LIST OF TABLES .................................................................................................................. 1
LIST OF TABLES .................................................................................................................. 3
LIST OF FIGURES .............................................................................................................. 4
STRESSOR IMPACTS ON SALMONIDS .............................................................................. 5
Heat-Shock Proteins Indicate Thermal Stress in Juvenile Steelhead Trout ...................... 5
Methods ............................................................................................................................ 7
Results .............................................................................................................................. 10
Discussion ....................................................................................................................... 12
Fluctuating Asymmetry .................................................................................................. 23
Study Areas And Methods ............................................................................................. 24
  Meristic Traits ............................................................................................................. 25
  Metric Traits ............................................................................................................... 25
  Measurement Error ..................................................................................................... 26
Results .............................................................................................................................. 27
  Quantitative Estimates Of Asymmetry ........................................................................ 27
  Spatial And Temporal Extent Of Asymmetry ............................................................ 29
Discussion ....................................................................................................................... 30
Physiological and Behavioral Effects of Zinc and Temperature on Coho Salmon
  (Oncorhynchus kisutch) .............................................................................................. 40
Methods ............................................................................................................................ 41
Results .............................................................................................................................. 44
  Growth and Condition Factor ................................................................................. 44
  HSP and Zinc .......................................................................................................... 45
  Behavior .................................................................................................................... 45
Discussion ....................................................................................................................... 46
LITERATURE CITED ...................................................................................................... Error! Bookmark not defined.
LIST OF TABLES

Table 3-1. Steelhead trout sampling sites in the Navarro River watershed......................17

Table 3-2. Presence of FA and DA in the two sample periods ........................................36

Table 3-3. Indices proposed for developing a composite measure of fluctuating asymmetry................................................................................................................36

Table 3-4. Basic parameter values for the four treatments .................................................50

Table 3-5 Between-subjects effects of diet and temperature on coho salmon ..................51
LIST OF FIGURES

Figure 3-1a, b. Relationship of stream temperature longitude, and percent shade………………19

Figure 3-2. Relationship of hsp72 with average monthly maximum temperature
mean monthly average temperatures…………………………………………………………20

Figure 3-3. Relationship of hsp72 levels with mean weekly maximum temperature
and mean weekly average temperatures for the 7 days preceding and
including the sampling dates ………………………………………………………………21

Figure 3-4. Relationship of hsp72 levels with mean daily temperature ranges
during the month of July 2000 ………………………………………………………………22
STRESSOR IMPACTS ON SALMONIDS
The primary stressor in the Navarro watershed appears to be high water temperatures that occur during both the spring period of larval fish development and throughout the late summer and fall. Juvenile fish are often isolated in pools and temperatures increase causing both acute and chronic temperature stress on the fish. Chronic temperature stress has been identified through the production of heat shock proteins by the fish, and we are investigating the potential tradeoff between maintenance of the inducible enzyme system and the potential decline in growth. Also, stress during larval development is indicated by the presence of fluctuating asymmetry of several skeletal characters in steelhead.

Heat-Shock Proteins Indicate Thermal Stress in Juvenile Steelhead Trout
Water temperature is one of the most important environmental variables influencing the distribution and success of salmonid populations (Dunham et al. 2001, Myrick and Cech 2001, Sullivan et al 2000, Baltz et al. 1987). Over the past three decades, these populations, among them steelhead rainbow trout (O. mykiss), have substantially declined along the West Coast of the United States (Lichatowich 1999, NMFS 2002). West Coast steelhead are presently distributed from the U.S.-Canada border south to San Mateo Creek, San Diego County, California (Moyle 2002). Populations south of Point Conception are endangered, whereas runs north of there are classified as threatened (Pt. Conception to Russian River, including Central Valley) or have been proposed for listing (NMFS, 2002). Efforts to conserve salmonid stocks include a variety of regulatory efforts to quantify and limit anthropogenic stressors such as increased thermal loading. To establish appropriate guidelines, there is an immediate need for information on temperature thresholds above which harm accrues to salmonid species.
The preferred temperature range for steelhead trout is reported to be 13-21°C (Coutant 1977) with a critical thermal limit of 27.5-32°C depending on acclimation temperature (10-25°C; Myrick and Cech 2001). Chronic lethal limits range from 22.8 to 27°C (Threader and Houston 1983) depending on acclimation history and body size with large fish being less tolerant than small fish. Evaluations of thermal tolerance in fishes are generally conducted in laboratory settings, and information on the physiological consequences of elevated but sublethal water temperatures under field conditions is rare. Juveniles may rear from one to several years (1-3 years; Moyle 2002) in freshwater streams before smolting and migrating to the ocean, and are therefore potentially most affected by high water temperatures. In small streams, thermal heterogeneity significantly complicates the task of identifying and quantifying thermal stress. Diurnal temperature fluctuations can be substantial, and the availability of thermal refuges, such as deep-water pools or shaded areas, determines if the fish experience harmful temperatures (Nielsen 1994). It is often unclear, if fish are able to avoid the areas of higher temperature and find thermal refuges, or if they are exposed to thermal stress. If exposed, it is not clear how individual fish respond to the temperature stress.

In an effort to detect and quantify thermal stress in steelhead trout in their natural habitat, we determined cellular levels of two heat-shock proteins (hsp), hsp72 and hsp76, in muscle tissue of parr (age group: 0+), and established threshold temperatures for increased hsp expression. Heat-shock proteins (hsp, stress proteins) play a major role in thermotolerance, and increased hsp levels are generally indicative of a disruption of cellular protein homeostasis (Parsell and Lindquist 1994, Coleman et al. 1995, Feder and
Hofmann 1999, Bierkens 2000). Hsps are important in protecting organisms against the cytotoxic consequences of protein denaturation (Feige et al. 1996). Recent research shows that hsps also interact with multiple key components of signaling pathways that regulate growth and development (Nollen and Morimoto, 2002). Among the major hsp protein families, hsp70 is the most prominent group. Hsp70s are involved in folding, repair and trafficking of intracellular proteins. Increased synthesis of hsp70s in response to thermal stress has been reported for numerous species of teleosts (Iwama et al. 1998) and many other organisms ranging from bacteria to humans (Morimoto et al. 1990).

Methods
Fish were collected between July 25 and August 4, 2000 at eleven sites located in the Navarro River watershed (lat. 39\(^\circ\) 10' 20", long. 123\(^\circ\) 40' 06"). Sampling sites covered all major sub-drainages including 2\(^{nd}\), 3\(^{rd}\) and 4\(^{th}\) order streams (Figure 1-1, Table 3-1). Steelhead parr (age group: 0+) were collected from both riffles and pools with beach seines at all sites except Upper Anderson Creek, Lower Indian Creek and Middle Rancheria Creek. Fish from those sites were collected using a Smith-Root Model 12-B backpack electrofisher. A random sample of 10 steelhead parr was removed from collections and flash frozen on dry ice within one half hour of collection. Only 5 fish were removed from Middle Flynn Creek and only one from Middle Anderson Creek due to the small size of the steelhead populations there. Samples were returned to the lab on dry ice, dissected and kept at –80\(^\circ\)C until processed.

Temperature was recorded at each site with a HOBO data logger fastened to a small piece of iron reinforcing bar and left throughout the summer on the substrate of a shaded run
within each site. No temperature data was collected for Upper Rancheria Creek because the site became dewatered early in the year. Temperature data used in our statistical data analyses were measurements from 1 July 2000 to the date when fish were sampled at individual sites. Conductivity and pH measured in early (June) and late (September) summer across the watershed ranged from 181 - 299 µScm$^{-1}$, and pH 6.6 - 7.9, respectively. Flow, percent shade and width-to-depth ratio were measured for sampling sites on Anderson, Indian, Rancheria Creeks and the North Fork of the Navarro River. Percent shade was measured using a convex spherical densiometer held 0.3m above the surface of the water in the middle of the channel. The mean percent of shade was calculated from three measurements taken at the downstream end, upstream end, and middle of the reach. To obtain the wetted channel width-to-depth ratio, eleven transects were spaced evenly along the length of the reach. The wetted width of the channel and the depth of the water were measured at three locations spaced evenly along each transect. The means of 11 width measurements and 33 depth measurements were used to calculate wetted channel width-to-depth ratio. Flow was measured using an electronic flow meter (Marsh-McBirney, Inc. Flo-Mate 2000) and the velocity-area method for measuring volumetric flow.

Hsp70 proteins were analyzed using western blotting techniques. Muscle samples were homogenized (1 min., glass on glass) on ice in a hypotonic solution containing 66 mM Tris-HCl (pH 7.5), 0.1% Nonidet, 10 mM EDTA, 10 mM DTT and protease inhibitors (10 mM benzamidine, 5 µM pepstatin, 0.001% aprotinin, and 0.1 mM phenylmethysulfonyl fluoride (PMSF)). Liver samples were analyzed initially, but did
not show a clear response pattern. Homogenates were centrifuged for 30 min. at 4000 g to remove large particulate material. Supernatants were collected, sample buffer (Laemmli, 1970) was immediately added, and samples were heated to 95°C for 2 minutes. Total protein concentration in each fraction was determined using the Biorad DC Protein Assay based on Lowry et al. (1951). Subsamples of equal total protein content (25 µg) were separated by SDS-PAGE on 12.5% polyacrylamide gels with 5% stacking gels (Blattler et al., 1972) using the buffer system described by Laemmli (1970). Hsp70 antigen (Stressgen, Victoria, BC, Canada) was applied to one lane per gel to serve as an internal standard for blotting efficiency. Proteins were separated at 25 mA per gel, then electroblotted onto Immobilon-P membrane at constant voltage (40 V) over night. Membranes were blocked with 5% skim milk in 20 mM Tris buffer and 0.4 M NaCl (pH 7.5) with 0.05% Tween-20 for 30 minutes. A monoclonal antibody for hsp70 (dilution 1:500; Affinity Bioreagents, MA3-001) was used as probe. In *O. mykiss*, this antibody recognizes two hsp70 isoforms of MW 72 and 76 kDa. Blots were incubated for 1 hour 30 minutes with primary antibody, then washed three times for 30 minutes in tris-buffered saline solution containing 0.05% Tween-20. Alkaline phosphatase-conjugated goat-anti-rat IgG (1:30000; Sigma) was used to detect the hsp70 probe. Bound antibody was visualized by a chemiluminescent substrate (CDP-Star; Tropix, Bedford, MA), and protein bands were quantified by densitometry (Biorad GS710).

All regression analyses were conducted with SPSS 7.0 and SigmaStat 2.0 (SPSS Inc., Chicago, IL). Since only one fish sample was obtained at Middle Anderson Creek (MAC), hsp results from this site were excluded from statistical analysis.
Results
Stream water temperatures in the Navarro River watershed are primarily dictated by air temperature and the degree of shading. Both geographic locations (as decimalized degrees longitude) reflecting the distance from the Pacific Ocean and percent shade were highly correlated with water temperatures at our sampling sites. Mean monthly maximum water temperatures ($\text{MMT}_{\text{max}}$) ranged from 15.4°C near the coast to 26.6°C at the most easterly sites ($r=0.94$, $p<0.001$; Fig. 3-1a). A similar relationship was seen for mean monthly average temperatures in July ($\text{MMAT}$) also increased from west to east, ranging from 14.6°C near the coast to 21.4°C further inland ($r=0.86$, $p=0.003$; Table 3-1).

Mean weekly average temperatures ($\text{MWAT}$) and mean weekly maximum temperatures ($\text{MWMT}$) for the 7 days preceding and including the sampling days ranged from 15.6°C-22.5°C and 16.6°C-27.4°C, respectively. Mean daily temperature ranges ($\text{MDTR}$) also increased from west to east ($r=0.94$, $p<0.001$) ranging from 1.7-10.6°C (Table 3-1).

Percent shade at our sampling sites was highest near the coast (approx. 80%) and decreased steadily to approximately 20% from west to east ($r=0.91$, $p=0.004$). Shade was inversely correlated with water temperatures ($\text{MMT}_{\text{max}}$: $r=0.83$, $p=0.010$; $\text{MMAT}$: $r=0.72$, $p=0.045$; Fig. 3-1b) and the mean daily temperature range ($r=0.79$, $p=0.020$). Average monthly minimum temperatures ($\text{MMT}_{\text{min}}$) for the same time period were not significantly correlated with geographic location ($r=0.58$, $p=0.1$) or percent shade ($r=0.03$, $p=0.713$). Neither the wetted channel width-to-depth ratio nor flow was associated with water temperatures at our field sites (data not shown).
Two hsp70 isoforms, hsp72 and hsp76, were detected in steelhead parr. Hsp72 showed a 3rd order sigmoid relationship with MMAT ($r=0.96$, $p=0.0004$; Fig. 3-2a) and with MMT$_{\text{max}}$ ($r=0.99$, $p<0.0001$; Fig. 3-2b). The threshold for the hsp72 increase was 18-19°C (minimum to maximum hsp72 levels) for MMAT, and 20-24°C for MMT$_{\text{max}}$.

Standard errors for the upper plateaus of the two curves, hsp72$_{\text{max}}$=12.98 for MMAT and hsp72$_{\text{max}}$=14.03 for MMT$_{\text{max}}$, are relatively large (8.76% and 8.03%, respectively), reflecting the degree of variation for these maximum values. The weekly temperature averages MWAT and MWMT showed similar but statistically weaker relationships with hsp72 (Fig. 3-3a, b). The threshold for MWMT was at 20-22°C ($r=0.96$, $p=0.0004$). The threshold temperature for MWAT was approximately 18°C, but MWAT was poorly associated with hsp72 levels, and did not appear to be an adequate parameter to characterize site-specific temperature regimes. For example, sites LIC and UIC (18.19-18.37) had similar MWAT values as LNF and MNF (18.22-18.30°C), but there was a significant difference in hsp72 levels. All other temperature parameters, especially MDTR, reflected this difference in hsp72 (Table 3-1). The second hsp isoform, hsp76, showed much weaker, but significant linear correlations with MMAT ($r=0.66$, $p=0.051$), MMT$_{\text{max}}$ ($r=0.76$, $p=0.019$) and MWMT ($r=0.69$, $p=0.04$). There was no significant correlation with MWAT ($r=0.56$, $p>0.05$). The maximum water temperatures were more strongly correlated with both hsp72 and hsp76 expression than the respective average water temperatures. Sampling sites with highest maximum temperatures also had the largest daily temperature fluctuations. The linear correlation between daily temperature fluctuation, expressed as MDTR, and hsp72 levels was highly significant ($r=0.95$, $p<0.001$; Fig. 3-4) and moderately significant for hsp76 ($r=0.76$, $p=0.019$). Hsp70 protein
levels were not significantly correlated with MMT min (hsp72: r=0.52, p>0.05; hsp76: r=0.44, p>0.05).

**Discussion**
The Navarro River watershed is representative of numerous streams along the west coast of North America where elevated water temperature and sediment load have become major stress factors for aquatic ecosystems. We have shown that where temperature is the dominant stressor, hsp proteins can be powerful tools to detect sublethal cellular stress. For Navarro River steelhead juveniles, thermal stress occurs at and above a monthly temperature average of 18-19°C (MMAT) and an average monthly maximum temperature of 20-24°C (MMT max). Threshold temperatures for the weekly averages preceding fish sampling were 18°C (MWAT) and 20-22°C (MWMT). The temperature thresholds established in this study concur with what little is known about the sublethal consequences of exposure to elevated temperatures in *O. mykiss*. Generally, it is assumed that steelhead trout experience thermal stress when temperatures exceed their preferred range of 13-21°C (Coutant 1977). In experiments on thermal preferences of steelhead trout (Myrick and Cech 2000 a), hatchery fish acclimated to constant (16°C) and diel cycling temperature regimes (16°C +/-2°C) selected temperatures in the 18-19°C range, while wild (Feather River, CA) fish, which were probably acclimated to lower temperatures, selected slightly cooler temperatures (approx. 17°C). Interestingly, the selected temperatures closely matched the temperature where growth rates were highest (Myrick and Cech 2000 a, b). In several studies, sublethal effects have been shown to occur at or below 21°C. In a 3-month study, Pankhurst et al. (1996) found that in adult rainbow trout, holding temperatures at and above 18°C had deleterious effects on
ovulation, egg production and embryo survival. A 3-hour exposure to 20-22°C (increased from 15°C in 30 min.) caused pathological changes in the epidermis of *O. mykiss* (Iger et al. 1994), and Magoulick et al. (1998) demonstrated that an exposure to 18°C (increased from 13°C) caused significant behavioral changes in juvenile brook trout and rainbow trout. Temperature can also influence physiological parameters such as the cholesterol-to-phospholipid ratios (C/P) in cellular membranes (Robertson and Hazel, 1995). In addition, susceptibility of *O. mykiss* to disease appears to increase at warmer water temperatures (Lyholt and Buchmann 1996, Schisler et al. 2000).

The induction of hsp70 by temperature stress has been studied in the laboratory in a variety of salmonids, but to our knowledge was never applied to fish populations in the field (Iwama et al. 1998, Bierkens 2000). Although we solely analyzed hsp70 isoforms in muscle tissue, it is possible that most other organs and tissues over-expressed hsp70s and members of other hsp families in these fish. For example, rainbow trout erythrocytes acclimated to 10°C, showed a significant increase in hsp70 when exposed for 2 hours to 25°C, but not at 15°C or 20°C (Currie and Tufts, 1997). In studying the whole-body response of Chinook salmon (*O. tshawytscha*) to various stressors, Palmisano et al. (2000) found that 5-h exposure to elevated temp. (21.6°C; +10.6°C over ambient) induced a marked increase in hsp90 mRNA accumulation in heart, brain, gill, muscle, liver, kidney, and tail fin tissues. The most vital tissues (heart, brain, gill, and muscle) showed the greatest hsp response, with heart tissue increasing approximately 35-fold. Smith et al. (1999) investigated the hsp response in isolated erythrocytes, branchial lamellae and hepatic tissue of *Salmo salar*. A 4-hour heat-shock at 20°C increased hsp65/66
(equivalent to hsp72/73) levels in branchial lamellae and erythrocytes, whereas the increase in hepatic tissue was seen at 24°C but not at 20°C. In *O. mykiss* primary cultures of hepatocytes, gill epithelial cells and fibroblast-like RTG-2 cells, hsp67, 69 and 92 were elevated when exposed to 26°C (from 18°C). Once induced, cellular hsp70 concentrations in salmonids may stay elevated for several days. Cutthroat trout (*O. clarki*) erythrocytes and gill tissue have been shown to maintain high levels of hsp70 proteins for at least 5 days after a 2-hour heat-shock at 22.4°C (from 6.2°C; Bierkens 2000).

Given the extent and potential duration of the thermal stress response in juvenile steelhead, we must assume that organisms exposed to prolonged temperature stress experience metabolic energy deficits. Protein synthesis and repair are energy intensive processes. Roberts *et al.* (1997) and Hofmann and Somero (1995) estimate the total cost of protein synthesis under non-stressful conditions constitutes 20-25% of the energy budget of the bay mussel, *Mytilus edulis*, and re-folding of one protein molecule requires as much as 100 ATP molecules (Roberts *et al.* 1997). A reduction in thermotolerance and the capacity to produce hsp70 observed in species adapted to cold or stenothermal conditions may be an indication, that this ability comes at some kind of cost to other organism functions (Sanders et al. 1991a, Coleman et al. 1995, Goto and Kimura, 1998).

Further evidence is presented by Krebs and Loeschcke (1994) who showed that fruit flies (*Drosophila melanogaster*) produced fewer offspring when exposed repeatedly to non-lethal temperature stress, although this treatment enabled the flies to survive severe temperature stress better than previously unexposed flies. Similarly, *Drosophila* cells
that over-expressed hsp70 at normal temperatures grew slower than normal cells (Feder et al. 1992). Hoffmann and Rinas (2001) determined that the increased energy demand resulting from the synthesis of plasmid-encoded and heat-shock proteins led to a reduction of growth rate in E. coli. In a study on the effects of β-naphthoflavone in rainbow trout Vijayan et al. (1997) found increased hsp70 expression and decreased metabolic capacity in the liver and suggested that hsp70 expression may be at the expense of other biochemical pathways. Sanders et al. (1991b) measured a reduction in the scope-for-growth of mussels (Mytilus edulis) along with elevated hsp60 in response to copper exposure. More recent findings show that several hsp proteins are associated with cellular signaling molecules and receptors, which regulate growth and development. Thus, an imbalance in cellular homeostasis caused by a temperature triggered stress response could lead to disruption of normal development and growth (Nollen and Morimoto 2002, Queitsch et al. 2002).

Based on the existing information on thermal tolerance of steelhead trout, the relative lack of toxicological stressors in the Navarro watershed and the pattern of increased hsp72 levels in fish at warmer sites, we conclude that the juvenile fish caught at Lower, Middle and Upper Anderson Creek (LAC, MAC, UAC), Lower and Upper Indian Creek (LIC, UIC) and Middle and Upper Rancheria Creek (MRC, URC) were experiencing temperature stress. All of these sites are located in the eastern part of the watershed and characterized by a lack of riparian vegetation, high water temperatures and large daily temperature fluctuations. Although we know that an induction of hsp70 proteins signals a disruption of cellular homeostasis, a full synthesis of the ecological and evolutionary
understanding of the implications of this stress has not yet emerged. Juvenile steelhead expressing high concentrations of hsp72, were consistently smaller than fish from cool water locations (unpublished data), but this potentially significant correlation is confounded by the lack of information on food availability and other factors at our field sites. All fish were alive when collected indicating that their protective responses were at least temporarily able to cope with exposure to the respective water temperatures. We know that exposure to mild heat-shock can enable the organism to survive previously lethal doses or temperatures, and that hsps play an important physiological role in this so-called “acquired tolerance” (Kapron-Bras and Hales 1991, Sanders 1993, Parsell and Lindquist 1994, Iwama et al. 1998). However, the energetic expense of continuous or repeated hsp production and cellular repair processes – especially over prolonged periods - may ultimately compromise the organism’s fitness and survival.
Table 3-1 Steelhead trout sampling sites in the Navarro River watershed: Geographic location, temperature data and heat-shock protein 72/76 levels in muscle tissue of fish. All monthly temperature averages are for the period of July 1, 2000 until the day of fish collection. All weekly averages are for the period preceding and including the sampling date. MMAT=mean monthly average temperature; \( MMT_{max} \)=mean monthly maximum temperature, MWAT=mean weekly average temperature; \( MWMT \)=mean weekly maximum temperature; \( MT_{min} \)=mean monthly minimum temperature; MDTR=mean daily temperature range for July 1 to fish collection; SE=standard error of the mean; n=10, except for MFC (n=5) and MAC (n=1).

<table>
<thead>
<tr>
<th>Sampling Site</th>
<th>Sampling Date</th>
<th>GPS Coordinates (<em>Lat/Long)</em></th>
<th>MMAT (°C)</th>
<th>( MMT_{max} ) (°C)</th>
<th>MWAT (°C)</th>
<th>MWMT (°C)</th>
<th>( MT_{min} ) (°C)</th>
<th>MDTR (°C)</th>
<th>Shade %</th>
<th>HSP72 ± SE (relative density)</th>
<th>HSP76 ± SE (relative density)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle Flynn Creek (MFC)</td>
<td>8/3/00</td>
<td>39.1847/123.5981</td>
<td>14.55</td>
<td>15.37</td>
<td>15.63</td>
<td>16.57</td>
<td>13.65</td>
<td>1.73</td>
<td>-</td>
<td>-</td>
<td>5.97±0.97</td>
</tr>
<tr>
<td>Lower Flynn Creek (LFC)</td>
<td>8/1/00</td>
<td>39.1615/123.5823</td>
<td>15.19</td>
<td>16.69</td>
<td>15.64</td>
<td>16.99</td>
<td>14.30</td>
<td>2.39</td>
<td>-</td>
<td>0.17±0.07</td>
<td>9.15±0.9</td>
</tr>
<tr>
<td>Lower North Fork (LNF)</td>
<td>8/1/00</td>
<td>39.1545/123.6197</td>
<td>17.59</td>
<td>18.99</td>
<td>18.22</td>
<td>19.71</td>
<td>16.39</td>
<td>2.59</td>
<td>81.73</td>
<td>0.14±0.07</td>
<td>10.58±1.42</td>
</tr>
<tr>
<td>Middle North Fork (MNF)</td>
<td>8/4/00</td>
<td>39.1735/123.5602</td>
<td>17.99</td>
<td>19.59</td>
<td>18.3</td>
<td>19.86</td>
<td>16.77</td>
<td>2.81</td>
<td>65.00</td>
<td>0.06±0.04</td>
<td>7.16±0.56</td>
</tr>
<tr>
<td>Lower Indian Creek (LIC)</td>
<td>7/25/00</td>
<td>39.0590/123.4397</td>
<td>18.72</td>
<td>22.83</td>
<td>18.19</td>
<td>22.27</td>
<td>16.29</td>
<td>6.53</td>
<td>49.40</td>
<td>10.47±0.82</td>
<td>11.36±1.35</td>
</tr>
<tr>
<td>Upper Indian Creek (UIC)</td>
<td>7/26/00</td>
<td>39.0776/123.3748</td>
<td>19.05</td>
<td>23.82</td>
<td>18.37</td>
<td>23.32</td>
<td>16.39</td>
<td>7.43</td>
<td>35.19</td>
<td>9.81±0.67</td>
<td>10.65±0.72</td>
</tr>
<tr>
<td>Lower Anderson Creek (LAC)</td>
<td>8/2/00</td>
<td>39.0538/123.4330</td>
<td>19.43</td>
<td>22.86</td>
<td>20.29</td>
<td>24.38</td>
<td>17.27</td>
<td>5.59</td>
<td>63.44</td>
<td>8.35±1.14</td>
<td>8.42±0.87</td>
</tr>
<tr>
<td>Middle Anderson Creek (MAC)</td>
<td>8/2/00</td>
<td>39.0140/123.3724</td>
<td>21.39</td>
<td>25.52</td>
<td>22.49</td>
<td>26.53</td>
<td>18.13</td>
<td>7.40</td>
<td>39.35</td>
<td>16.0</td>
<td>16.4</td>
</tr>
<tr>
<td>Upper Anderson Creek (UAC)</td>
<td>8/1/00</td>
<td>39.9904/123.3146</td>
<td>19.69</td>
<td>26.57</td>
<td>20.41</td>
<td>27.37</td>
<td>15.95</td>
<td>10.61</td>
<td>26.17</td>
<td>14.24±0.89</td>
<td>12.13±0.75</td>
</tr>
<tr>
<td>Middle Rancheria Creek (MRC)</td>
<td>7/28/00</td>
<td>39.9483/123.3242</td>
<td>20.46</td>
<td>24.43</td>
<td>21.22</td>
<td>25.38</td>
<td>17.62</td>
<td>6.80</td>
<td>10.75</td>
<td>13.04±0.8</td>
<td>11.24±0.92</td>
</tr>
<tr>
<td>Upper Rancheria Creek (URC)</td>
<td>7/31/00</td>
<td>39.8503/123.2392</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.59±0.92</td>
<td>13.54±1.56</td>
</tr>
</tbody>
</table>
Figure 3-1 a

![Graph showing the relationship between Degree Longitude and MMTmax (°C). The correlation coefficient (r) is 0.95, and the p-value is less than 0.001.](image-url)
Figure 3-1. Relationship of stream temperature (average monthly maximum temperature) with a) longitude reflecting high temperatures at easterly, inland sites and low temperatures at sites closer to the Pacific Ocean, and b) with percent shade. Error bands represent 95% confidence intervals.
Figure 3-2. Relationship of hsp72 levels measured in steelhead trout parr with a) average monthly maximum temperature (MMT\textsubscript{max}) and b) mean monthly average temperatures (MMAT) for the month of July 2000 at our sampling sites. Hsp72 values represent densitometer measurements of protein bands detected by western blotting.
Figure 3-3. Relationship of hsp72 levels measured in steelhead trout parr with a) mean weekly maximum temperature (MWMT) and b) mean weekly average temperatures (MMAT) for the 7 days preceding and including the sampling dates. Hsp72 values represent densitometer measurements of protein bands detected by western blotting.
Figure 3-4. Relationship of hsp72 levels measured in steelhead trout parr with mean daily temperature ranges (MDTR) during the month of July 2000. Hsp72 values represent densitometer measurements of protein bands detected by western blotting.
Fluctuating Asymmetry
Asymmetry is used as an indicator of developmental stability in a broad range of animals from humans to freshwater mussels (see review by Palmer and Strobeck 1986). Measures of asymmetry are typically expressed as variation between right and left (R-L) metric and meristic bilateral traits. Asymmetry is known to be a robust predictor of growth, survival ability, and fecundity (see review in Moller and Shykoff 1999) and has been negatively correlated with fitness in rainbow trout (O. mykiss) (Leary 1984).
Aquatic ecologists have since used asymmetry to examine the health and stability of fish populations including studies on the potential effects of inbreeding in salmonid broodstocks (Wagner 1996), recruitment in anchovies (Engraulis encrasicolus) (Somarakis et al 1997) and dietary differences in stickleback (Gasterosteus aculeatus) (Reimchen and Nosil 2001).

Palmer and Strobeck (1986) describe three types of asymmetry observable in nature: directional asymmetry (DA), antisymmetry (AA), and fluctuating asymmetry (FA). Traits exhibiting fluctuating asymmetry are bimodally distributed with a mean trait difference of zero, and occur when an organism is unable to develop in a predetermined path (VanValen 1962, Palmer and Strobeck 1986). Directional asymmetry occurs when a trait on one side of a bilateral plane is consistently larger across individuals in the population than the same trait on the other side, resulting in a distribution of right and left values whose mean is not centered on zero. Antisymmetric traits generally have a platykurtic distribution with a mean centered on zero and occurs when symmetry is not expected under optimal conditions but neither the right or left side is favored. Palmer and Strobeck (1986, 1992) argue that FA is the only form of asymmetry that shows no genetic
basis and reflects decreased developmental stability. FA is the only form of asymmetry that is commonly reported as an indicator of environmental stress (see review in Zakharov and Graham 1992). However, mathematical simulations performed by Graham et al. (1993) highlighted the possibility of phase-lagged periodicity due to morphogen concentrations fluctuating from left to right over time. From these simulations, it is concluded that both DA and AA may occur due to alterations in feedback or inhibition provoked by a disturbance in the environment (Graham et al. 1993). Therefore, we have chosen to include all asymmetry data present in the Navarro watershed in our analysis.

Fish are most vulnerable to both natural and anthropogenic environmental stressors in early life history stages. Even the egg stage is susceptible to stressors including temperature (Ali and Lindsey 1974, Campbell et al. 1998), light (MacCrimmon 1968), low dissolved oxygen (Alekseeva et al. 1992), and contaminants (Zakharov and Ruban 1985, Jagoe and Haines 1984). Any environmental stress experienced during ontogeny may reduce the efficiency of the normal developmental process (Clarke 1992). Measurements of asymmetry offer a tool by which we can assess chronic stress during early development that may impact the long-term success of that year class (Pottinger and Mosuwe 1994). This study was designed to examine relationships between asymmetry and selected individual fitness measures in *O. mykiss* as indicators of environmental stressors.

**Study Areas And Methods**
Fish from each of the sampled sites were collected during both a spring sampling period (May 24-26, June 6-9) and summer sampling period (July 25-31, August 1-16) in 2000. A Smith-Root Model 12-B electrofisher was used to collect fish from all sites except
within the North Fork and Flynn Creek drainages where permit restrictions necessitated the use of dip nets and seines the fish. Where possible, 20 fish were collected except when population sizes were low. Fish were placed on ice or dry ice immediately upon capture and then transferred to a -30°C freezer within 6-10 hours. Specimens were later thawed, cleared and stained following the protocols outlined by Snyder (1990). Meristic traits (pelvic fin rays, pectoral fin rays, and brachiostegal rays) were determined by direct counts before mounting. Pelvic fins, pectoral fins and dentary bones were mounted onto microscope slides with Cytoseal and digitally photographed a microscope-mounted Spot® camera at 1-4x magnification. The images were created, manipulated and measurements taken using Spot® RT Software (Diagnostic Instruments, version 3.0, 1999).

_Meristic Traits_
The number of pelvic rays (Pv), number of pectoral rays (Pt), and number of brachiostegal rays (Br) were counted by two individuals and recounted until there was mutual agreement on the number of rays present. This procedure ensured that small or lightly dyed rays were not overlooked and that there were no double counts of rays. Frequency plots were conducted to visually determine the difference, if any, between right and left sides exhibited FA, DA, or AA for meristic traits (Palmer and Strobeck 1992).

_Metric Traits_
The length of the fourth pelvic ray (PvL), the fourth pectoral ray (PtL), and the dentary bones (Dent) were obtained for each fish using the corresponding digital image and the
Spot® imaging software. The fourth ray of the pelvic and pectoral fins was identified by counting from the ray farthest from the auxiliary process. To decrease subjectivity, the base of the fin ray was determined to be the point at which the ray curved out from the body of the fish. The length of each dentary bone was measured as the straight-line distance between the farthest posterior point of one side of the dentary bone and the point where the right and left dentary bones join. All measurements were performed twice to reduce measurement error.

A series of two-way GLM ANOVA tests using NCSS (2000/2001) statistical software were conducted on metric traits using fish and sides as factors to detect the presence of asymmetry. If the interaction term (fish x sides) of the ANOVA test was not significant then tests for asymmetry were not justified (Palmer and Strobeck 1986). If both the interaction term and the side factor were significant then the variance between sides is attributed to directional asymmetry (DA). In the case of fluctuating asymmetry (FA), the interaction term must be significant and the sides factor not significant. Additionally, D’Agostino skewness and kurtosis tests were performed on these fish from populations (i.e. sites) exhibiting FA and DA to eliminate cases of antisymmetry (Palmer and Strobeck 1992). No size scaling was needed in any of the traits examined since there was no correlation between sides (R-L) and fish size as determined from scatter plots developed for each trait and site (Palmer and Strobeck 1986).

Measurement Error
In order to identify FA and DA, the observed variability in a trait must be discernable from the variability due to sampling error (Palmer and Strobeck 1986). Therefore, the
presence and extent of measurement error was examined using a large series of repeated measures for metric traits from 22 fish. Six measurements of each trait were determined on different dates by each of two researchers so that measurement error both within and among researchers could be identified. Two one-way ANOVA tests were performed to determine the presence of significant differences in measurements associated with fish (all repeated measures for each trait) and investigators (six measurements by each of two researchers). The one-way ANOVA test results revealed no significant difference ($\alpha \geq 0.05$) between researchers and no significant difference between measurements for an individual trait with the fish sample (22 individuals). Power analyses (NCSS 2000/2001) was also performed on these data to determine the number of measurements needed to achieve a power of at least 0.80 with which to detect a 1% difference in trait means at $p < 0.05$. The power analyses indicated that two measurements of each metric trait were sufficient to reduce measurement error and allow for the detection of differences between the right and left sides of individuals.

**Results**

*Quantitative Estimates Of Asymmetry*

The initial examination for the presence/absence of asymmetry indicated that some form of asymmetry occurred in some traits at all sites (Figure1-1). Traits exhibiting asymmetry were not consistent across sites or sample periods. We decided to combine the asymmetry found in the various traits into a single index of asymmetry for an individual fish. Numerous indices have been proposed as overall measures of asymmetry in a population (Palmer and Strobeck 1986). Each index is based on some measure of variance between the right and left sides for a particular trait. Palmer and Strobeck
(1994) discuss the advantages and disadvantages of using each index. Of the thirteen indices discussed, we used six (Table 3-2) to examine the relationship among asymmetry, measures of individual fish fitness and potential environmental stressors. These indices are based on either absolute $|R-L|$ values or $(R-L)^2$ and include the standard deviation of the difference between sides, coefficient of variation (CV), $1-r$ (1- Pearson’s correlation coefficient), and the F value from ANOVA analysis for metric traits.

We calculated the six indices for each fish and then examined the correlation among indices for each trait across fish. Index 5 was the most highly correlated with the other indices, and this index was selected to quantify asymmetry across all traits. We created composite scores for several traits by combining trait index values within each fish. The general form for this composite index is:

$$CSI_n = \sum T_n$$

where $CSI_n$ = composite score index

$T_n$ = FA or DA score for trait n

The value of each individual index ($T_n$) increases as the prevalence and/or severity of the FA and DA increases for that trait. Three composite indices were used to examine potential relationships with fish fitness variables, stream conditions, and landscape attributes associated with the watershed drainage. The three composite indices are: 1) all-trait index ($CSI_{all}$), 2) metric trait index ($CSI_{metric}$), and 3) meristic trait index ($CSI_{meristic}$). The proposed all-trait index is composed of calculated index values for FA and DA for
all six traits using each of the six different indices (i.e. statistical measure). Similarly, a metric trait index consisting of the three metric traits measured (i.e. \(P_v, P_t, B_r\)) and a meristic trait index of the composite scores for \(P_vL, P_tL, \) and \(D_{ent} \) were calculated using the six different index statistics.

"Spatial And Temporal Extent Of Asymmetry"
Fluctuating and directional asymmetry were observed in fish populations throughout the watershed in both the headwater and larger streams (Table 3-2, Appendix 3-1) during summer and spring sample periods (Figure 1-1). \(P_vL\) and \(P_tL\) demonstrated only FA for spring and summer. \(D_{ent}\) showed FA and DA present in spring but only FA in the summer. Both FA and DA were present in \(P_v\) in the spring and only DA in the summer. FA and DA were observed in \(P_t\) in both sampling events. \(B_r\) showed directional asymmetry at all but one site in spring and at all sites in summer. \(B_r\) was the only trait exhibiting asymmetry in all steelhead populations during both spring and summer. It appears that FA and/or DA manifests itself in one or more traits during either the spring or summer periods (or both).

To help characterize the relationship between time and asymmetry, scatter plots were created with \((R-L)/(R+L)\) values for individual traits versus length for all fish collected within the Navarro watershed during both sampling periods. There were no visual relationships and no significant correlations using a Pearson’s correlation matrix between length and the difference in right and left traits standardized by the length of those traits. When examining the 5 drainages within the Navarro river watershed, there were three significant correlations between standardized right and left difference and the length of
fish. However, when the scatter plots were examined no relationship was evident and two of the correlations were from the same drainage with different signs indicating that due to small sample sizes these correlations are biologically insignificant.

**Discussion**
Eight out of thirteen sites exhibited FA in meristic traits, 10 out of 13 sites exhibited FA in metric traits, and 12 out of 13 sites exhibited FA over all traits. Examining both meristic and metric traits allows us to look at different physiological responses to stress. Asymmetry in metric traits has the potential to increase from the first stages of development all the way through adulthood. On the other hand, fish would have to encounter environmental stress during the first stages of development to express asymmetry in meristic traits. Environmental factors would not affect meristic traits if a stress occurred after the number of rays had been developed. Metric traits, however, may prove different. Since fish continue to grow at a rapid rate beyond the early development stages, metric traits may show the affect of stress during embryogenesis, hatching, emergence, and early growth. Metric traits continue to grow with the fish, emphasizing previous differences in right and left traits and possibly increasing that difference if a new stress is added.

We were unable to obtain temperatures during times of spawning but we assume that the variation between sub-watershed maximum temperatures was consistent year round. There is a relationship between meristic traits (excluding Br) exhibiting either fluctuating or directional asymmetry and maximum temperature. This relationship was consistent.
when we looked at the individual meristic traits, though not as strong. Sites having consistently higher temperatures may cause alevins to emerge earlier. Changes in light intensity during various stages of egg development of rainbow trout (*Salmo gairdneri*) affect metabolic rates, vertebrae number, anal and dorsal fin ray numbers, mortality, and time to hatch (MacCrimmon and Kwain 1968). Variance in canopy cover, maximum temperature and width to depth ratio all affect light intensity and all positively correlate with meristic asymmetry. Average depth negatively correlated with total asymmetry and not with meristic or metric asymmetry. Average depth may affect the survivability of fish after emerging from the gravel rather than affect development prior to emergence. An increase in average depth is not correlated with dissolved oxygen or flow at that site. It may be that average depth is a physical parameter affecting the choice of spawning location by the hen to ensure adequate habitat for emerging fish. This environmental factor is less specific but more consistent temporally than other parameters such as dissolved oxygen, which varies with temperature and flow.

By examining individual traits, we found pelvic fin ray counts and lengths to be the most sensitive to environmental factors. Temperature range correlated with Pv counts and Pt counts. Campbell (1998) found that thermal treatment on coho salmon crosses during embryogenesis had no affect on pectoral fin ray counts and different affects on pelvic fin rays and gillraker counts of the lower brachiostegal rays. He reported that pelvic fin rays had higher levels of FA at fluctuating temperature whereas gillrakers had higher levels of FA at ambient temperatures (Campbell et al. 1998). Individual characters may differ in magnitudes of asymmetry due to various stress sensitivities resulting from alternate
timing in character development (Campbell et al. 1998). In lab experiments with rainbow trout, incubation temperature did not affect all characters equally (Leary et al. 1992). Leary et al. (1992) reported FA in pelvic, pectoral, and mandibular pore counts. Our findings that meristic traits, not including brachioseptal counts, correlated with temperature variation are consistent with controlled lab experiments on closely related fish species.

Fish development has been researched mostly using zebra fish due to the transparency of the embryo and the ease of genetic manipulation (Kimmel et al. 1995). From these studies it is found that the pectoral fins rays are the first rays to develop, followed by the brachioseptal rays. The brachioseptal rays differentiate from the primordial that also forms the jaws, operculum and the gills. By the end of 48 hours after the eggs are fertilized, collagenous fin rays are formed and the pectoral fin bud develops. Seventy-two hours after fertilization, rapid development in the pectoral fins, jaws, and gills occurs from the embryo rudiments. The cartilage development in the jaw is slower than in the pectoral fin but develops before the brachioseptal cartilages. During the first fry stage (post-emergence), fin rays appear in the caudal and pectoral fins. The pelvic fin rays form in the third fry stage (Kimmel et al. 1995).

The development of the pectoral fins first, before emergence from the gravel, emphasizes the importance of these fins for survival. Pelvic fins may be less canalized than pectoral fins since they are less important for movement involved in prey capture and predator avoidance than the pectoral fin rays. It would therefore seem likely that pelvic fins
would show the most sensitivity to stress if they are the least canalized. We found no
correlation with brachioseagal ray counts and temperature, which corresponds to
Campbell’s finding in coho salmon (1998). It may be that brachioseagal ray asymmetry
in steelhead is more sensitive to ambient temperatures than to fluctuating temperatures.
This may be true for the dentary bone, which develops from the same tissue as the
brachioseagal rays.

The variation in site-specific canopy cover had a higher correlation with asymmetry in
pelvic counts than temperature had with asymmetry in pelvic counts. A variation in
canopy cover may have caused greater variation in light intensity and increased
stratification within the water column. This variability may be a source of developmental
stress to young of year fish.

We found that using a composite index with Pv counts and Pt counts (including sites that
exhibited DA) produced the highest and most biologically significant relationships. All
traits, except PtL and PvL, showed DA at one or more sites. We have chosen to include
both DA and FA based on the following reasons: 1) small sample sizes may show DA
whereas if we had been able to have a large sample size we would have seen FA 2) there
may be visual bias for meristic traits since frequency plots are not a statistical way to test
for skewness (DA) and 3) DA may not always be genetically based (Graham et al. 1993).

Debate continues about the implications of using asymmetry other than FA as an
indicator of developmental instability. Presumably directional and antisymmetry are
genetically controlled since they do not show normal, bimodal distributions (Van Valen 1962). However, FA may switch to DA depending on the amount of stress inflicted (Palmer 1994). This was seen in the mandibles of mice, which were given doses of an insecticide derived from DDT. Characters of the mandible expressing FA changed to DA at a higher dosage (Leamy et al. 1999). Of these mandibular characters expressing DA, only one character (out of 10) proved to have significant heritability (Leamy 1999).

There is still evidence that some traits are controlled genetically to produce DA and therefore are not good indicators of developmental stability. Brachiostegals counts are a common trait that expresses DA in fish and not included in asymmetry results (Jagoe 1985, Campbell 1998, Bryden and Heath 2000). Based on the literature and individual trait correlation matrices, we found that brachiostegal rays were not correlated with other traits and resulted in conflicting correlations. The largest breeding program, as to date, to assess the heritability of FA in wild and hatchery chinook salmon found that FA was not heritable, however, excluded traits exhibiting DA, including brachiostegal rays, maxillary length, and head length (Bryden and Heath 2000). Although not discussed in detail, it was not shown in this study that either brachiostegal ray or maxillary length DA was significantly heritable. Future lab experiments would prove useful in determining if brachiostegal rays normally show DA and if this DA changes with stress (i.e. fluctuating temperatures) to FA or antisymmetry. We have shown here that excluding groups or traits exhibiting DA does not portray an accurate picture of the health of the population. More research needs to be conducted to understand individual trait sensitivity, heritability of asymmetry, and the biological implications for all types of asymmetry.
Small sample size is an issue for research conducted on populations with low numbers. It is important to understand the limits as well as the implications of conducting studies on small populations, especially on a species of concern. Further research needs to be conducted to further understand the importance of temperature on individual traits and types of asymmetry for steelhead trout. All three types of asymmetry may be interactive, change over time, and be present in the same populations. Therefore, more research needs to be conducted and reported for all three types of asymmetry to further understand their relations to each other and developmental stability.
Table 3-2: Presence of FA and DA in the two sample periods in at least one site within the Navarro watershed.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Spring</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br</td>
<td>DA</td>
<td>DA</td>
</tr>
<tr>
<td>PvL</td>
<td>FA</td>
<td>FA</td>
</tr>
<tr>
<td>PtL</td>
<td>FA</td>
<td>FA</td>
</tr>
<tr>
<td>Pv</td>
<td>FA, DA</td>
<td>DA</td>
</tr>
<tr>
<td>Pt</td>
<td>FA, DA</td>
<td>FA, DA</td>
</tr>
<tr>
<td>Dent</td>
<td>FA, DA</td>
<td>FA</td>
</tr>
</tbody>
</table>

Table 3-3. Indices proposed for developing a composite measure of fluctuating asymmetry.

<table>
<thead>
<tr>
<th>Index</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$(</td>
</tr>
<tr>
<td>2</td>
<td>CV of $(</td>
</tr>
<tr>
<td>3</td>
<td>$(1-r)$ from correlation matrix of right and left values</td>
</tr>
<tr>
<td>4</td>
<td>$(R-L)^2/SD$</td>
</tr>
<tr>
<td>5</td>
<td>CV of $(R-L)^2$</td>
</tr>
<tr>
<td>6</td>
<td>F ratio from ANOVA table for metric traits</td>
</tr>
</tbody>
</table>

SD=standard deviation  
CV=coefficient of variation  
r=Pearson’s correlation coefficient
Appendix 1. ANOVA results for FA and DA for metric traits in the two sample periods.

<table>
<thead>
<tr>
<th>Sub-watershed</th>
<th>Site</th>
<th>Sample Size</th>
<th>Side</th>
<th>Interaction</th>
<th>Asymmetry</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flynn Creek</td>
<td>LFC- Summer</td>
<td>8</td>
<td>NS</td>
<td>0.0049 FA</td>
<td>Accept</td>
<td>Accept</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MFC- Spring</td>
<td>11</td>
<td>NS</td>
<td>0.013445 FA</td>
<td>Accept</td>
<td>Accept</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MFC- Summer</td>
<td>3</td>
<td>NS</td>
<td>0.047662 FA</td>
<td>NA</td>
<td>Accept</td>
<td></td>
</tr>
<tr>
<td>North Fork</td>
<td>LNF- Spring</td>
<td>6</td>
<td>NS</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>LNF- Summer</td>
<td>9</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MNF- Spring</td>
<td>15</td>
<td>NS</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>JSC- Spring</td>
<td>4</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Indian Creek</td>
<td>LIC- Spring</td>
<td>7</td>
<td>NS</td>
<td>0.00519 FA</td>
<td>Accept</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>MIC- Spring</td>
<td>11</td>
<td>NS</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>UIC- Spring</td>
<td>9</td>
<td>NS</td>
<td>0.017942 FA</td>
<td>Accept</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>UIC- Summer</td>
<td>8</td>
<td>NS</td>
<td>0.006884 FA</td>
<td>FA</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td>Anderson Creek</td>
<td>LAC- Spring</td>
<td>13</td>
<td>NS</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>MAC- Spring</td>
<td>22</td>
<td>NS</td>
<td>0</td>
<td>FA</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>UAC- Spring</td>
<td>7</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Rancheria Creek</td>
<td>LRC- Spring</td>
<td>10</td>
<td>NS</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>LRC- Summer</td>
<td>4</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRC- Spring</td>
<td>9</td>
<td>NS</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>MRC- Summer</td>
<td>4</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>URC- Spring</td>
<td>14</td>
<td>NS</td>
<td>0.014395 FA</td>
<td>Accept</td>
<td>Accept</td>
<td></td>
</tr>
<tr>
<td></td>
<td>URC- Summer</td>
<td>8</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>
2-way ANOVA test for PtLength. Significance is alpha p <.05

<table>
<thead>
<tr>
<th>Sub-watershed</th>
<th>Site</th>
<th>Sample Size</th>
<th>Side</th>
<th>Interaction Asymmetry</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flynn Creek</td>
<td>LFC-Summer</td>
<td>7</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MFC-Spring</td>
<td>11</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MFC-Summer</td>
<td>3</td>
<td>NS</td>
<td>0.031666</td>
<td>FA</td>
<td>NA</td>
</tr>
<tr>
<td>North Fork</td>
<td>LNF-Spring</td>
<td>3</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LNF-Summer</td>
<td>6</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MNF-Spring</td>
<td>20</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>JSC-Spring</td>
<td>5</td>
<td>NS</td>
<td>0.00015</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td>Indian Creek</td>
<td>LIC-Spring</td>
<td>13</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIC-Spring</td>
<td>12</td>
<td>NS</td>
<td>0.004496</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>UIC-Spring</td>
<td>8</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UIC-Summer</td>
<td>8</td>
<td>NS</td>
<td>0.02675</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td>Anderson Creek</td>
<td>LAC-Spring</td>
<td>10</td>
<td>NS</td>
<td>0.004306</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>MAC-Spring</td>
<td>18</td>
<td>NS</td>
<td>0.005962</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>UAC-Spring</td>
<td>11</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rancheria Creek</td>
<td>LRC-Spring</td>
<td>14</td>
<td>NS</td>
<td>0.032852</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>LRC-Summer</td>
<td>5</td>
<td>NS</td>
<td>0.000283</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>MRC-Spring</td>
<td>7</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRC-Summer</td>
<td>3</td>
<td>NS</td>
<td>0.000229</td>
<td>FA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>URC-Spring</td>
<td>18</td>
<td>NS</td>
<td>0.003038</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>URC-Summer</td>
<td>8</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dent

<table>
<thead>
<tr>
<th>Sub-watershed</th>
<th>Site</th>
<th>Sample Size</th>
<th>Side</th>
<th>Interaction Asymmetry</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flynn Creek</td>
<td>LFC-Summer</td>
<td>8</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MFC-Spring</td>
<td>16</td>
<td>0.014975</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MFC-Summer</td>
<td>5</td>
<td>NS</td>
<td>0.003349</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td>North Fork</td>
<td>LNF-Spring</td>
<td>17</td>
<td>0.001339</td>
<td>0.001811</td>
<td>DA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>LNF-Summer</td>
<td>10</td>
<td>NS</td>
<td>0.000032</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td>Location</td>
<td>Season</td>
<td>Sample Size</td>
<td>p-value</td>
<td>Statistic</td>
<td>Decision</td>
<td>Action</td>
</tr>
<tr>
<td>---------------</td>
<td>--------</td>
<td>-------------</td>
<td>---------</td>
<td>-----------</td>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>MNF- Spring</td>
<td></td>
<td>24</td>
<td>0.00349</td>
<td>NS</td>
<td>FA</td>
<td>Reject</td>
</tr>
<tr>
<td>JSC- Spring</td>
<td></td>
<td>12</td>
<td>NS</td>
<td>0.000123</td>
<td>FA</td>
<td>Reject</td>
</tr>
<tr>
<td>Indian Creek</td>
<td>LIC- Spring</td>
<td>19</td>
<td>NS</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>MIC- Spring</td>
<td>19</td>
<td>0.006039</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>UIC- Spring</td>
<td>18</td>
<td>NS</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>UIC- Summer</td>
<td>9</td>
<td>0.000165</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td>Anderson Creek</td>
<td>LAC- Spring</td>
<td>15</td>
<td>0.006605</td>
<td>0.029759</td>
<td>DA</td>
<td>Accept</td>
</tr>
<tr>
<td>Rancheria Creek</td>
<td>MAC- Spring</td>
<td>22</td>
<td>NS</td>
<td>0.000673</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>UAC- Spring</td>
<td>20</td>
<td>0.024821</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>LRC- Spring</td>
<td>17</td>
<td>NS</td>
<td>NS</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>LRC- Summer</td>
<td>6</td>
<td>NS</td>
<td>0.007766</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>MRC- Spring</td>
<td>18</td>
<td>NS</td>
<td>0.000016</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>MRC- Summer</td>
<td>6</td>
<td>NS</td>
<td>0.000008</td>
<td>FA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>URC- Spring</td>
<td>24</td>
<td>0.005265</td>
<td>0.000001</td>
<td>DA</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>URC- Summer</td>
<td>8</td>
<td>NS</td>
<td>0.001931</td>
<td>FA</td>
<td>Accept</td>
</tr>
</tbody>
</table>
Physiological and Behavioral Effects of Zinc and Temperature on Coho Salmon 
(*Oncorhynchus kisutch*)

Incidences of increased contaminant loads, including concentrations of heavy metals, are found in many coastal Northern California watersheds. Zinc is one of the most common contaminants and is associated with urban runoff, soil erosion, industrial discharges, pharmaceuticals, and pesticides (Krenkel 1975, Irwin 1997). In some areas up to 50% of the zinc comes from highway runoff (Krenkel 1975). Recent studies have demonstrated that fish fed diets contaminated with metals exhibit reduced survival, growth, and health (Farag et al. 1994, Balasubramanian et al. 1995).

Increased temperature regimes are common in most Northern California coastal watersheds due to riparian degradation and sediment deposition (Brown and Moyle 1991). Anadromous fishes in particular, may be subjected to sublethal heat stress due to temperature fluctuations (Wedemeyer 1973). Studies of the lethality of the more extreme temperature changes have been numerous but the metabolic consequences of sublethal heat stress (thermal additions) have received less attention (Wedemeyer 1973).

One of the most common features of the cellular stress response is the production of heat shock proteins (hsp) in response to stressors that threaten the life of the cell (Iwama et al 1998, Iwama et al. 1999). Hsps play vital roles in maintenance of protein integrity, preventing premature folding and aggregation of proteins, protein translocation, and mediating steroid and receptor binding (Iwama et al 1998, Iwama et al. 1999). Under normal conditions, healthy cells produce only small amount of hsp. However, the level of hsp induction increases and regular protein synthesis is repressed during environmental conditions that result in stress to an organism (Fader et al. 1994).
Temperature may act as a stress factor in synergy with a toxicant. Zinc has been found to be more acutely toxic to fish at higher temperatures than at lower temperatures (Hodson and Sprague 1975). Water temperature can alter the toxicity of zinc in a variety of ways (Hodson and Sprague 1975, Donker et al. 1998). Increased water temperature may reduce locomotor and feeding activity and reduce nutrient uptake, increase metabolism of the animal and increase elimination or detoxification, or change the physiological state of the animal (e.g. by induction of heat shock proteins) which may increase susceptibility to toxicants (Donker et al. 1998).

Stressors such as temperature and toxicity may also affect overall survival and reproduction of an organism indirectly by modifying behavior (Shumway 1999). Metals have been shown to reduce aggression at sublethal concentrations (Henry and Atchison 1986, Atchison et al. 1987). Toxicants can also affect swimming performance and compromise the ability to escape predators, impair predator detection abilities, and increase conspicuousness due to erratic behavior or hyperactivity (Mesa 1994, Weis et al. 1999). The objective of this study was to examine the physiological, biochemical, and behavioral responses of coho salmon to excess dietary zinc and increased temperatures.

**Methods**
The experimental metal and temperature exposures were performed on juvenile coho salmon. Fish were obtained from the Cascade Fish Hatchery in Cascade Locks, Oregon, and transported in chilled water to the University of California, Davis. Fish were acclimated at 10°C for 7 days and then randomly separated into 16 tanks (40 liters), 6 fish
per tank at 10°C. Temperature in eight tanks was increased by 1°C per day to a final
temperature of 15°C. Fish were acclimated to final temperature regimes for 14 days.
During acclimatization, all fish were fed 2.5% of their body weight twice per day with
Biodiet Oregon 1 mm pellet size. Prior to introducing the experimental diet, all fish were
weighed to the nearest 0.5 g and measured to the nearest millimeter. No fish were
individually marked and all differences in measurements between pretreatment and post-
treatment fish were based on averages for each replicate tank.

On day 1 of the experiment, diet in eight tanks was changed to a zinc-enhanced diet. The
zinc-enhanced diet was produced by mixing distilled water and ZnCl$_2$ with 1mm pellet
size Biodiet Oregon. The mixture was then freeze-dried, ground, and passed through a 1
mm mesh sieve. The final zinc concentration was 1900 ppm as analyzed by CVDLS at
the University of California at Davis. During the experimental exposures fish were fed
2.5% of their body weight twice per day of either Biodiet Oregon or zinc-enhanced
Biodiet Oregon. Fish were exposed for 21 days to one of four combinations of diet and
temperature, high zinc/high temperature (15°C), low zinc/high temperature, high zinc/low
temperature (10°C), and low zinc/low temperature. End points included length, weight,
tissue metal accumulation, heat shock protein induction, aggression, and feeding
frequency.

Each fish was observed for a total of 5 hours over the course of the 21-day experiment.
Fish were monitored for aggression as strikes/minute against conspecifics and for feeding
as strikes/minutes at food pellets.
On day 21, fish were removed from the tanks, individually weighed, measured, and placed in liquid nitrogen for immediate freezing. Condition factor was determined by the equation: $C.F. = \frac{\text{body weight (g)}}{\text{length}^3 \text{(cm)}}$ according to NOAA Technical Memorandum NMFS-NWFSC-1. All fish were stored in a $-80^\circ\text{C}$ freezer until dissection for heavy metal and hsp analysis. Fish were dissected and gill, muscle, and $\frac{1}{2}$ liver were removed for analysis of hsp induction. The remaining portion of liver was sent to CVDLS for heavy metals analysis.

Fish livers were composited by tank and sent to the California Veterinary Diagnostic Laboratory (CVDLS) at the University of California at Davis for analysis. Livers were analyzed according to methods described by Martin et al. (1998). Briefly, livers were analyzed using an ICP analytical procedure for nine metals. Samples were prepared by nitric acid/hydrochloric acid digestion. Based on a one gram sample size, this screen quantitates for Fe$>0.2$ ppm, Mn$>0.04$ ppm, Cu$>0.1$ ppm, Zn$>0.1$ ppm, Cd$>0.3$ ppm, and Mo$>0.4$ ppm, while semi-quantitative results were obtained for Pb$>1$ ppm, and Hg$>1$ ppm. Hsp70 proteins were analyzed using Western blotting techniques as described above. To reduce handling stress, fish were not individually marked. Instead, all behavioral, growth and condition factor differences were based on averages of individuals in each tank.

Data were analyzed using a 2-way Analysis of Variance with temperature and zinc as the main treatment effects. A Bonferroni correction was made to account for repeated testing.
of multiple response variables; significance was established as $P \leq 0.0036$). In tests of the effects of diet and temperature on post-trial condition factor and aggression (strikes/min), pretrial condition factor and post-trial condition factor, respectively, were used as covariates. Removing the effect of the covariates eliminated any possibility that differences in post-trial condition factor could be due to pre-trial condition or that changes in aggression could result from differences in body size of the fish.

**Results**

**Growth and Condition Factor**

Fish in the four treatments did not differ from each other prior to the exposures in length, body mass, or condition factor (Tables 3-3 and 3-4). Growth in length of fish in the four treatments differed slightly. Fish not exposed to zinc grew approximately 15% during the course of the experiment (17% at 10°C, 14% at 15°C), while fish on zinc-supplemented diets experienced lower growth rate of approximately 6% (8% at 10°C, 5% at 15°C). The two-way ANOVA indicated that the diet difference was significant ($F = 23.8$, $df = 1,12$, $p = 0.000$), temperature was not significant ($F = 1.46$, $p = 0.250$) and there was no interaction ($F = 0.031$, $p = 0.863$). There were no significant differences in growth of body mass between the treatments, although the differences due to temperature were nearly significant ($F = 8.9$, $df = 1,12$, $p = 0.011$). Although there was no statistically significant effect of temperature on either growth in length or body mass, the trend was for higher growth at the lower temperature. The change in condition factor between pre-treatment and post-treatment was positive (increase in condition factor) in all treatments. The increase was significantly smaller in the high zinc treatments ($F = 14.33$, $df = 1,11$, $p = 0.003$, pretrial condition factor used as a covariate) and there was no effect of temperature on change in condition factor.
**HSP and Zinc**

No pretreatment zinc measurements were available due to the limited number of fish. However, those fish not exposed to zinc in the experiment can be considered as the control for measurements of zinc and iron in the liver. There were no significant differences in the non-zinc exposure fish between the temperature treatments. Zinc in liver increased slightly ($F = 5.00$, $df = 1,12$, $p = 0.045$) with exposure to a high zinc diet. Fe in the liver increased with exposure to high zinc and high temperature. The increase due diet was nearly significant ($F = 5.63$, $df = 1,12$, $p = 0.035$), and the increase due to temperature was significant ($F = 17.98$, $df = 1,12$, $p = 0.001$).

Interestingly, expression of hsp-70 in both muscle and liver supernatant extractions was higher in the 10°C treatments than in the 15°C treatments. Expression of hsp-70 (liver supernatant lower band) was lower in the high zinc exposure treatments. None of the differences was statistically significant.

**Behavior**

Exposure to zinc diet decreased aggression as measured by the number of strikes per minute at other fish in the tank, although the differences were not quite significant ($F = 11.65$, $df = 1,12$, $p = 0.005$). Condition factor used as a covariate was not significant indicating that larger fish were not more aggressive. Feeding rate increased with exposure to zinc ($F = 15.41$, $df = 1,12$, $p = 0.002$).
Discussion
Coho salmon used in this experiment were obtained from hatchery stock and were considered genetically distinct from wild coho salmon. Differences in tolerance to environmental factors appear to have a genetic basis; results therefore may not be directly applicable to field situations (Weis et al. 1999). Additionally, methods used to incorporate metals into fish diets can influence the degree of toxicity caused by metals (Farag et al. 1994). When metals were added surficially to commercial diets, the toxicity to trout was less than when zinc occurred naturally in the diet. Thus, although the concentrations of metals in diets may be similar, toxicological effects of those diets can differ (Farag et al. 1994).

One of the primary results of this experiment was the lack of any significant interaction between temperature and zinc. In fact, for no endpoint was the interaction even close to significant indicating that there are no synergistic effects between exposure to zinc and a moderate increase in temperature. Increased zinc in diets caused changes in many experimental endpoints, some of them apparently confounding. For example, feeding rate was higher for fish exposed to increased zinc while growth under zinc exposure was lower than growth in control fish. There is a large cost expenditure for production of detoxifying proteins (Barton and Schreck 1987). Changes may occur as a result of energy repartitioning by diverting energy substrates to cope with the enhanced energy demand and away from anabolic activity such as growth and reproduction. Therefore, long-term exposure to a stressor can lead to decreased growth, disease resistance, reproductive success, smolting, and swimming performance (Iwama et al. 1999). Although we analyzed for production of heat shock proteins, there are other detoxifying
systems such as metallothioneins that are likely to be induced in the presence of zinc. It is possible that increased amounts of zinc induced detoxifying systems, which, in turn, created an energy deficit and elicited increased feeding behavior, yet was not capable of supplying enough additional nutrients to maintain comparable growth. An alternate hypothesis reflects an indirect effect involving changes in aggressive behaviors. Increased zinc in diets reduced incidents of intraspecific aggressive behaviors (Table 3-3). Environmental stressors have been shown to impair predator avoidance (Weis et al. 1999) and would potentially affect competitive behaviors in general. Less time spent defending resources would allow for additional time spent feeding.

Temperature clearly reduced growth rate, a common result in studies of this type. Increasing temperature results generally in an increase in metabolic rate and consequently, energy demand. If food is supplied at a greater rate, growth rate can in fact increase. However, we kept the feeding rate constant at 2.5% body mass and therefore would have placed the fish in an energetic deficit resulting in a reduced rate of growth.

It is generally believed that juvenile salmonids cannot tolerate temperatures greater than 23-26°C, and the preferred temperature of juvenile coho salmon is about 10-12°C (Konecki et al. 1995). We chose treatment temperatures of 10°C (control) and 15°C (experimental thermal stress) in order to investigate continuous thermal stress. Increased treatment temperatures lead to less growth (g) than in control temperature fish. 15°C is at the high end of the range of thermal tolerance for most salmonid species (Konecki et al. 1995), and continued exposure may result in thermal stress and subsequent reduced growth. Expression of heat shock proteins was actually lower in the 10° treatments than
in the 15° treatments. During the course of the experiments, ambient air temperatures reached 110°F, contributing to a breakdown of the water-cooling machinery. As a result, temperatures in the 10° tanks averaged between 8.1° and 13.1° on a daily basis prior to the beginning of the experiment and between 8.6° and 11.5° during the experiment. Temperatures in the 15° tanks averaged between 13.8° and 16.2° prior to the experiment and between 14.6° and 15.9° during the experiment. Organisms can acclimate to temperature variations over even relatively short periods of time; any residual hsps produced would not be detectable after approximately 24 hours. Induction of hsps may be dependent more upon the relative increase in environmental temperature than upon the absolute temperature experienced by these fish (Fader et al. 1994). Therefore, the continually changing temperatures in the 10° treatments would not allow for acclimation and would be more likely to induce hsps.

Increasing concentrations of iron in the liver in response to the presence of zinc and high temperatures have been used as an indicator of liver damage (Skibba and Gwartney 1997, Mori and Hirayama 2000). Mammalian hepatotoxicity in response to hyperthermia may be the result of oxidative stress from superoxide generation. Ferritin released from the liver appeared to play a central role in hyperthermic toxicity (Martin et al. 1998). Whether this same phenomenon is occurring in fish and by what mechanism is unknown, but livers did accumulate iron in response to increasing temperature and increasing zinc indicating that hepatotoxicity was occurring.
Ultimately, population survival depends on behavior across the continuum of heritable and environmental influences (Shumway 1999). As populations of coho salmon in many Pacific North coast watersheds are declining rapidly, field experimentation and investigation of this species is not advisable. Alternately, species with similar life history strategies and habitat requirements such as steelhead may be studied in the field to gain insights into the complex interactions and subsequent population consequences of environmental stressors. In the Navarro River, for example, we have examined steelhead in an attempt to gain insight into the decline of the coho salmon populations in that watershed. Preliminary investigations have shown levels of zinc in steelhead livers averaging 32.8 ppm, approximately 1.5 times the levels found in livers of our experimental coho. Levels of iron in livers taken from Navarro steelhead averaged 114 ppm, while values from our experimental coho averaged 66 ppm (10° + Zn), 51 ppm (10°), 120 ppm (15° + Zn), and 86 ppm (15°). Hsp levels in a small sample of Navarro steelhead averaged 9.6, while levels in our experimental coho averaged approximately 8.05 (muscle supernatant) and 7.05 (muscle pellet). These values demonstrate strong responses to environmental stressors present in the Navarro River. Clearly, however, further research is necessary in order to begin to separate the multiple factors involved in declines of fish species and populations.
Table 3-3. Basic parameter values for the four treatments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>10°C – no zinc</th>
<th>10°C - zinc</th>
<th>15°C – no zinc</th>
<th>15°C - zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment length (mm)</td>
<td>60.1</td>
<td>60.9</td>
<td>64.5</td>
<td>65.0</td>
</tr>
<tr>
<td>Post-treatment length (mm)</td>
<td>70.3</td>
<td>65.7</td>
<td>73.5</td>
<td>68.1</td>
</tr>
<tr>
<td>Growth (mm)</td>
<td>10.2</td>
<td>4.7</td>
<td>9.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Pretreatment mass (g)</td>
<td>4.6</td>
<td>4.6</td>
<td>5.5</td>
<td>5.2</td>
</tr>
<tr>
<td>Post-treatment mass (g)</td>
<td>14.2</td>
<td>14.2</td>
<td>14.3</td>
<td>14.1</td>
</tr>
<tr>
<td>Growth (g)</td>
<td>9.6</td>
<td>9.5</td>
<td>8.8</td>
<td>9.0</td>
</tr>
<tr>
<td>Pretreatment condition factor</td>
<td>0.021</td>
<td>0.020</td>
<td>0.021</td>
<td>0.019</td>
</tr>
<tr>
<td>Post-treatment condition factor*</td>
<td>0.041</td>
<td>0.050</td>
<td>0.036</td>
<td>0.045</td>
</tr>
<tr>
<td>Zinc in liver (ppm)</td>
<td>20.0</td>
<td>21.5</td>
<td>19.5</td>
<td>23.0</td>
</tr>
<tr>
<td>Fe in liver (ppm)</td>
<td>51.0</td>
<td>66.3</td>
<td>85.8</td>
<td>120.0</td>
</tr>
<tr>
<td>Aggression (Strikes/minute)</td>
<td>3.6</td>
<td>1.3</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Feeding (Strikes/minute)**</td>
<td>9.5</td>
<td>15.2</td>
<td>11.2</td>
<td>18.0</td>
</tr>
<tr>
<td>Hsp-70 (muscle supernatant)</td>
<td>8.41 (1.26)</td>
<td>9.00 (1.76)</td>
<td>6.9 (2.02)</td>
<td>7.9 (1.12)</td>
</tr>
<tr>
<td>Hsp-70 (liver supernatant)</td>
<td>17.38 (2.76)</td>
<td>15.44 (3.21)</td>
<td>16.67 (3.93)</td>
<td>12.66 (3.55)</td>
</tr>
</tbody>
</table>

* Pretrial condition factor as a covariate
** Post-trial condition factor
Table 3-4. Between-subjects effects of diet and temperature on coho salmon.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model</th>
<th>Covariate</th>
<th>Diet</th>
<th>Temp</th>
<th>Diet x Temp (Interaction Term)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-trial condition factor</td>
<td>0.124</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Growth (length/mm)</td>
<td>0.003</td>
<td>N/A</td>
<td><strong>0.000</strong></td>
<td>0.250</td>
<td>0.863</td>
</tr>
<tr>
<td>Growth (weight/g)</td>
<td>0.066</td>
<td>N/A</td>
<td>0.782</td>
<td>0.011</td>
<td>0.532</td>
</tr>
<tr>
<td>Post-trial condition factor</td>
<td>0.007</td>
<td>0.446*</td>
<td>0.005</td>
<td><strong>0.033</strong></td>
<td>0.959</td>
</tr>
<tr>
<td>Zinc in liver</td>
<td>0.168</td>
<td>N/A</td>
<td>0.045</td>
<td>0.663</td>
<td>0.389</td>
</tr>
<tr>
<td>Iron in liver</td>
<td><strong>0.003</strong></td>
<td>N/A</td>
<td><strong>0.035</strong></td>
<td><strong>0.001</strong></td>
<td>0.381</td>
</tr>
<tr>
<td>Hsp-70 Muscle supernatant</td>
<td>0.036</td>
<td>N/A</td>
<td>0.102</td>
<td>0.008</td>
<td>0.680</td>
</tr>
<tr>
<td>Hsp-70 Muscle pellet</td>
<td><strong>0.000</strong></td>
<td>N/A</td>
<td>0.753</td>
<td>0.234</td>
<td>0.016</td>
</tr>
<tr>
<td>Hsp-70 Gill supernatant</td>
<td>0.182</td>
<td>N/A</td>
<td>0.947</td>
<td>0.110</td>
<td>0.227</td>
</tr>
<tr>
<td>Hsp-70 Gill pellet</td>
<td>0.772</td>
<td>N/A</td>
<td>0.351</td>
<td>0.729</td>
<td>0.493</td>
</tr>
<tr>
<td>Hsp-70 Liver supernatant upper band</td>
<td>0.580</td>
<td>N/A</td>
<td>0.425</td>
<td>0.453</td>
<td>0.327</td>
</tr>
<tr>
<td>Hsp-70 Liver supernatant lower band</td>
<td>0.009</td>
<td>N/A</td>
<td>0.004</td>
<td>0.072</td>
<td>0.259</td>
</tr>
<tr>
<td>Aggression (strikes/min)</td>
<td>0.052</td>
<td>0.590**</td>
<td>0.133</td>
<td>0.266</td>
<td>0.344</td>
</tr>
<tr>
<td>Aggression (strikes/min)</td>
<td>0.023</td>
<td>N/A</td>
<td>0.005</td>
<td>0.305</td>
<td>0.349</td>
</tr>
<tr>
<td>Feeding (strikes/min)</td>
<td>0.011</td>
<td>N/A</td>
<td><strong>0.002</strong></td>
<td>0.192</td>
<td>0.736</td>
</tr>
</tbody>
</table>

* Pre-trial condition factor

** Post-trial condition factor
LITERATURE CITED


Cousins, S. H.  1987.  The decline of the trophic level concept.  TREE 2:312-316


juvenile Chinook salmon and steelhead trout in two Idaho streams. J Fish Res Board Can 29:91-100


Moyle, P. B., R. M. Yoshiyama, J. E. Williams, and E. D. Wikramanayake. 1995. Fish species of special concern in California. 2nd ed. Final report prepared for the State of California, the Resource Agency, Department of Fish and Game, Inland Fisheries Division.


Nielsen, J. L. and M. C. Fountain. 1999. Microsatellite diversity in sympatric reproductive ecotypes of Pacific steelhead (Oncorhynchus mykiss) from the Middle Fork Eel River, California. Ecol. Fresh. Fishes. 8:159-168.


Palmer, L. 1967. History of Mendocino County, California, comprising its geography, geology, topography, climatology, springs and timber (Mendocino County Historical Society, Ukiah.


Pankhurst, N. W., G. J. Purser, G. Van Der Kraak, P. M. Thomas, and G. N. R. Forteath. 1996. Effect of holding temperature on ovulation, egg fertility, plasma levels of
reproductive hormones and in vitro ovarian steroidogenesis in the rainbow trout
*Oncorhynchus mykiss*. Aquaculture 146(3-4):277-290.


Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin ver. 2.000: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.


