were chosen over line transects because variation in ^{87}Sr:^{86}Sr can be monitored with sampling depth to ensure that material deposited later in the fish's life was not inadvertently sampled.[46] Data corrections included: measuring background 86 Kr voltages for 30 s prior to each batch of analysis for blank subtraction of the Krypton interference, monitoring 85 Rb to correct for and remove the 87 Rb influence on the measured 87 Sr value, and accounting for instrument bias by systematically analyzing a marine carbonate standard (*Anomia nobilis*). The measured value in the standard was normalized to 0.70918, and this correction was applied to all analyses. The accuracy (average ^{87}Sr:^{86}Sr) and precision (1 standard deviation) of 18 measurements were (0.709042 ± 0.000078) during the analytical session.

^{87}Sr:^{86}Sr measurements previously reported from water collected in splittail spawning habitats on the Sacramento River and San Joaquin Rivers were compiled (Data file S1[19,45,46]). Otoliths from juvenile salmon collected on the San Joaquin River upstream of the confluence of the Merced River were analyzed per established technique.[18,46,48] These samples were used in this study to characterize additional ^{87}Sr:^{86}Sr inputs that could influence ^{87}Sr:^{86}Sr in downstream splittail spawning habitats on the San Joaquin and floodplains.[47] Briefly, for otoliths, the ^{87}Sr:^{86}Sr values in the spot measurements in the portion of the otolith >250 μm from the primordia represent the average and variance (1SD) for the natal rearing habitat. To assess the extent to which the Sacramento and San Joaquin Rivers differed isotopically, a *t*-test was conducted (JMP Pro 14, SAS Institute, Cary, NC, 1989−2019).

Chemical Chronology Integration and Statistical Analysis. Transects used to generate daily ages in individual fish (Figure S3) were georeferenced in the GIS chemistry layer. Daily elemental chemistry data (Se/Ca and Sr/Ca) along the growth transect from the core to the edge were extracted (Figures S4 and S5 and Data file S2). ^{87}Sr:^{86}Sr isotope data were also georeferenced to the same daily growth transect (Figure S6). Because otoliths grow incrementally throughout a fish's lifetime, these transects are analogous to a time series of chemistry and exposure histories. The otolith time series was further categorized into three developmental stages. (1) Eembryo: otolith primordia to visible hatch mark corresponding to embryonic development prior to hatching, (2) yolk-feeding: hatch to 10 days post-hatch during which larvae are nourished through maternal yolk, and (3) exogenous: 10 days after hatch when larvae begin to feed on aquatic prey. Both the "embryo" and "yolk" portions would be under the maternal influence of Se/Ca, while 'exogenous' would represent Se/Ca directly consumed through diet.

We developed a Bayesian mixed-effect model to investigate variation in otolith Se/Ca concentration across life stages (embryo, yolk, and exogenous) and treatment groups (deformed, undiagnosed, control-culture, and control-wild).

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Table 2. Parameter Estimates from Bayesian Mixed-Effect Model[a]

	estimate	est. error	lower 95% CI	upper 95% CI	eff.sample	Rhat
intercept	**1.1**	**0.12**	**0.86**	**1.33**	**3229**	**1**
Life Stage (Embryo)						
yolk	−0.01	0.03	−0.07	0.06	12 000	1
exogenous	**1.11**	**0.02**	**1.06**	**1.16**	**12 000**	**1**
Group (Deformed)						
undiagnosed	0.17	0.24	−0.3	0.65	4465	1
control wild	**−0.57**	**0.28**	**−1.13**	**−0.01**	**5589**	**1**
control culture	**−0.95**	**0.38**	**−1.72**	**−0.23**	**5431**	**1**
Life Stage (Embryo): Group (Deformed)						
yolk: undiagnosed	−0.02	0.07	−0.15	0.12	12 000	1
exogenous: undiagnosed	**−0.46**	**0.06**	**−0.58**	**−0.33**	**12 000**	**1**
yolk: control wild	0.02	0.15	−0.26	0.31	12 000	1
exogenous: control wild	**−0.57**	**0.13**	**−0.8**	**−0.3**	**12 000**	**1**
yolk: control culture	0.41	0.24	−0.01	0.92	6127	1
exogenous: control culture	**−0.13**	**0.23**	**−0.51**	**0.37**	**5426**	**1**

[a]There is strong evidence suggesting that Se/Ca values are elevated in the treatment groups (deformed and undiagnosed) relative to the control groups (wild and culture), and Se/Ca values for the treatment groups (deformed and undiagnosed) in the exogenous life stage are elevated relative to all others. Life stage and treatment groups that had lower and upper 95% credible intervals[34] and did not overlap "0" were considered to have a significant influence on the Se/Ca distributions (bolded). For each parameter, eff.sample is a crude measure of the effective sample size, and Rhat is the potential scale reduction factor on split chains (at convergence, Rhat = 1).

The underlying hypothesis of interest was to test if Se/Ca concentration varied across life stages and treatment groups after accounting for variation among individuals. There was no correlation between Se/Ca and daily age so daily age was not included in the model (Figure 3). The model included Se/Ca concentration as the response variable (λ), life stage, and treatment group as interacting fixed factors (denoted as β in eq 1) and individual (Fish_ID) as a random factor (denoted as α in eq 1) as follows

$$\lambda = \alpha + \alpha_{\text{individual}} + \beta \text{ lifestage} \times \beta \text{ treatment group} \quad (1)$$

Including individual as a random factor allowed for varying intercepts to account for the fact that repeated measurements within an individual were likely to be more similar to each other than to those of another individual. The model treated Se exposure in the exogenous life stages of each individual as a temporary phenomenon, the timing of which varied across individuals. Therefore, evaluation of the exogenous period was not biased by data points where there was no Se exposure. The data examined included the 10 embryonic life stage data points, 10 yolk life stage data points (days), and the 10 highest Se/Ca values observed in the exogenous life stage for each individual. Modeling was implemented in the "brms" package in the R statistical computing environment[49] using a skew-normal distribution with a log link function. Weakly-informative priors were assigned to fixed effects (μ = 0, σ = 5) and random effects (μ = 0, σ = 1). Modeling was implemented with four chains with iterations = 4000 and warmup = 1000. Model details, including R code and assessment of fit, are provided in Supporting Information. Briefly, the model converged and performed consistently across all chains. Model predictions are strongly correlated with the original data set. Predictions deviated from the original data set mostly at the highest extreme values (Supporting Information).

To calculate the duration of time individuals were exposed to elevated Se/Ca, the number of days individuals had Se/Ca values > 5×10^{-6} were counted (Table 1). The daily ages of some of the fish were challenging to read after day 65. To standardize the analysis across fish, the number of days from hatch to 65 was used and is therefore an underestimate for the total days of exposure. To assign the deformed fish to either the Sacramento or San Joaquin River, a linear discriminant function was developed using the water and otolith ^{87}Sr:^{86}Sr collected between the two watersheds (Data file S1). The classification strength of the model was determined using a jackknife approach. The model was then used to classify unknown-origin splittail exposed to elevated Se, and their posterior probabilities of the strength of assignments were calculated (Table 1).

RESULTS

Deformities Observed in Wild Splittail. Splittail were observed to have three primary categories of spinal deformities that ranged in severity (Figure 2 and Table 1). The most common diagnoses were lordosis (38%; 6 of 16), kyphosis (31%; 5 of 16), and scoliosis (6%; 1 of 16). The remaining fish (25%; 4 of 16) appeared to have normal radiographs but were considered undiagnosed given limited resolution in this study. For example, previous research on vertebrae malformations found inconsistent distances among vertebrae as diagnostic aberrations using high-resolution radiography that may be undetected in these individuals (Figure S1 and Table 1).[39] Visually, prior to radiographs, 50% (8 of 16) were thought to be deformed based on external morphology (Fish_ID codes with "C" as a suffix) and 50% normal (8 of 16; Fish_ID codes with "N" as a suffix).

Selenium Chronologies to Diagnose the Se Pathway. To evaluate the life stages that deformed and undiagnosed juveniles (collectively the treatment group) were exposed to elevated Se, the spatial distribution of Se/Ca in otoliths was measured using SXRF across three different life stages discernible in the otoliths: embryo, yolk, and exogenous. The protein in the embryo and yolk portions of the otoliths originate from maternal resources (Figure 1A), while the exogenous portion is derived from the direct diet of juveniles (Figure 1B). We evaluated the Se chronologies using a Bayesian generalized linear mixed model and found that Se/Ca

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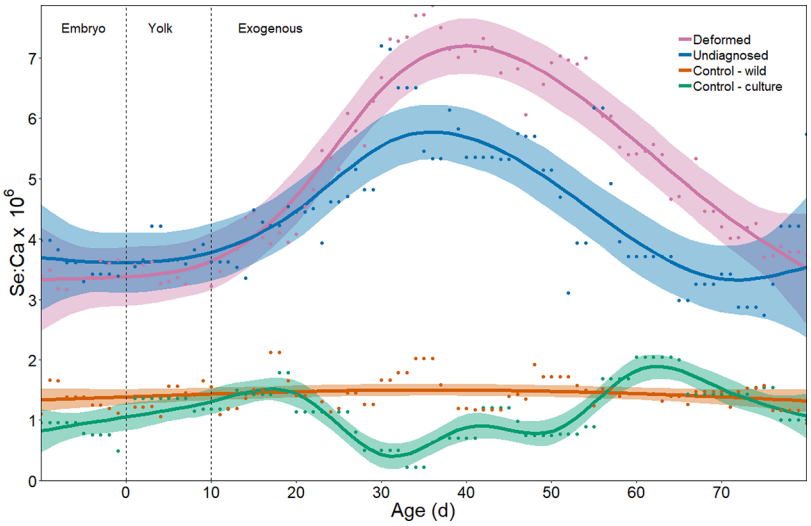

Figure 3. Ratio of selenium to calcium in splittail otoliths. Smoothed function of the selenium/calcium ratio across life stages (embryo, yolk, and exogenous) in deformed (purple; $N = 12$), undiagnosed (blue; $N = 4$), cultured individuals that were spawned and reared in captivity (green; $N = 2$), and wild-caught adults from the estuary (orange; $N = 2$). Shaded area around smoothed function represent 95% confidence interval for predictions. Se/Ca values are elevated in treatment groups (deformed and undiagnosed) relative to control groups (wild and culture), and Se/Ca values for the treatment groups (deformed and undiagnosed) in the exogenous life stage (10 days after hatch; vertical line) are elevated relative to all others.

values were substantially higher in the treatment group, relative to adult splittail collected from the estuary in 2010 or born and reared in captivity (collectively the control group) (Table 2, 95% credible intervals (CI) in parameter estimates did not overlap the number zero[40]). The highest concentrations of Se in the treatment group occurred following the initiation of exogenous feeding (>10 days post-hatch and beyond yolk absorption), indicating an increase in Se exposure from direct ingestion of contaminated prey (Figure 3 and Table 1 and Figures S7 and S4, 95% CI nonoverlap with (0)). Additionally, individuals in the treatment group also exhibited elevated Se concentrations in the period of life prior to exogenous feeding (embryo and yolk-feeding period), suggesting maternal transfer of Se (Figure 3 and Table 2, 95% CI nonoverlap with (0)). The Se/Ca values in the maternal portion of the otoliths in the treatment group were variable, with some individuals exhibiting values as high as 8×10^{-6}, rivaling elevated values in the exogenous portion (Figure 3 and Table 2). The model results collectively suggest that deformed and undiagnosed individuals were exposed to multiple Se exposure pathways (maternal transfer and direct feeding) across aquatic ecosystems (estuary and river), supporting both pathways and habitat sources in the conceptual model (Figures 1A,B and 3 and Table 2, 95% CI nonoverlap with (0)).

Strontium Isotopes to Link Se Exposure to Habitat Sources. To further confirm the aquatic habitat contributing to Se toxicity in deformed and undiagnosed juveniles, we measured otolith strontium isotopes (^{87}Sr:^{86}Sr) across life stages. Female splittail forage in the estuary, where ^{87}Sr:^{86}Sr reflects higher saline and isotopic values (>0.7075[38]) prior to migrating and spawning in either the Sacramento (0.70576 ± 0.00051) (1 standard deviation, SD) or the San Joaquin Rivers (0.70720 ± 0.00027, SD) and their associated floodplains, which are isotopically distinct from one another (*t*-test, $p < 0.001$; Data file S1[19,45,46]). Otolith ^{87}Sr:^{86}Sr chronologies in the treatment group (13 of 15 individuals) showed an estuary ^{87}Sr:^{86}Sr value in the embryo and yolk portion of the otolith reflecting water ^{87}Sr:^{86}Sr experienced by females during maturation followed by San Joaquin River ^{87}Sr:^{86}Sr values in the exogenous feeding portion reflecting the juvenile rearing habitat (Table 1, posterior probabilities 0.99−1.00). Otolith ^{87}Sr:^{86}Sr values converged on the range of San Joaquin River values coincident with the main elevated Se/Ca peaks in the exogenous portion of the otolith, around day 50 post-hatch (Figure 3 and Table 1). These data suggest that individuals acquired Se toxicity while feeding in the freshwaters of the San Joaquin River but already started with significantly higher Se burdens from females maturing in the estuary (Figure 3, Table 1 and Supporting Information).

Duration of Se Exposure. Daily depositional chronology in otoliths showed that individuals varied widely in the number of days exposed to Se/Ca above a threshold $>5 \times 10^{-6}$ (Table 1). The majority of deformed and undiagnosed individuals had the greatest Se/Ca exposures 25−80 days after hatch, with some individuals experiencing as many as 64 days of Se/Ca ratios $> 5 \times 10^{-6}$ (Figure 3 and Table 1). This is illustrated by the distinct halo of the increased Se/Ca exterior of the center of the otolith, while the variation in exposure duration is reflected by the width of the halo along the growth axis (Figures 2 and S3). There was more variation among individuals in the exposure history during embryo and yolk-feeding, likely attributed to variation in female feeding ecology in the estuary (Table 1).[50]

■ DISCUSSION

Water bodies are increasingly threatened by several human-mediated sources of contaminants. For migratory species that occupy multiple, often distant aquatic habitats over their lives, understanding where in their life cycle they encounter toxic levels of contaminants that can originate from different sources is vital to understanding where vulnerabilities occur for a species. Se toxicity that results in significant deformities has primarily been shown to occur transgenerationally through parents to progeny[23] with fewer examples of deformities arising somatically due to individuals directly exposed to Se-enriched food webs[29] and none examining the potential cumulative

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influence of both. The extent to which splittail are threatened by point sources of Se in the San Francisco Bay estuary and/or freshwater (San Joaquin River) has a direct bearing on which water bodies are considered impaired for fish and wildlife and at what threshold levels.

The temporal sequences of Se/Ca and ^{87}Sr:^{86}Sr in the otoliths and the presence of multiple spinal malformations all corroborate that females transferred elevated Se to developing juveniles that were then exposed to even higher levels of Se while feeding and rearing in the San Joaquin River (Figure 3 and Table 1). Larval development studies confirm otoliths in the splittail form prior to hatching and that yolk absorption occurs approximately 10 days after hatching.[51] These laboratory studies also highlight several important developmental transitions that occur between exogenous feeding and 50 days, i.e., when juveniles form their adult fin-structures but still lack scales.[51] This falls within the developmental window of splittail (days 25−80) wherein we estimate the greatest Se exposure and toxicity to have occurred. Additionally, splittail 7 months of age and fed Se-enriched diets ≥2.7 mg of Se kg^{-1} for 5−9 months produced spinal deformities in the laboratory identical to those observed in nature, so exposure within 0−80 days is not obligatory to produce deformities.[29] Further, the differences between deformed and undiagnosed groups diverge with the level of Se/Ca observed during exogenous feeding. The levels of Se/Ca measured in otoliths in our study (individuals >22 × 10^{-6}; Data file S2) exceed those recorded in Walleye (*Sander vitreus*) and White Sucker (*Catostomus commersonii*) with no spinal deformities in polluted lakes in New York, which exhibited Se/Ca < 10 × 10^{-6}.[14] Three cold water fish species downstream of coal mines in British Columbia known to produce high levels of selenium reported varying levels of Se with cutthroat being the most vulnerable to high Se exposure.[52] Further work is needed to develop thresholds of Se/Ca in otoliths that relate to tissue values and spinal deformities across species.

Se transferred from females to progeny likely played an important role in how dietary Se from the San Joaquin River reached threshold levels to produce deformities. Embryo and larval life stages in the otoliths of deformed and undiagnosed fish showed elevated Se and ^{87}Sr:^{86}Sr values attributed to juveniles endogenously feeding on Se-rich yolk from females feeding in the estuary. Long-term (>17 year) monthly observations of Se levels in the invasive Asian clam, a common food item for splittail in the estuary, indicate that Se concentrations vary spatially, with levels highest near Carquinez Strait, and temporally, peaking in seasons and years when the freshwater inflow from the Sacramento River is low (<500 m^3/s).[28] This variation in elevated Se has also been observed in reproductive female splittail in the estuary in the fall prior to spawning, with concentrations ranging from 2 to 59 and from 3 to 29 $\mu g/g$ dry weight in ovary and liver tissues, respectively.[50] These data highlight that some females in the estuary contain potentially toxic values of Se in ovarian tissues, used to produce the protein in eggs and yolk.[12,50] We see this variation reflected in the embryo and yolk portions of otoliths in this study with deformed and undiagnosed fish having values significantly higher than nondeformed splittail collected in the estuary.[50]

Several factors besides Se toxicity can result in spinal deformities such as kyphosis, lordosis, and/or scoliosis in fish. These include elevated temperatures,[53] diseases,[54] other contaminants,[55] nutritional deficiencies,[56] as well as interactions among multiple stressors.[53,55] While it is possible that fish in this study were exposed to other stressors that could be linked to their skeletal deformities, the otolith chemistry, in combination with age determination from counting daily growth rings, provides strong, direct evidence of juvenile exposure to elevated Se. These data suggest that Se is a plausible and quantifiable stressor for those individuals across life stages. Other studies suggest that minnows in the family Cyprinidae, like splittail, may be more susceptible to Se toxicity due to their diet that comprises Se-accumulating prey and perhaps their physiology.[57,58] Additional investigations on the prevalence of multiple stressors would assist in characterizing the extent to which other factors coincident with Se might augment a stress linked to spinal deformities in splittail and other fishes in the wild.

Because Se is an essential nutrient, it is commonly present in elevated concentrations in protein-rich eggs.[59] Maternal transfer of Se at levels that do not result in spinal deformities has been observed in embryo and yolk portions of otolith cores of other fish species, which may be expected given the high protein concentrations of this part of the otolith.[60] Female splittail exposed to elevated Se in the estuary during vitellogenesis would likely produce eggs enriched in Se, which would in turn raise Se in embryonic otolith cores that would record this maternal exposure. While all deformed and undiagnosed individuals did show elevated levels outside of maternal influence, there was significant variation among individuals in the Se/Ca values in the embryo and yolk portions of the otoliths. For example, one individual had a Se:Ca value of 8 × 10^{-6} within the first 10 days post-hatch, comparable to the elevated values observed post-exogenous feeding in some deformed individuals. Therefore, it is likely that female body burdens (Se exposure in the estuary) may also be a contributing factor in the observed deformities. Further laboratory and field studies are necessary to understand the relationship between deformities and cumulative environmental exposure to elevated Se during multiple life stages and corresponding Se/Ca reflected in otoliths.

The abnormalities found in this study are not unique for this geographic region. Wild aquatic birds exposed to selenium in agricultural irrigation drainage water at Kesterson Reservoir in the San Joaquin River were observed to have beak, eyes, and limb malformations in the 1980s.[33] We know from laboratory studies on splittail that spinal deformities can be induced in juveniles as old as 7-month-old by feeding them Se-enriched diets.[29] Direct exposure to waterborne selenite or selenate is not the dominant route for Se accumulation in fish tissues, yet dissolved forms of Se influence dietary concentrations and exposures to high levels of aqueous Se (>10 $\mu g/L$) can result in accumulation of Se in tissues and oxidative stress.[61,62] The Grasslands Bypass was created as a solution to divert the Se-enriched soils and irrigation water around the Kesterson Reservoir to reduce wildlife impacts. This bypass effort significantly reduced the concentrations of Se that enter the San Joaquin River downstream of Mud Slough.[30] However, in wet years such as that experienced in 2010−2011 (our study year), portions of floodplain habitats accessible to spawning and rearing splittail may expose splittail to elevated Se levels. Splittail have been documented spawning in regions near Mud Slough where Se levels in water still exceed National USEPA criteria of <3.1 $\mu g/L$ monthly average in flowing waters and 1.5 $\mu g/L$ in standing waters.[48,63]

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Among the greatest challenges in aquatic ecotoxicology is the detection of sublethal exposures of organisms to environmental contaminants. Exposed individuals are likely more susceptible to predation or are out-competed for food, and thus are rarely encountered in routine sampling. This study provided a rare opportunity to use otoliths of fish that exhibited skeletal deformities to test hypotheses about which life stages, and in what habitats, Se toxicity may be occurring. The variation in timing of Se exposure for these fish suggests that traditional soft tissue concentrations, while useful indicators of recent exposure, cannot provide insight into the multiple and likely cumulative risks of exposures over the lifetime of individuals. Further, these data suggest the importance of considering the influence of cumulative exposure of Se across aquatic ecosystems that individually may not exceed thresholds that result in Se toxicity, but together could result in vulnerabilities to species. Empirical data, coupled with population modeling and cohort reconstructions, are necessary to quantify potential population-level effects of Se toxicity in this imperiled species in the watershed. Indeed, conditions producing skeletal deformities could be an important, but easily overlooked phenomenon, contributing to recruitment failure in Se-contaminated aquatic habitats. This novel approach is a significant advancement in the field of ecotoxicology and paves the way for diagnosing lifetime exposures of sublethal contaminants across broad landscapes relevant for multispecies conservation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b06419.

Modeling Se/Ca concentration variation across life stage and treatment groups; graphical posterior predictive checks; photographs capturing external morphology of juvenile splittail (Figure S1); radiographs capturing vertebral condition of splittail (Figure S2) (PDF)

Compiled Strontium isotope data for locations in the Sacramento and San Joaquin Rivers (Data file S1) (XLSX)

Daily Se/Ca and Sr/Ca values measured in splittail otoliths in each life stage derived by linking daily ages and chemistry from the synchrotron (Data file S2) (XLSX)

Daily ^{87}Sr:^{86}Sr values measured in splittail otoliths derived by linking daily growth measurements and chemistry from the LA-MC-ICPMS (Data file S3) (XLSX)

Daily increment distances measured from the otolith primordia along the growth axis of splittail otoliths (Data file S4) (XLSX)

Readme metadata (TXT)

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Author Contributions

R.C.J. and F.F. were responsible for the intellectual inception of the use of otoliths for this study; A.R.S. and F.F. wrote the proposal and secured project funding; R.C.J., K.E.L., F.F., and R.H. conducted Se measurements, analysis, and data interpretation; R.C.J. supervised Sr isotope and daily growth measurements; F.F. and R.C.J. conducted data analysis and designed figures. All co-authors contributed to the integration of the data to reveal the story narrative that was captured by R.C.J. as the lead author.

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Notes

The authors declare no competing financial interest.

[▽]R.C.J. and F.F. contributed equally to this work.

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