

**U.S. Fish & Wildlife Service**

**California Nevada Fish Health Center**

**FY2011 Technical Report:**

**Juvenile Stanislaus River Chinook salmon pathogen and physiology assessment: January – May 2011.**

**J.Scott Foott\* and R. Fogerty**



U.S. Fish and Wildlife Service  
California-Nevada Fish Health Center  
24411 Coleman Hatchery Road  
Anderson, CA 96007  
PH: (530) 365-4271 FAX: (530) 365-7150  
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\*direct correspondence

**Summary:** A total of 194 juvenile Chinook salmon were collected from the Oakdale (n=163) and Caswell (n=31) rotary screw traps between 25 January and 03 May 2011. High river flows impaired capture efficiency for parr and smolt. Both Oakdale and Caswell parr and smolts were judged to be in good health and condition. No site specific abnormality (histological marker for contaminate exposure) was observed in the sampled population. A low prevalence of sub-clinical *R. salmoninarum* infection was detected by direct fluorescent antibody test but not confirmed by PCR. Unlike 2010, the kidney parasite *Tetracapsuloides bryosalmonae* was not observed in the fish. It is likely that higher flows and cooler water temperatures were associated with this change. No viral agents or significant parasite or systemic bacterial infections were detected in the salmon. As expected, Stanislaus salmon showed increase growth and energy reserve content over time. Gill ATPase activity was slightly lower than observed in 2010 but considered normal for smolts.

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**Background** – The role disease, xenobiotic exposure, and impaired growth on low egg to smolt survival of Stanislaus River Fall-run Chinook salmon, is currently not understood. The USFWS California - Nevada Fish Health Center (FHC) has performed health and physiology evaluations of juvenile salmonids in the Sacramento, San Joaquin, and Klamath River basins since the early 1990's (reports at <http://fws.gov/canvfhc>). A 2010 survey of 259 Stanislaus R. juveniles demonstrated a low prevalence of infectious agents and apparently healthy population (Nichols 2010).

In 2011, our objective was to examine Stanislaus R. juvenile Chinook salmon (*Oncorhynchus tshawytscha*) collected at an upper (Oakdale, rkm 64.5) and lower river (Caswell, rkm 13.8) site for the following:

- 1) Comparison of smolt quality of 0+ *smolts* between Oakdale and Caswell.
  - a. Gill Na-K-ATPase activity indicative of smoltification ( $> 2SD$  of mean parr activity, approximately 6-7  $\mu\text{moles ADP/mg protein/hr}$  or greater).
  - b. Condition factor ( $KFL > 0.90$ ,  $KFL = WT/FL^3 \times 10^5$ ) and presence of visceral fat (gross observation rating 0-2)
  - c. Prevalence of disease -pathogen infection or non-infectious tissue abnormality (data from culture assays or histology)
- 2) Compare energy reserves and growth indicators of *parr* between Oakdale and Caswell.
  - a. Whole body triglyceride & protein content
  - b. Caudal muscle RNA:DNA ratio
  - c. Condition factor ( $KFL = WT/FL^3 \times 10^5$ )
- 3) Determine energy reserves and growth indicators of *fry* at Oakdale.
  - a. Whole body triglyceride & protein content
  - b. Caudal muscle RNA:DNA ratio
  - c. Condition factor ( $KFL = WT/FL^3 \times 10^5$ )
- 4) Determine prevalence and severity of infectious and/or non-infectious disease.
  - a. Viral assay of fry and parr
  - b. Systemic bacteria (kidney) from parr and smolt
  - c. *Renibacterium salmoninarum* from parr and smolt
  - d. Internal / external parasites (histological examination) all 3 life stages
  - e. Tissue abnormalities (histological examination) all 3 life stages

## **Methods:**

*Field collection* - Juvenile fall-run Chinook salmon were collected at two 2.5m rotary screw trap (RST) sites on the Stanislaus River between 25January – 03May 2011. The upper trap (Oakdale, operated by FISHBIO) and lower trap (Caswell, operated by Cramer Fish Sciences) were sampled five times to target the fry, parr and smolt life stages. Subsamples of each collection group were weighted to the nearest 0.01g and fork length measured with calipers (1mm). Condition factor was calculated as  $KFL = Wt / FL^3 \times 10^5$ . A qualitative visceral fat score (0= not visible by eye, 1 =  $< 50\%$  of peritoneum with adipose tissue, 2=

>50% of peritoneum with adipose tissue) was recorded for a subset of dissected fish in each collection group. Fry were sampled for viral assay, whole body triglyceride and protein, caudal muscle RNA: DNA, and histology. In addition to the above samples, the following were obtained from parr and smolts: gill for Na-K-ATPase, a portion of liver for lipid peroxidation, and kidney inoculum for bacterial isolation and *R. salmoninarum* testing. Low abundance of parr and smolts resulted in trap crews freezing fish to increase sample size for triglyceride, protein, and *R. salmoninarum* testing.

*Microbiology and histology* - Kidney samples were inoculated onto BHIA media and isolates identified to genus by standard biochemical methods, 5 pooled kidney or whole body (fry) samples were inoculated onto EPC and CHSE214 cell lines for 18d (15°C) virus isolation, and kidney imprints were examined for bacteria by gram stain and direct fluorescent antibody test (DFAT) for *R. salmoninarum* using polyclonal antibody followed by PCR of archived materials from presumptive positive samples (USFWS and AFS-FHS 2007). Histological samples were held in Davidson's fixative for 48h, processed for 5µm paraffin sections, and stained with hematoxylin and eosin. Sagittal sections were made of smaller fish with tissues examined including skin, muscle, gill, thymus, olfactory pit, eye, brain, liver, intestine, adipose tissue, acinar cells, kidney, and the peritoneal cavity. Fish larger than 70mm were dissected and specific organs (kidney, gastrointestinal tract, gill, and liver) processed for histology.

*RNA: DNA assay* - The method of Kaplan et al. (2001) was modified to determine RNA-DNA ratio of caudal muscle. Briefly, a 1-2mm thick section of the caudal peduncle was dissected and placed into 0.5 mL of RNAlater storage solution, held at 4°C for 24h and stored at -20°C until processed. Caudal muscle sections (0.02 – 0.10 g), with skin and vertebrae, were later removed from the storage solution, blotted, weighed to the nearest 0.01g, placed into 1.5mL snapcap tube containing 250µL lysis buffer and 0.1g of 0.5mm ZrO<sub>2</sub> beads, homogenized in a bullet blender (striated muscle setting, NextAdvance Inc.), 10µL samples of digest was analyzed with a BioTek FL600 fluorescence reader using Invitrogen Quant-IT™ dsDNA (high sensitivity Q33120) and RNA (high sensitivity Q33140) kits with DNA and RNA standard curves. The lysis buffer contained 5.1M guanidine thiocyanate, 50mM NaCl, 50mM EDTA, and 0.5% B-mercaptoethanol. This index is based on the concept that a fish undergoing growth will have increased protein synthesis and associated increased cellular RNA levels while cellular DNA concentration remains constant (MacLean et al. 2008). A high ratio indicates recent growth.

*ATPase and Lipid peroxide assay* - Gill Na-K-ATPase activity of gill tissue was assayed by the enzymatic method of McCormick (1993). Malondialdehyde (MDA), a lipid peroxidation product, was measured in liver tissue by the method of Almroth et al. (2008) using OxisResearch LPO-586 kit (OxisResearch, 1499 Rollins Road, Burlingame CA 94010). Briefly, a small sample of liver (10 –

20mg) was placed into 0.5mL buffer (PBS, 0.5M butylated hydroxytoluene in acetonitrile), frozen on dry ice, stored at -70°C, defrosted and rapidly homogenized by sonication (Microson™ Ultrasonic Cell Disruptor), centrifuge (15,000 x g, 4°C, 10 min), supernatant reacted with N-methyl-2-phenylindole in acetonitrile and 37% HCl, and its absorbance at 586 nm compared to a MDA standard curve. Lipid peroxidation is a result of oxidative stress on lipid components of the cell membrane and can be induced by xenobiotics (Porter et al 1995). When antioxidant mechanisms are overwhelmed, peroxidation will tend to occur in the fish.

*Triglyceride and protein* - Cold distilled water was added to a 20 mL tube containing the fish (1:1 w / v) and blended for 30 – 90s with a Biospec M133 homogenizer. Two aliquots (100- 200µl) of the homogenate (2xWB) were placed into tared 2mL centrifuge tubes, weighed to the nearest 0.01g to determine tissue weight (homogenate wt. divided by 2), and assayed for triglyceride and protein.

Tissue triglyceride content (mg TG / g tissue) was assayed by a modification of Weber et al. 2003. Absolute isopropanol was added (5x dilution w/v) to an aliquot of homogenate, mixed at room temperature for 20 min, centrifuged at 4500xg for 10 min, and replicate 10 µL samples of the supernatant used in an enzyme assay for triglyceride (Pointe Scientific triglyceride GPO kit). Standards were diluted in a 1:4 mixture of distilled water and isopropanol (same as blank solution).

Protein content (mg protein / g tissue) of the homogenate was assayed by a modification of the alkaline digestion method reported by Woo et al. 1978. Briefly, 0.5N NaOH was added to the homogenate (5x or 10x dilution w/v), mixed at 45°C for 120 min, centrifuged at 4500xg for 10 min, and replicate 10 µL samples of the supernatant assayed for total protein by the Biuret method (Pierce BCA protein assay kit, Rockford IL). The blank consisted of 1:4 or 1:9 mixture of distilled water and 0.5N NaOH. Albumin diluted in the blank 0.5M NaOH solution was used as the protein standard.

*Statistical analysis* - Analysis was performed with SigmaStat 3.1 software on raw data. Normality was tested by 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-Wallis ANOVA or Mann-Whitney U test on ranks (non-parametric analysis) with subsequent multiple comparison procedures (Holm-Sidak or Dunns method respectively,  $\alpha \leq 0.05$ ) was used to compare groups.

### **Results:**

A total of 194 juvenile Chinook salmon were collected by the FHC or RST staff from the Oakdale (n=163 and Caswell (n=31) traps. Catches of parr and smolts at both traps were low (<5 / d) after flow increases in late March (Fig. 1 and 2).

Water temperature ranged from 9 – 14°C during the 25January – 03May study period (FishBio newsletters, <http://fishbio.com/archives>).

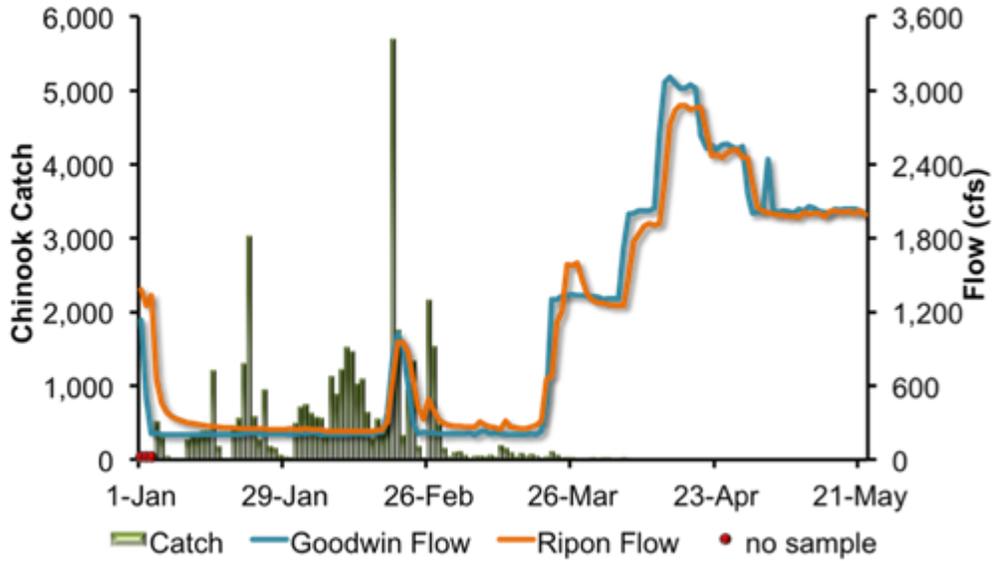


Figure 1. Daily Chinook salmon catch at Oakdale, and Stanislaus River flow recorded at Goodwin Dam (GDW) and Ripon (RIP) in 2011. FISHBIO.COM San Joaquin Basin update 6-3-2011.

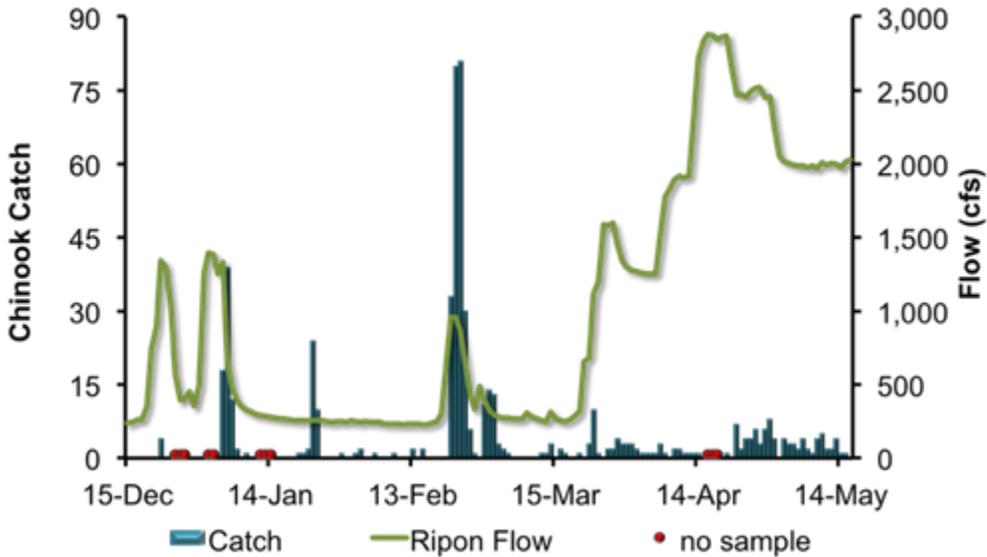


Figure 2. Daily Chinook salmon catch at Caswell, and Stanislaus River flow recorded at Ripon (RIP) in 2011. FISHBIO.COM San Joaquin Basin update 6-3-2011.

*Fish size* – Fry collected at Oakdale on 25January averaged 35mm FL with relatively low mean condition factor of 0.769 (Table 1). Parr and smolt sizes were similar at the 2 sites except for the 31March collection in which Caswell smolts were larger. The condition factor of the 31March Caswell parr was also larger than the Oakdale parr (T-test of 31March fork lengths had a P-value of P=0.057). In general, no size differences were observed between the sites and smolt showed normal condition factors.

Table 1. Fork length (FL, mm), weight (WT, 0.01g), and condition factor (KFL =  $WT / FL^3 \times 10^5$ ) for fry, parr and smolt stage salmon sampled at Oakdale or Caswell between 25January and 03May 2011. Data recorded as mean (standard error of the mean) and number of fish measured (No.).

Date	Site / life stage	No.	FL	WT	KFL
25JAN	<u>Oakdale</u> Fry	20	35 (0.4)	0.32 (0.01)	0.769 (0.016)
18MAR	<u>Oakdale</u> Parr	16	60 (1)	1.88 (0.13)	0.856 (0.025)
	Smolt	3	71 (0.3)	3.22 (0.23)	0.911 (0.055)
	<u>Caswell</u> Parr	1	51	1.15	0.867
	Smolt	2	77 (3)	4.45 (0.45)	0.971 (0.016)
31MAR	<u>Oakdale</u> Parr	37	61 (1)	2.09 (0.11)	0.948 (0.014) <sup>b</sup>
	Smolt	6	72 (1) <sup>b</sup>	3.59 (0.24) <sup>b</sup>	0.986 (0.041)
	<u>Caswell</u> Parr	3	67 (1)	3.22 (0.26)	1.083 (0.027) <sup>a</sup>
	Smolt	10	85 (3) <sup>a</sup>	7.25 (0.87) <sup>a</sup>	1.111 (0.037)
7APR	<u>Oakdale</u> Smolt	5	77 (5)	4.64 (0.51)	0.991 (0.042)
	<u>Caswell</u> Smolt	4	84 (4)	5.91 (1.72)	0.907 (0.062)
3MAY	<u>Oakdale</u> Smolt	10	88 (1)	7.41 (0.24)	1.075 (0.021)
	<u>Caswell</u> Smolt	7	94 (3)	8.47 (0.89)	0.998 (0.046)

Letters indicate significant difference between the specific life stage and collection date cohort (P<0.05)

*Pathogens*- No viral agents were detected in 76 fry or parr sampled in January or March (Table 2). A relatively low prevalence of common aquatic bacteria (14% *Aeromonas* / *Pseudomonas*, 3% *Staphylococcus* sp.) were isolated throughout the collection period with no fish showing clinical signs of infection (Table 2). It is likely that a low prevalence of sub-clinical *Renibacterium salmoninarum* infection is present in the juvenile population however this assertion cannot be confirmed. The direct fluorescent antibody screening test (DFAT) for *R. salmoninarum* infection detected low numbers of characteristic bacteria in 12 kidney samples (11% presumptive prevalence) with the majority of positive samples collected on 31March at both locations (Table 2). PCR assays of archived kidney material, from the DFAT positive samples, did not amplify *R. salmoninarum* DNA. It is likely that the number of bacteria per milligram of tissue was too low for confirmation.

*Tetracapsuloides bryosalmonae* was not observed in 62 kidney sections from fry, parr, or smolts collected at both locations (Table 2). Nematode or trematode infection of the small intestine (pyloric ceca) was observed in 15% of the samples however there was no associated lesion or inflammation (Fig. 3). Focal necrosis in the liver of one Caswell smolt was further analyzed by gram stain. No bacteria were observed in the lesion and it is unclear the cause of the necrosis. Similarly, we do not understand the cause of eosinophilic vacuolization in hepatocytes of an Oakdale smolt collected on 03May (Fig. 4). It is likely that the vacuoles contain excess protein. The majority of livers were normal and showed little to no hepatocyte vacuolization (Vac0 or Vac1 in Table 2). These vacuoles likely contained fat as Periodic Acid – Schiff (PAS) stain showed only minor amounts of PAS+ glycogen in the cytoplasm (Fig. 5).

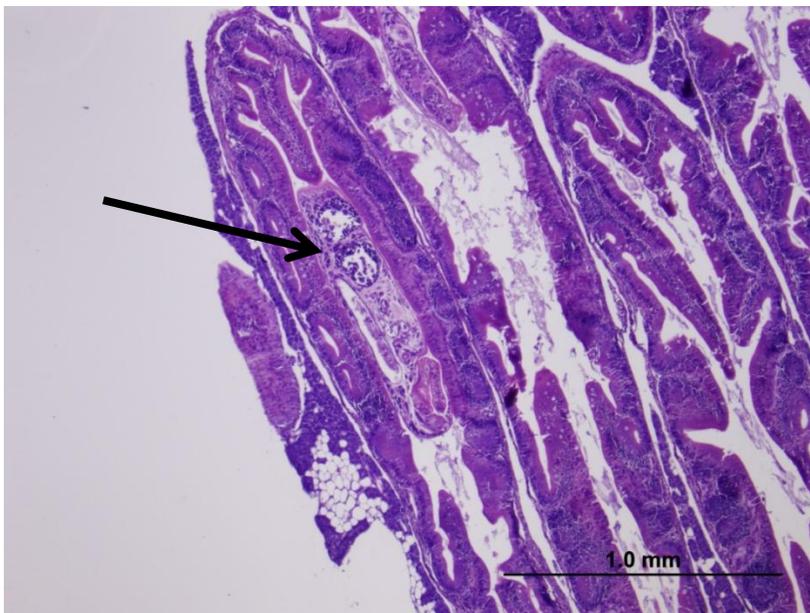


Figure 3. Trematode within pyloric ceca of 31March Oakdale parr.

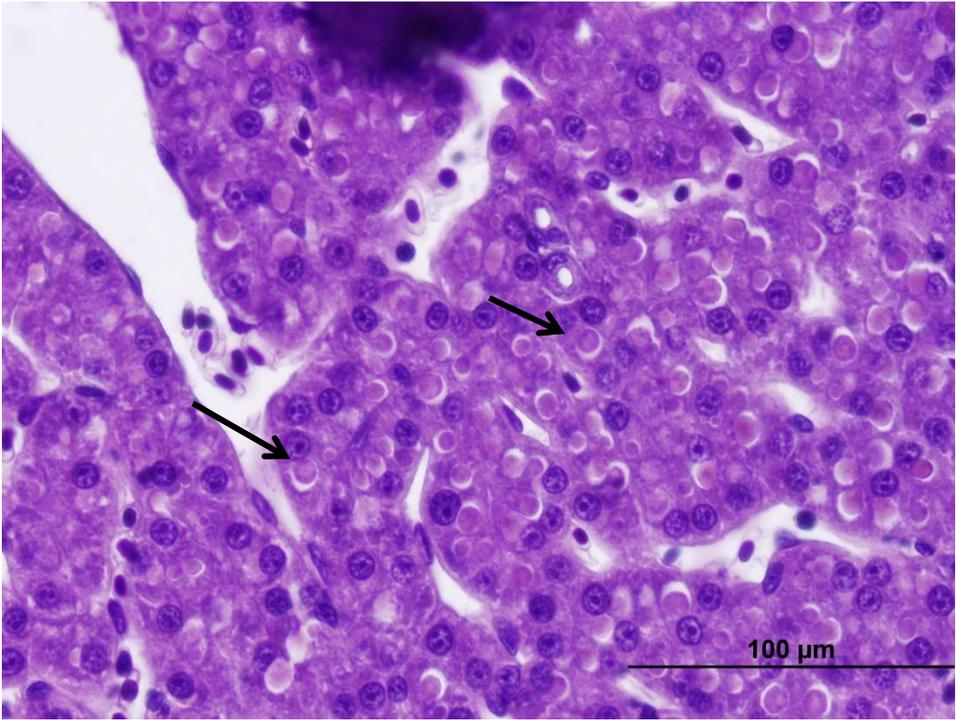


Figure 4. Eosinophilic inclusions (arrows) with hepatocyte of 03May Oakdale smolt liver.

Table 2. Pathogen detection in juvenile Chinook fry, parr, and smolts captured between 25Jan and 03May 2011 at Oakdale and Caswell traps. Data recorded as number of samples positive / total samples (%) and overall prevalence of infection for all samples. Viral samples are pools of fish (Total fish) and forty *R. salmoninarum* DFAT samples (36 Oakdale, 4 Caswell) were from frozen carcasses of parr and smolts collected by rotary screw trap crews in April.

Date	25-Jan	17-Mar	17-Mar	31-Mar	31-Mar	7-Apr	7-Apr	3-May	3-May	April	
Location	Oakdale	Oakdale	Caswell	Oakdale	Caswell	Oakdale	Caswell	Oakdale	Caswell	Frozen RST	POI
Viral (pools, fish)	0 / 6, (30fish)	0 / 2, (10fish)	0 / 3, (3fish)	0 / 4, (20fish)	0 / 2, (13fish)	ND	ND	ND	ND		0 / 17 pools, 0 / 76 fish
<b>Bacteriology</b>											
A/P	ND	1 / 10	1 / 3	3 / 20	2 / 11	0 / 5	0 / 4	2 / 10	1 / 7		10 / 70 (14%)
Staphylococcus	ND	0 / 10	0 / 3	0 / 20	1 / 11	0 / 5	0 / 4	1 / 10	0 / 7		2 / 70 (3%)
Rs DFAT	ND	2 / 10	0 / 3	6 / 20	3 / 13	0 / 5	0 / 4	1 / 10	0 / 7	0 / 40	12 / 112 (11%)**
Rs PCR	ND	0 / 1 p	ND	0 / 3 p	0 / 1 p	ND	ND	0 / 1	ND	ND	0 / 6 **
<b>Histology</b>											
KID Tb	0 / 10	0 / 4	0 / 2	0 / 7	0 / 13	0 / 5	0 / 4	0 / 10	0 / 7		0 / 62
Smlntes Trem-Nem	0 / 10	1 / 4	0 / 2	5 / 7	0 / 13	0 / 5	1 / 4	1 / 10	1 / 7		9 / 62 (15%)
Hepatocyte Vac0	5 / 10	3 / 4	2 / 2	2 / 7	2 / 13	0 / 5	2 / 4	3 / 9	5 / 7		24 / 61 (39%)
Hepatocyte Vac1	5 / 10	1 / 4	0 / 2	4 / 7	7 / 13	5 / 5	2 / 4	5 / 9	1 / 7		30 / 61 (49%)
Hepatocyte Vac2	0 / 10	0 / 4	0 / 2	1 / 7	4 / 13	0 / 5	0 / 4	1 / 9	1 / 7		7 / 61 (12%)

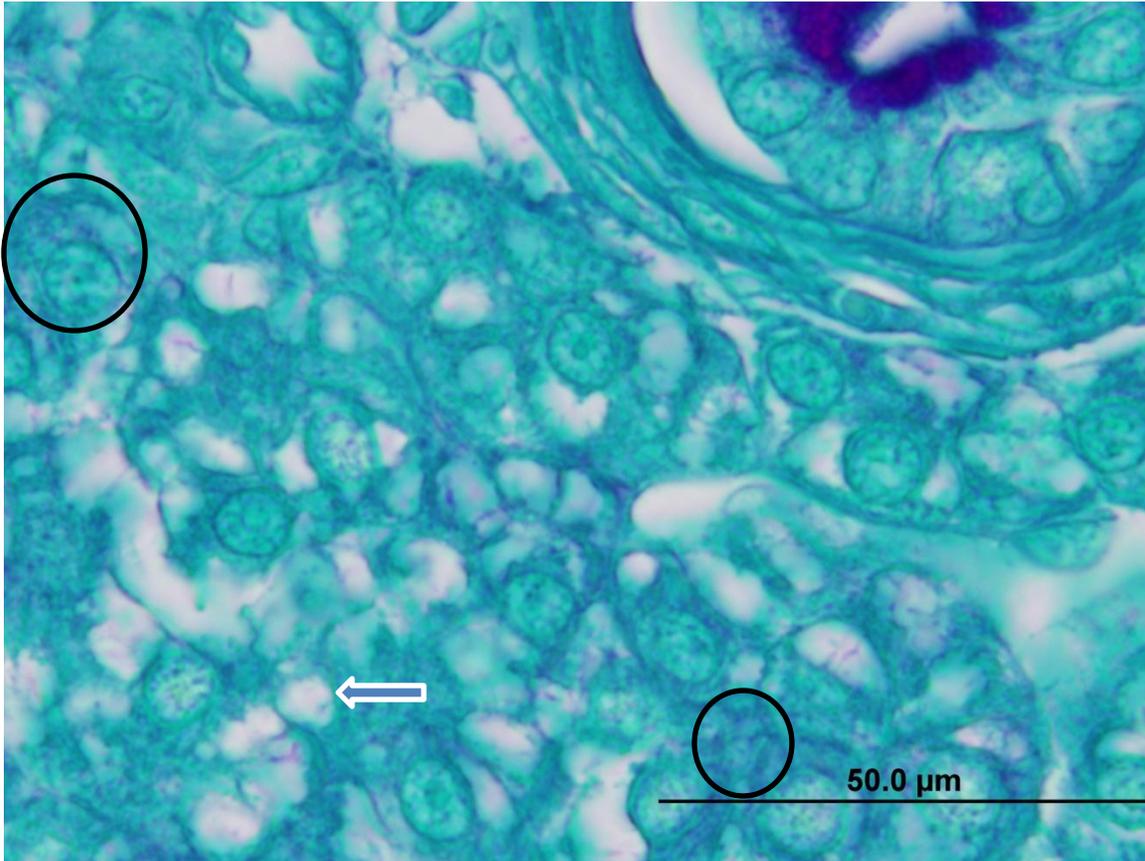


Figure 5. Minimal hepatocyte vacuole glycogen content by PAS stain. Cytoplasmic purple granules (indicative of glycogen are circled) are faint and relatively rare. The clear vacuoles indicate the presence of fat (open arrow)

*Gill ATPase*- Sodium-Potassium- Adenosine Triphosphotase activity increased as fish reached smolt size (Table 3, Fig. 6). No differences were detected between Oakdale and Caswell fish collected on any specific date. Oakdale smolts tended to have higher activity levels than Caswell (T-test,  $P=0.056$ , Fig. 6). No significant difference was observed in parr from both sites.

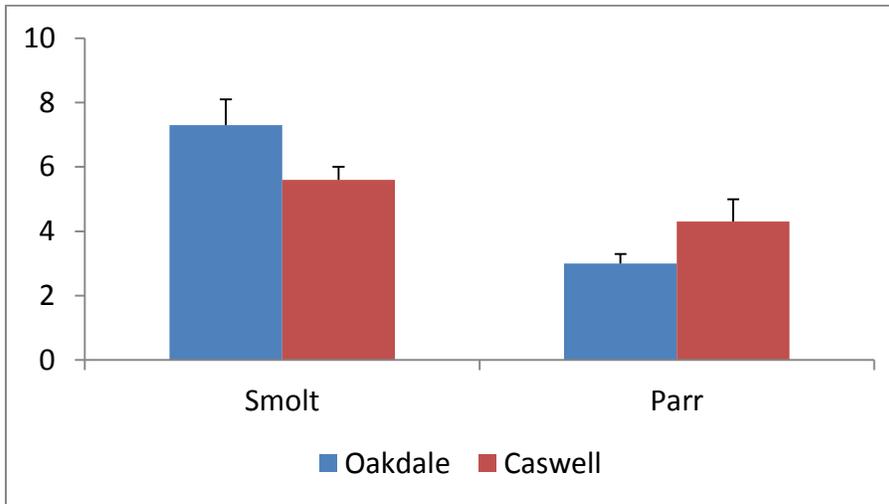


Figure 6. Mean gill Na-K-ATPase activity ( $\mu\text{M ADP/mg protein/hr}$ ) in smolts and parr collected throughout the study period at Oakdale or Caswell.

Table 3. Mean (SEM) values of RNA ( $\text{ng}/\mu\text{L}$ ): DNA ( $\text{ng}/\mu\text{L}$ ) ratio of caudal muscle, liver lipid peroxide ( $\mu\text{M MDA/g protein}$ ), and gill Na-K-ATPase activity ( $\mu\text{mole ADP/ mg protein/ hr}$ ). Number (n) of samples are indicated for each group.

Date	Site	Muscle	Liver	Gill ATPase	Gill ATPase
		RNA:DNA	Lipid peroxide	Smolt	Parr
25-Jan					
	OAKDALE	1.0 (0.1), n=24	ND	ND	ND
18-Mar					
	OAKDALE	0.5 (0.03), n= 10	17.5 (4.2), n= 3	ND	3.8 (0.9), n= 4
	CASWELL	0.6 (0.09), n=3	21.9 (3.4), n= 3	3.9 (1.0), n= 2	4.9, n= 1
31-Mar					
	OAKDALE	0.8 (0.03), n= 6	17.1 (4.1), n= 7	5.01, n=1	2.8 (0.2), n= 12
	CASWELL	0.7 (0.05), n= 13	41.1 (17.2), n= 7	4.7 (0.4), n= 11	4.1 (1.1), n= 2
7-Apr					
	OAKDALE	ND	ND	ND	ND
	CASWELL	ND	ND	ND	ND
3-May					
	OAKDALE	0.9 (0.07), n= 10 a	29.6 (5.8), n= 10	7.5 (0.9), n= 9	ND
	CASWELL	0.6 (0.05), n= 7 b	24.3 (2.5), n= 6	7.0 (0.8), n= 7	ND

ND = not done

*Energy and growth-* The RNA:DNA (R:D) ratio of caudal muscle ranged from 0.4 to 2.5 with means  $\leq 1.0$  (Table 3). It appears that RNA was lost during processing thereby invalidating this data. Comparison of smolt and parr muscle samples between locations did not demonstrate significant ( $P < 0.05$ ) differences. Weber et al. (2003) reports that juvenile rainbow trout starved for 5 – 18d had muscle R:D ratios of 1 – 2 with fed cohorts R:D ratios ranging from 3 -4. Similarly, MacLean et al. (2008) reported R:D values of 2 – 8 in juvenile Atlantic salmon.

Both triglyceride and protein content increased with life stage (Fry < Parr < Smolt) (Table 4, Fig. 7). There was no significant difference between the 4 smolts collected at Caswell and the 12 Oakdale smolts (T-test,  $P = 0.201$ ). A median visceral fat score of #1 was observed in parr and smolts at both locations with fewer #2 score fish at Oakdale (15%) than at Caswell (30%). Percent #0 score was 4% and 9% at Caswell and Oakdale respectively.

Table 4. Whole body triglyceride and protein (mg / g fish) content of fry, parr, and smolts captured between 25Jan and 20April. Caswell collection only included 4 smolts. Data reported as mean (SEM) and sample number (n).

<u>Dates</u>	<u>Group</u>	<u>mg Triglyceride / g fish</u>	<u>mg Protein / g fish</u>
25-Jan			
	Oakdale Fry	4.8 (0.4), n = 20	23.1 (0.9), n = 20
31Mar - 01Apr			
	Oakdale Parr	11.6 (1.2), n = 25	40.3 (0.7), n = 25
01Apr - 20Apr			
	Oakdale Smolt	23.8 (2.2), n = 12	46.4 (1.1), n = 12
	Caswell Smolt	29.7 (3.5), n = 4	48.2 (1.4), n = 4

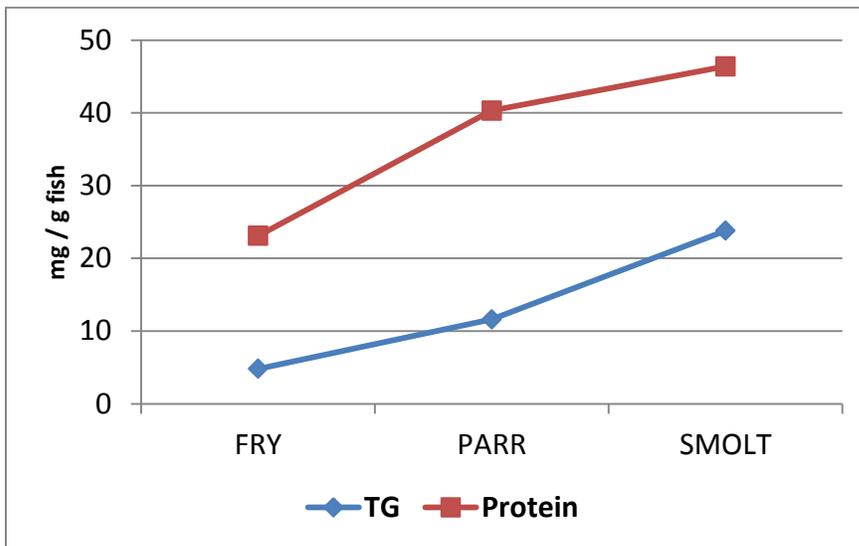


Figure 7. Mean whole body triglyceride and protein content of fry, parr, and smolts from Oakdale trap.

*Liver lipid peroxide (LPO)* – A total of 36 liver samples were collected from both sites between 18 March and 3 May and assayed for LPO (Table 3). No significant site differences were detected between fish collected on a specific date or particular life stage (parr or smolt). Coefficient of variation was quite large (33 – 119%) for the site specific data sets. We have located one report that used this method with fish tissue and their highest reported value is 48 – 1490x lower than that measured in this study. Further validation work will be required to gain confidence in this assay and relevance to juvenile salmon health.

### **Discussion:**

Neither infectious nor non-infectious (contaminate lesion) disease was observed in sampled salmon. It is likely that the population has a low prevalence of sub-clinical *R. salmoninarum* infection but the long term prognosis of these infections is unknown. *R. salmoninarum* was not detected in 2010. In contrast, the kidney parasite *Tetracapsuloides bryosalmonae* was seen in 2010 but not in 2011. High flows and cool temperatures in 2011 could be factors in this change. This parasite has caused significant disease problems in Merced River Hatchery Chinook salmon and has been detected in San Joaquin R. tributaries (Foott et al. 2007, Nichols and Foott 2002). Our first attempt at the LPO assay for liver lipid peroxidation was inconclusive and will require further validation work. Similarly, RNA:DNA data was inconclusive due to poor RNA recovery.

Oakdale and Caswell smolts were judged to be in good health and condition. Smolt ATPase activity was slightly higher (mean > 7.0) in May 2010 than observed in the 2011 (mean 7.0 and 7.5, Caswell and Oakdale respectively). ATPase activity is quite plastic in juvenile salmonids and the cooler water

temperatures in the spring of 2011 could have slowed smolt development. Overall, the 2011 smolts showed increased activity over time and had activities within expected ranges. As expected, Stanislaus salmon showed increase growth and energy reserve content over time. No site specific abnormality or consistent difference was observed in the sampled population.

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