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PATHOGEN SURVEY, GILL Na-K-ATPase ACTIVITY, AND LEUKOCYTE PROFILE OF ADULT DELTA SMELT

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Delta smelt abundance has declined drastically and the species was listed as threatened in 1993. The role of disease in this decline is largely unknown. One hundred and five adult smelt, collected from the lower Sacramento River between January and May 2010, were surveyed for infectious agents, blood leukocyte profile, and gill Na-K-ATPase activity. Few tissue changes or significant parasitic infections were observed in histological specimens and there was a low incidence of bacterial isolations. Asymptomatic *Mycobacterium sp.* infection was detected in 54% of the samples by PCR; however, this bacterial group was not isolated in culture. Gill Na-K-ATPase activity was lower in the January sample than subsequent month collections. The number of blood granulocytes increased between March and May. Disease did not appear to be an overt influence on the surveyed population in the spring of 2010.

Keywords: California, delta smelt, *Hypomesus transpacificus*, parasites, pathology, potassium, sodium, PCR, Sacramento River Delta

INTRODUCTION

The delta smelt (*Hypomesus transpacificus*) was listed as threatened under the Endangered Species Act in 1993 and is endemic to the upper San Francisco Bay Estuary (Moyle et al. 1992). In the last decade, a clade of pelagic organisms in the delta has declined in abundance (Feyrer et al. 2007). Potential factors associated with these declines in delta smelt include reduction in freshwater flows, entrainment losses at diversions and power plants, inadequate food base and competition for food from exotic species, environmental contaminants, and predation by exotic fishes. Only limited work has been reported on smelt disease (Antonio et al. 2000, Teh 2007). Our objective was to determine the presence of infectious pathogens (viruses, bacteria, or parasites), tissue abnormalities (gill, liver, intestinal tract), peripheral blood cell profile, and gill Na-K-ATPase activity in adult smelt captured in the lower Sacramento River between January and May 2010.

METHODS

One hundred and five adult smelt were sampled from the California Department of Fish and Game (CDFG) Bay-Delta Kodiak trawl collections in the Sacramento River during January–May 2010 (Table 1). After initial evaluation of maturity by a CDFG biologist, the following actions were taken: (1) fish were euthanized in 100 mg/L benzocaine; (2) measured by caliper (standard length; mm), weighed (Pesola micro-line 20010 hanging balance, 0.1g), and condition factor calculated ($(g/mm^3) \times 10^5$); (3) caudal peduncle severed and blood collected in microhematocrit tube; (4) blood was transferred into cold L-15 separation solution or smeared directly onto glass slides; (5) gill tissue placed into 100 microliter Sucrose-EDTA-Imidazole solution and frozen on dry ice; (6) liver aseptically removed with half placed into Brain Heart Infusion (BHI) broth transport media and the other half placed into a tube and frozen on dry ice; (7) anterior kidney removed and placed into cold antibiotic-

Table 1. Delta smelt collection dates in 2010, site numbers, (Montezuma slough [606]; Sacramento River from Brannon Island [704] upstream to the deep water ship channel [719]) and associated sample of delta smelt per site, January - April 2010. If no sample was collected it is designated as "NT". One 30 mm larva was included in the collection from site 719 on 5 May.

	Date					
Location	13 Jan	10 Feb	10 Mar	11 Mar	7 APR	5 MAY
606	NT	NT	NT	2	NT	NT
704	8	NT	5	NT	12	2
706	6	1	4	NT	NT	NT
707	NT	NT	2	NT	NT	NT
713	1	NT	4	NT	NT	NT
715	3	7	7	NT	NT	1
716	NT	2	6	NT	NT	1
719	5	2	12	NT	25	2

antimycotic solution; and, (8) gill and viscera placed into Davidson's fixative for 24 h and processed for 6 µm histological sections. The remaining carcass (head into ethanol, body and gonad into 10% formalin) was given to CDFG for use by other researchers.

Anterior kidney was pooled (1 to 5 fish) in 1 ml antibiotic /mycotic solution and held on ice for 18 - 24 h until processed (American Fisheries Society-Fish Health Section 2007). The samples were sonicated (Heat system Microson XL2005, Farmingdale NY) for 10 s at #3 setting to disrupt the tissue, centrifuged at 10,000 x g x 4 °C, supernatant diluted 2x and 4x in Hanks Buffered Salt Solution, and 100 microliter samples inoculated onto duplicate wells of EPC, CHSE214, BF2, and SSN1 cell cultures maintained in 48 well plates. Cultures were incubated for 18 d at either 15 °C (EPC and CHSE214) or 25 °C (BF2 and SSN1) and examined every 3 or 4 days.

Liver in BHI broth was inoculated onto BHI agar and incubated at 20 - 25°C for 48 - 72 h as well as Middlebrook 7H10 media. For the 5 May collection, Lowenstein-Jenson 5% NaCl media was also inoculated. Both Middlebrook and Lowenstein-Jenson media were incubated at 25°C for 18 – 20 d. Any colony obtained from either Middlebrook or Lowenstein-Jenson media was acid-fast stained to presumptively identify *Mycobacterium* (Antonio et al. 2000, Stine et al. 2010). An ATCC culture of *Mycobacterium marinum* (ATCC 11565) was

grown on Middlebrook 7H10 media to verify its suitability for the survey. Isolates from BHIA were identified to genus or clade (American Fisheries Society-Fish Health Section 2007). DNA from frozen liver was extracted with a Qiagen Cube using the manufacturer's procedure and sent to the Real-time PCR Research and Diagnostics Core Facility, School of Veterinary Medicine, Department of Medicine and Epidemiology, University of California, Davis to be analyzed in their proprietary MTC assay for multiple *Mycobacterium* species.

Fixed tissue was processed for 6 µm paraffin sections and stained with hematoxylin and eosin (H&E). Sagittal sections, of a 30 mm larvae collected on 5 May, were stained by both H&E and Nissel stain for neurons. Tissues examined microscopically included gill, liver, intestine, adipose tissue, pancreatic acinar cells, spleen, and heart. Not all tissues were sectioned for each fish.

In March, blood smears were immediately prepared using a cover slip, air-dried, fixed in cold methanol for 5 minutes, and protected from light until further processing. During April and May, blood was dispensed from the hematocrit tubes into Eppendorf tubes containing L-15 separation solution (1,000 µl of Lebowitz L-15 media, 75 µl of 7.5% fetal bovine serum, and 2 µl of 5 KU / ml heparin). Blood smears were then prepared 4-8 h later by brief vortexing, placing 200 µl of the suspension into a single well cyto-spin chamber, centrifuged at 1,600 rpm for 4 min in a Statspin Cytofuge (StatSpin, Inc., Norwood, MA), air-dried and fixed in cold methanol for 5 minutes. For panoptical staining, blood smears were treated according to a modified Leishman-Giemsa staining protocol of Yasutake and Wales (1983). For each peripheral blood smear, 81-115 leukocytes were classified as lymphocytes, granulocytes, or thrombocytes on the basis of panoptical staining characteristics (Blaxhall and Daisley 1973) by examination at 100x magnification and expressed as a proportion of the total number of leukocytes classified. A lymphocyte to granulocyte ratio was calculated for each sample (Modra et al. 1998). Gill Sodium-Potassium-Adenosine Triphosphatase activity (ATPase = µmoles ADP/mg protein/hr) was assayed by the method of McCormick and Bern (1989). Statistical analysis was performed with SigmaStat 3.1 software on raw data. Normality was tested with the Kolmogorov–Smirnov method at the $P = 0.05$ level. One-way ANOVA or T-test (data with normal distribution, reported with F or t value) or Kruskal-Wallace ANOVA or Mann-Whitney U test with subsequent multiple comparison procedures (Holm-Sidak or Dunns method respectively, $alpha \leq 0.05$) was used to compare groups.

RESULTS

Male smelt averaged 62 mm in standard length (SL) while the mean female SL was 66 mm (Table 2). Similarly, female wet weight and condition factor were higher than males due to their gravid condition. No abnormalities (cytopathic effects) were observed in the 4 cell lines over the 18 d incubation periods (Table 3). The eighteen anterior kidney pools (1-5 fish / pool) tested contained a total of 79 smelt. It was necessary to add fresh media to SSN1 and BF2 cultures to maintain them for the full 18 days. Liver samples inoculated onto the general purpose media, Brain Heart Infusion Agar, isolated a low incidence of aquatic bacteria (*Aeromonas / Pseudomonas* sp. and *Staphlococcus / Micrococcus* sp.). Middlebrook cultures, collected on 13 January, were contaminated with fungi and environmental bacteria related to the outside inoculation conditions and discarded. On subsequent trips, fish dissection and tissue collection were done within the boat's cabin. No acid-fast bacteria were isolated from either the Middlebrook 7H10 or Lowenstein-Jenson 5% NaCl media (Table 3). In contrast, 54% of the livers tested positive for *Mycobacterium* sp. DNA by PCR

Table 2. Mean (SE) standard length, weight, and condition factor ($(g/mm^3) \times 10^5$) of delta smelt collected from the lower Sacramento River, California, January - May, 2010. Sample number (N) for fish identified to sex is less than total number of fish collected in 2010.

Metric	Male	Female
Standard length (mm)	62 (4), N = 30	66 (4), N = 60
Weight (g)	2.1 (0.3), N = 26	2.7 (0.6), N = 59
Condition factor	0.895 (0.119), N = 26	0.936 (0.115), N = 59

assay. These *Mycobacterium* data indicate a high incidence of asymptomatic, carrier-state infections but not a significant health problem for the sample population.

Table 3. Viral assay, bacterial culture (Brain heart infusion agar [BHIA] and mycobacterium media Middlebrook 7H10 [MB]), and *Mycobacterium sp.* (Mycob) PCR results for delta smelt collected from the lower Sacramento River, California, January - May 2010. Data are reported as number positive/monthly sample number (or viral pools) collected in Kodiak trawls and incidence (total positive/total samples [%]). If sample was not performed or collected it is designated as "ND".

Assay	13 Jan	10 Feb	10 Mar	11 Mar	7 Apr	5 May	Incidence
Viral (pools)	0 / 5	0 / 3	ND	0 / 1	0 / 8	0 / 1	0 / 18
BHIA	5 / 23 [a]	0 / 12	ND	0 / 2	2 / 33 [a] 2 / 33 [b]	0 / 5	7 / 75 (9) [a] 2 / 75 (3) [b]
MB	ND	0 / 12	ND	0 / 2	0 / 33	0 / 5 [c]	0 / 19
Mycob PCR	18 / 23 (78)	6 / 11 (55)	ND	2 / 2 (100)	9 / 27 (33)	1 / 4 (25)	36 / 67 (54)

[a] *Aeromonas / Pseudomonas* sp.
[b] *Staphlococcus / Micrococcus* sp.
[c] Lowenstein – Jenson 5%NaCl media also used for *Mycobacterium* isolation

Few tissue abnormalities and no overt infectious disease was observed in 72 histological specimens (Table 4). Helminth parasites (nematode and trematode) were seen in < 10% of intestine, visceral adipose, or liver sections. No lesions were associated with the parasites. No parasitic infection was seen in gill sections. Focal liquefactive necrosis was observed in 7% of the liver sections and occurred primarily in the 10 February collection. The cause of the lesion is unclear, as there was no association with bacterial or parasitic infection, but post-mortem artifact cannot be ruled out. No acid-fast bacteria (e.g, *Mycobacterium*) were observed in a pancreatic acinar tissue granuloma from a 13 January

sample. Spleen contained multifocal regions of endogenous pigments / melanomacrophage centers. Small foci of lipofuscin were seen in the visceral adipose tissue of 2 fish. No necrotic neurons were seen in the brain and retina (Nissel stain) of the 30 mm larvae collected on 5 May.

Table 4. Histological observation of parasites or tissue abnormalities such as nematode (nem), trematode (trm), granulomatous foci (grn), inflammatiory cell foci (icf), liquefactive necrosis foci (lqn), and endogenous brown pigment foci (bpf) among delta smelt collected from the lower Sacramento River. January - May 2010. Data are reported as the number of specific tissues positive for either parasites or abnormality/total sections by date of collection and incidence (total positive/total samples [%]). If no specific tissue in sections of a given collection it is designated as "NT".

	Date						
Tissue	13 Jan	10 Feb	10Mar	11Mar	7 Apr	5 May	Incidence
Gill	0 / 12	0 / 12	0 / 21	0 / 2	0 / 14[a]	0 / 4	0 / 65 (0)
Heart	0 / 6	0 / 4	0 / 6	NT	0 / 5	NT	0 / 21 (0)
Intestine	2/15 nem	0 /12	0 / 23	0 / 2	0 / 16	0 / 4	2 / 72 (3) nem
Stomach	0 / 16	0 / 12	0 / 19	0 / 2	0 / 12	0 / 3	0 / 64 (0)
Acinar cells	1 / 15 grn	NT	0 / 21	NT	0 / 12	0 / 3	1 / 51 (2) grn[b]
Visceral	0 / 15	0 / 11	1 / 21 nem	0/ 1	1 / 11 nem	1 / 3 trm	2 / 62 (3) nem
Adipose							1 / 62 (2) trm
Liver	1 / 15 icf	4 / 7 lqn	1 / 21 trm	0 / 1	0 / 2	0 / 3	1 / 59 (2) trm 4 / 59 (7) lqn 1 / 59 (2) icr
Spleen	3/3 bpf	2 /2 bpf	NT	NT	2 / 2 bpf	NT	7 / 7 (100) bpf

[a] Late fixation resulted in variable levels of post-mortem changes in tissue morphology.
[b] No acid fast bacteria observed in granuloma

Gill Na-K-ATPase activity was significantly lower in the 13 January samples compared to subsequent collection groups, but low sample numbers reduced our confidence in its biological significance (Table 5, ANOVA $F= 6.984$, $P= 0.001$). Twenty-five percent of the samples did not produce enzymatic activity. Protein concentrations of these "no activity" samples were similar to the other samples. We suspect the quantity of muscle and bone was in excess of gill filament in these samples, but were not able to rule out other explanations. Eight of the ten "no activity" samples occurred in the January and February collection groups. Mean activities of smelt ranged from 10.7 to 20.9; in comparison, mean gill Na-K-ATPase activity ranges of juvenile Chinook smolts assayed by the same methods ranged from 7.0 - 11.2 µmole ADP/mg protein/hr (Foott et al. 2007).

Table 5. Mean (SE) Gill Na-K-ATPase activity (micromole ADP/mg protein/hr), total gill sample set, and number of samples showing no activity (not included in analysis) from adult delta smelt collected from the lower Sacramento River by sample date, January - May 2010. Letters in common indicate differences were not significant. The single sample from 11 March was not compared to other groups.

	Date				
	13 Jan	10 Feb	11 Mar	7 Apr	5 May
Mean (SE)	10.7 (1.2) A	20.9 (2.8) B	8.6	17.6 (0.9) B	19.0 (2.4) B
Total	13	12	2	8	5
No activity	3	5	1	1	0

Thrombocytes, lymphocytes, and granulocytes were present in proportions of 0.876, 0.119, and 0.006 in males, of which 8 of 9 males were captured during March (Table 6). Females displayed the same order of prevalence (thrombocyte>lymphocyte>granulocyte)

Table 6. Proportion of leukocytes (mean [SE], range, and coefficient of variation [CV]) in peripheral blood of delta smelt collected from the lower Sacramento River, California, January - May 2010.

Type of leukocyte	Males (n = 9)	Females (n = 24)	Both Sexes (n = 33)
Lymphocytes			
Mean (SE)	0.119 (0.0285)	0.167 (0.0336)	0.154 (0.0257)
Range	0.030 - 0.300	0.000 - 0.610	0.000 - 0.610
CV	0.719	0.989	0.962
Granulocytes			
Mean (SE)	0.006 (0.0024)	0.045 (0.0139)	0.034 (0.0105)
Range	0.000 - 0.020	0.000 - 0.291	0.000 - 0.291
CV	1.308	1.526	1.780
Thrombocytes			
Mean (SE)	0.876 (0.0300)	0.789 (0.0416)	0.813 (0.0318)
Range	0.690 - 0.970	0.301 - 1.000	0.301 - 1.000
CV	0.103	0.258	0.225

in peripheral blood leukocyte types as males during March, April, and May. Granulocyte numbers increased over time in the females, resulting in a lower lymphocyte:granulocyte ratio in May compared to March or April (Figure 1).

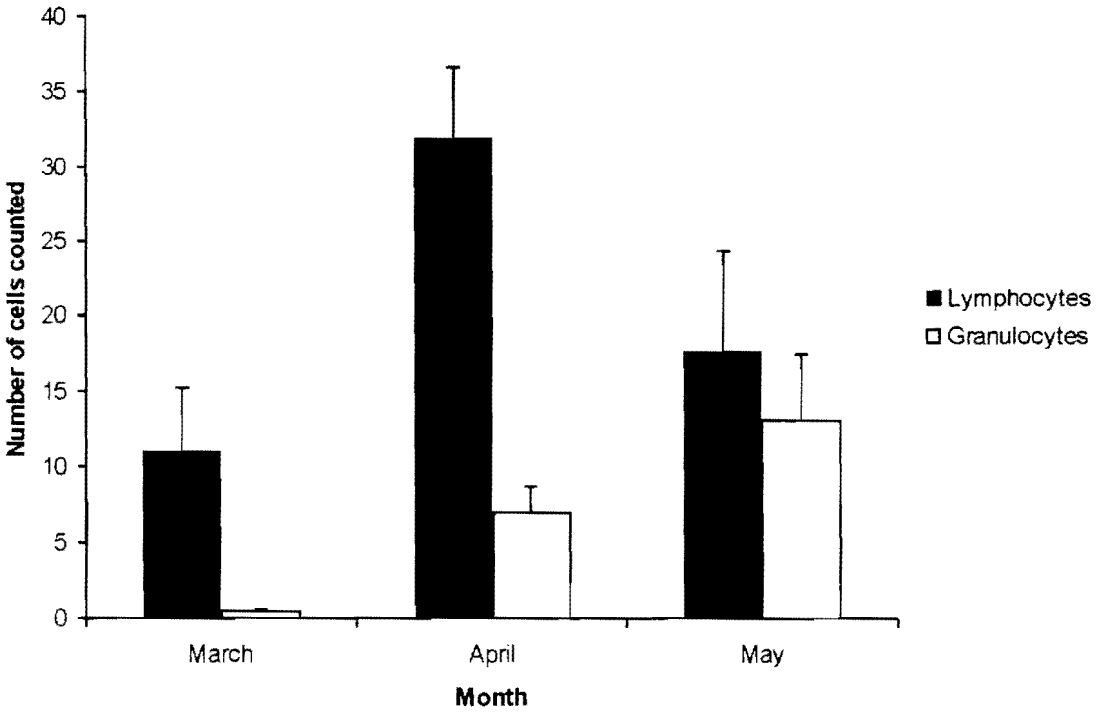

Figure 1. Mean number of lymphocytes and granulocytes counted (bar = SE) in peripheral blood of female delta smelt collected from the Sacramento River, California, January - May 2010. Insufficient male samples excluded them from temporal analysis.

DISCUSSION

Adult delta smelt, collected from the lower Sacramento River in the spring of 2010, were apparently healthy as demonstrated by lack of clinical signs or morbidity, few tissue changes or significant parasitic infections observed in histological specimens, or microbiological isolations. While a high incidence of asymptomatic *Mycobacterium* sp. infection was detected by PCR, this bacterial group was not isolated in culture. Antonio et al. (2000) reported the occurrence of *Mycobacterium* disease in delta smelt broodstock held at $\geq$ 16 °C and stressed by handling. Similar mycobacteria disease has occurred in captive post-spawning smelt held at Livingston Stone National Fish Hatchery. In contrast to a stressful culture situation, Antonio et al. (2000) were not able to culture *Mycobacterium* in recently caught smelt. Similar to the histological data, Teh (2007) reported no significant disease or parasitic infection observed in 385 adult smelt collected from all regions of the Delta in 2005. Teh (2007) noted that liver abnormalities (glycogen depletion, cytoplasm eosinophilic inclusion, fat vacuoles, single cell necrosis, and inflammation) were common observations.

The occurrence of melanomacrophage foci in the spleen has been reported in other fish. Melanomacrophage centers are common in fish hemopoietic tissues and act as depositories of oxidized materials, scavenged iron, and some micro-parasites (Aguis and

Roberts 2003). The macrophages contain a mixture of endogenous pigments such as lipofuscin/ceroid, melanin, and hemosiderin. The extent of such foci has been associated with contaminate exposure or hypoxic conditions (Fournie et al. 2001).

We do not have an adequate explanation for the "no activity" gill Na-K-ATPase samples collected in January and February. While collection, storage, and processing error could be the cause, metal exposure has been reported to impair Na-K-ATPase activity (Lauren and McDonald 1985, Roger et al. 2003). Delta smelt are exposed to a variety of contaminates that could affect their health and physiology (Kuivila and Moon 2004, Foe 1995).

Increased granulocyte numbers in the blood between March and May are counter to that observed by other workers where lymphocyte numbers and overall specific immune function tend to increase with increasing seasonal temperature. Alcorn et al. (2002) reported temperature influence on lymphocyte to granulocyte ratios, with greater numbers of peripheral blood lymphocytes in sockeye salmon reared at 12 C than 8 C. Similarly, Luskova (1998) noted the white blood cell count (leukocyte) increased with higher seasonal water temperatures in brown trout. Higher granulocyte counts result in low L:G ratio values, and can indicate infection, tissue damage, or seasonal blood cell changes (Modra et al 1998). It is unclear what influenced the higher granulocyte numbers in the May collection, given that water temperature increased throughout the spring and we did not detect clinical infections or tissue lesions in the gill or viscera.

The extreme stress of capture and handling associated with the Kodiak trawl would likely influence blood measurements. Delta smelt are extremely sensitive to handling stress (Swanson et al. 1996), and the effect on peripheral blood cells could induce an artifact. It is unlikely that leukocyte evaluation will be a valid biomarker for captured smelt. Physiological measurements that utilize tissue or blood are limited by the small size of smelt. The use of sentinel fish is one option for examining the effects of water quality on the health and physiology of these fish.

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