

# FISHERY RESEARCH



## IMPROVED NUTRITION TO INCREASE THE VIABILITY OF HATCHERY SMOLTS

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By:

Jim Congleton and Tom Welker  
Idaho Cooperative Fishery Research Unit &  
Department of Fish and Wildlife  
University of Idaho

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# Improved Nutrition to Increase the Viability of Hatchery Smolts

## Annual Report to the Idaho Department of Fish and Game

Jim Congleton and Tom Welker  
Idaho Cooperative Fishery Research Unit & Dept. of Fish and Wildlife  
University of Idaho  
Moscow, ID 83844-1141

### Year 1 (1996)

#### Introduction

Highly reactive oxygen radicals, produced as a consequence of normal cellular metabolism, have been implicated as causative and contributory agents in many diseases (Halliwell 1987 and 1991; Kehrler 1993; Martinez-Cayuela 1995). Free radicals are independent chemical species containing one or more unpaired electrons; most biological molecules are nonradicals containing only paired electrons (Halliwell 1991). Oxygen radicals and their metabolites are collectively referred to as reactive oxygen species (ROS).

Reactive oxygen species are more reactive than non-radicals and have the potential to produce damage in biological systems. Due to their unstable nature and potential to damage cells and tissues, organisms possess enzyme systems and small molecular weight molecules with antioxidant capabilities that can protect against the adverse effects of free radicals (Machlin and Bendich 1987). A delicate balance exists between free radical generation and antioxidant defenses (Machlin and Bendich 1987), and maintaining this balance is vital to the well-being of an organism.

The steady-state balance of ROS and antioxidants may be upset in an organism, and oxidative stress can occur when this balance tips in favor of ROS (Sies 1985). This

phenomenon can result in oxidative damage to all types of biological molecules, including nucleic acids, lipids, proteins, and carbohydrates. Therefore, oxidative stress may be involved in processes such as mutagenesis, carcinogenesis, membrane damage, lipid peroxidation, protein fragmentation and oxidation, and carbohydrate damage (Sies 1993). Because of its deleterious nature, oxidative stress has been implicated in the pathogenesis of various diseases in humans (Halliwell 1987 and 1991; Kehrer 1993; Martinez-Cayuela 1995) and also in fish, e.g. liver lipid degeneration (Roald et al. 1981).

Nutrition plays a vital role in maintaining the prooxidant-antioxidant balance of fish (Cowey 1986). Most antioxidant vitamins ( $\alpha$ -tocopherol, ascorbic acid, carotenoids, etc.) and trace metals (selenium, manganese, zinc, copper, and iron) are essential and must be obtained from the diet (Machlin and Bendich 1987). However, iron and copper, when in dietary excess, can act as prooxidants (Kehrer 1993; Martinez-Cayuela 1995; McCord 1996). Historically, iron levels have ranged from 200 to 1000 mg/kg diet in commercial salmon feeds (Desjardins et al. 1987). Currently, levels of iron in commercial salmon and trout feeds are often greater than 200 mg/kg feed. These levels exceed the recommended level of ~40 to 60 mg/kg diet (NRC 1993) and probably should be lowered. Excess cellular iron is quite toxic, even in healthy organisms (McCord 1996), and high levels of iron in fish feed may adversely affect the immune system and lead to increased disease incidence and reduced growth rates (Goksøyr et al. 1993) and can catalyze the oxidation of lipids (rancidity) in fish feeds

(Desjardins et al. 1987). In addition, "optimal" levels of most dietary factors and their interactions need to be refined and better delineated for most fish species (Fowler 1989), including the levels of antioxidant vitamins and minerals in the diet of cultured Pacific salmon.

Some of the physiological differences between hatchery-reared (H) and wild (W) smolts are known to be affected by diet composition, e.g., body fat and lipid composition (Ackman and Takeuchi 1986; Bergstrom 1989; Fujioka et al. 1991), fin erosion (Ackman and Takeuchi 1986), trace metal composition (Felton et al. 1990, 1994, 1996), and immune function (Landolt 1989; Felton et al. 1990, 1994, 1996). Wild salmon appear to be superior to H fish with regard to these differences. These physiological differences may have important implications regarding the superior health and survival of W as compared to H salmon.

## **Phase 1**

Objectives for Phase 1 were to organize a workshop on fish nutrition (held Feb. 25, 1997; summary in Appendix 1) and develop assays for the detection of oxidative stress. Development of assays began in 1996 and is continuing. The assays and their progress are listed below.

1. Red blood cell peroxidation resistance assay - Assays based on the resistance of red blood cells (RBCs) to oxidative lysis have been used for many years to evaluate the status of antioxidant defenses (e.g. Rose and Gyorgy 1952; Gordon et al. 1955;

Mino et al. 1985). In some of these studies, the antioxidant defenses of the RBC have been of primary interest; in others, the RBC membrane has been used as a convenient model system to examine the effects of prooxidant or antioxidant substances on the cell membrane. The assay is performed by incubating aliquots of a RBC suspension in saline solutions containing various concentrations of a mild oxidizing agent (such as hydrogen peroxide). The concentration producing 50% lysis is estimated spectrophotometrically. This assay has recently been used to demonstrate that the RBC membranes of channel catfish fed vitamin E-deficient diets were more susceptible to peroxidation than were cell membranes of fish fed diets that met or exceeded recommendations for vitamin E supplementation (Wise et al. 1993).

Status: The assay has been adapted for use with salmonid RBCs and successfully used in laboratory and field studies.

2. Lipid peroxidation assays - When lipids react with ROS, lipid peroxidation is a potential outcome (Martinez-Cayuela 1995). One oxygen radical can initiate a chain reaction resulting in the conversion of hundreds of fatty acid side-chains into lipid hydroperoxides (Halliwell 1991). Accumulation of lipid hydroperoxides in a cellular membrane alters its fluidity, disrupts its function, and eventually causes it to collapse (Halliwell 1991; Martinez-Cayuela 1995). Lipid peroxides (LPO) formed during the lipid peroxidation chain reaction can damage other cellular components (e.g., proteins and other lipids). In addition, these peroxides can decompose to yield a number of cytotoxic products (e.g., aldehydes, alcohols, volatile hydrocarbons, etc.) that are

capable of causing further physiological damage (Halliwell 1991; Martinez-Cayuela 1995).

a. Measurement of LPO

Lipid peroxides are an early marker of lipid peroxidation and oxidative stress. We chose an assay kit (**K-Assay**<sup>TM</sup> LPO-CC, Cat. No. CC-004) manufactured by Kamiya Biomedical Company, Tukwila, WA to quantify LPO in tissues and plasma. The LPO-CC kit is unique because it specifically and directly measures LPO. The conventional method to measure lipid peroxidation is the thiobarbituric acid (TBA) assay. However, the TBA assay measures the acid breakdown of lipid peroxides into malondialdehyde (MDA), which then reacts with TBA to produce a colored product that is measured spectrophotometrically (Halliwell and Chirico 1993). TBA also reacts with other lipid peroxidation breakdown products with varying sensitivities and does not, therefore, measure lipid peroxidation exclusively (Halliwell and Chirico 1993).

The LPO-CC assay measures lipid peroxides by reducing LPO in the presence of hemoglobin to hydroxyl derivatives (lipid alcohols) which oxidatively cleave colorless MCDP (10-N-Methylcarbamoyl-3, 7-dimethylamino-10 H-phenothiazine) chromagen to form methylene blue in an equal molar reaction. LPO are quantified by colorimetrically measuring the released methylene blue at 675 nm.

Status: Completed.

b. Thiobarbituric acid assay

Historically, the TBA assay has been the conventional method used to measure lipid

peroxidation. However, it has certain drawbacks (see LPO-CC assay description). The TBA assay is useful in studies of oxidative stress, because it gives a relative measure of intermediate stages of lipid peroxidation and oxidative stress. Lipid peroxide metabolites, such as aldehydes (e.g., MDA) and ketones, are known carcinogens and possess other deleterious effects (Bostoglou et al. 1994). In addition, measurement of LPO metabolites is important in determining feed rancidity where the intermediate stages of lipid peroxidation are most toxic (R. Hardy, pers. communication).

Thiobarbituric acid reactive substances are measured by the method of Bostoglou et al. (1994). However, third-derivative spectrophotometry is not employed.

Status: Completed.

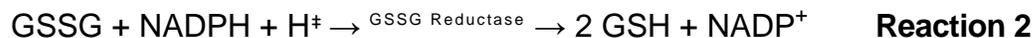
3. Glutathione Assay - Reduced glutathione (GSH) is a tripeptide found in all animal cells that evolved as a cellular protective mechanism against oxidation (Meister 1995). It serves as an antioxidant by directly reacting with free radicals and by acting as a substrate for the antioxidant enzymes glutathione peroxidase and glutathione transferase (Meister 1995). During oxidative stress, GSH is oxidized to GSSG (oxidized glutathione or glutathione disulfide). GSH levels are maintained by reduction of GSSG via the enzyme glutathione reductase. The GSH:GSSG ratio can be upset by oxidative stress (Smith 1992) or exposure to xenobiotics (Wallace 1989) where the ratio decreases. Therefore, GSH concentration and the GSH:GSSG ratio are important markers of oxidative stress (Smith 1992).

Reduced glutathione and GSSG are measured by the microplate method of Baker

et al. (1990).

a. Total glutathione (GSH + GSSG) - In short, GSH reacts with 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB) to give GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB; Reaction 1). GSSG in samples is reduced to GSH by the action of the highly specific glutathione reductase and NADPH (Anderson 1985; Reaction 2); the GSH formed then proceeds through Reaction 1. The rate of TNB formation is followed at 405 nm and is proportional to the sum of GSH and GSSG present.

b. Reduced glutathione (GSSG) - The procedure for measuring GSSG is identical to that for total glutathione except samples are treated with 2-vinylpyridine. 2-vinylpyridine removes GSH via conjugation; therefore, GSSG is measured exclusively (Baker et al. 1990).



Status: Initiated but not yet completed.

4. Carbonyl assay - Protein oxidation recently has been recognized as an important consequence of oxidative stress (Stadtman and Oliver 1991; Reznick and Packer 1994). Accumulation of oxidized proteins may be an early marker of oxygen radical-mediated tissue damage, and protein oxidation is implicated as an earlier indicator of oxidative stress than lipid peroxidation (Halliwell and Chirico 1993). Furthermore, oxygen radicals are suggested as an important cause of oxidative modification of proteins and enzymes, which may lead to their decreased activity and

degradation by various proteases (Stadtman and Oliver 1991).

Among the various oxidative modifications of amino acids in proteins, carbonyl formation is an early marker for protein oxidation through metal-catalyzed oxidation systems (Stadtman and Oliver 1991). Several methods are used to quantify the levels of carbonyls in purified and crude protein extracts of various tissues. The most extensively used method is the reaction of dinitrophenylhydrazine (DNPH) with protein carbonyls to form protein hydrazones. The protein hydrazone is detected spectrophotometrically at 355-390 nm. We will use the method of Reznick and Packer (1994).

Status: Not yet initiated.

5. Liver function assay - The liver, an important and multi-functional organ, is of interest here not only because it is important in glucose homeostasis, but also because it is the primary site for detoxification of xenobiotics, including various lipid peroxidation breakdown products (Stegman 1989). The most important liver enzymes for biotransformation of endobiotic and xenobiotic compounds are the multi-functional cytochrome P450 enzymes (CP450) (Flood et al. 1996). Recently, hatchery-reared red drum (*Sciaenops ocellatus*) have been shown to have reduced liver CP450 activity as compared to their wild counterparts (Flood et al. 1996). Greater levels of cellular lipid and lipid peroxides in hatchery red drum were strongly correlated with reduced CP450 activity (Flood et al. 1996). The authors suggested that higher levels of lipid peroxides inactivate CP450 enzymes and that feeding a potentially lipogenic diet to hatchery fish

may compromise their ability to biotransform various endobiotics and xenobiotics. Therefore, measurement of the detoxification capability of the liver provides a tangible means to directly assess the effects of oxidative stress on an important physiological function.

One of the liver-function assays evaluated by Hilton and Dixon (1982) will be used.

Status: Not yet initiated.

## **Phase 2**

A dietary study will begin at the University of Idaho in June 1997 and will attempt to determine the effects of dietary vitamin E, vitamin C, selenium, and iron on various indices of oxidative stress (listed above) and possibly other physiological functions as well (e.g., immune function) in spring chinook salmon. It also will examine possible interactions between these dietary nutrients.

### Experimental fish and diets

Semi-purified test diets based on the Pacific salmon modified H-440 formulation (NRC 1993; Table 1) will be manufactured at the Hagerman Fish Culture Station, Hagerman, ID. Only the levels of vitamin E, vitamin C, selenium, and iron will vary among the individual test diets; all other dietary components will remain constant. Super Refined<sup>®</sup> Menhaden Oil NP (Croda, Inc., Fullerton, Calif.) will be used as the dietary oil source. The super refining process removes polar impurities (e.g., vitamin E, carotenoids, oxidation products, monoglycerides, diglycerides, and free fatty acids). No

artificial antioxidants are added. Fifteen test diets will be used during the study.

Individual diets and the corresponding levels of vitamin E, vitamin C, selenium, and iron are given below (Table 2). Spring chinook salmon parr (Willamette River stock) were obtained from Smith Farm Aquaculture Facility at Oregon State University in February 1997 for use in this experiment.

**Table 1.**Pacific salmon modified H-440 diet

<u>Ingredient</u>	<u>%</u>	<u>Selected Minerals</u>	<u>mg/kg feed</u>
Casein (vitamin free)	40.8	CuSO <sub>4</sub>	~ 5
Gelatin	8.0	ZnSO <sub>4</sub>	30-50
Dextrin	19.5	MnSO <sub>4</sub>	20
Alpha-cellulose	4.7	KI	10
Amino acid mixture	4.4		
Vitamin premix	3.1		
Mineral premix	4.0		
Choline	0.5		
Super-refined <sup>®</sup> menhaden oil	15.0		

**Table 2.**

<u>Statistical Level</u>				<u>Dietary Level (mg/kg) &amp; Statistical Level<sup>a</sup></u>			
<u>Diet #</u>	<u>Vit. E &amp; Vit. C</u>	<u>Se</u>	<u>Fe</u>	<u>Diet #</u>	<u>Vit. E &amp; Vit. C</u>	<u>Se</u>	<u>Fe</u>
<b>3 replicate tanks/diet</b>							
1	+1	+1	+1	1	608.1 (+1)	7.20 (+1)	606.1 (+1)
2	+1	-1	-1	2	608.1 (+1)	1.90 (-1)	183.9 (-1)
3	-1	+1	-1	3	191.9 (-1)	7.20 (+1)	183.9 (-1)
4	-1	-1	+1	4	191.9 (-1)	1.90 (-1)	606.1 (+1)
5	+1	+1	-1	5	608.1 (+1)	7.20 (+1)	183.9 (-1)
6	+1	-1	+1	6	608.1 (+1)	1.90 (-1)	606.1 (+1)
7	-1	+1	+1	7	191.9 (-1)	7.20 (+1)	606.1 (+1)
8	-1	-1	-1	8	191.9 (-1)	1.90 (-1)	183.9 (-1)
<b>1 tank/diet</b>							
9	-α	0	0	9	50.0 (-α)	4.55 (0)	395.0 (0)
10	+α	0	0	10	750.0 (+α)	4.55 (0)	395.0 (0)
11	0	-α	0	11	400.0 (0)	0.10 (-α)	395.0 (0)
12	0	+α	0	12	400.0 (0)	9.00 (+α)	395.0 (0)
13	0	0	-α	13	400.0 (0)	4.55 (0)	40.0 (-α)
14	0	0	+α	14	400.0 (0)	4.55 (0)	750.0 (+α)
<b>2 replicate tanks/diet</b>							
15	0	0	0	15	400.0 (0)	4.55 (0)	395.0 (0)

<sup>a</sup>See text for explanation.

## Statistical design

The statistical design is a  $2^3$  factorial and central composite-surface response hybrid (Chris Williams, Statistics Consulting Center, University of Idaho). Thirty-two tanks will be used in the study (experimental units). Experimental diets will be allocated randomly to each tank. Twenty-four tanks (3 replicates of diets #1-8) will receive dietary combinations used in the factorial portion of the design (corner points in Fig. 1). Six tanks will receive a diet associated with the 6 axial points of the central composite (1 tank/diet; diets #9-14), and the dietary combination associated with the central composite center point will be assigned to 2 tanks (2 replicates of diet #15) (Fig. 1).

The attached schematic (Fig. 1) depicts the central composite design and the associated dietary combinations.

The following is a brief description of response surface methodology and central composite design (Clancy and King 1993).

Response surface methodology encompasses a group of techniques used in empirical studies of the relationships between one or more measured responses (e.g., oxidative stress bioassays) and a number of independent variables that may have important effects on the responses (e.g., concentrations of antioxidant nutrients in test diets).

A strength of response methodology is that it requires many fewer treatments than equivalent factorial designs to evaluate how biological phenomena respond when independent variables (e.g., antioxidant nutrients) are changed, not individually, but together (Box et al. 1978).

A geometric illustration of a three-variable response surface design is shown on the attached schematic (Fig. 1), using five different levels of treatment variable A (vitamin E + vitamin C), treatment variable B (selenium), and treatment variable C (iron) in chinook salmon diets as the independent variables. The eight corner points ( $A^{\pm 1} B^{\pm 1} C^{\pm 1}$ ) of the cube are the usual factorial design points for fitting a linear (i.e., first-order) model of the form:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \epsilon.$$

The center point of the design ( $A^0 B^0 C^0$ ) is usually replicated so that lack of fit of the response model can be estimated (Box et al. 1978). The six remaining points ( $A^{\pm \alpha} B^0 C^0$ ,  $A^0 B^{\pm \alpha} C^0$ ,  $A^0 B^0 C^{\pm \alpha}$ ) in Fig. 1 are arranged a distance  $\alpha$  ( $= 1.682$ ) from the center point along the axes of the variables and symmetrically positioned with respect to the factorial cube (Box and Draper 1987) and are referred to as the axial points (also called star points) of the cube, which allow fitting a second-order model of the form:

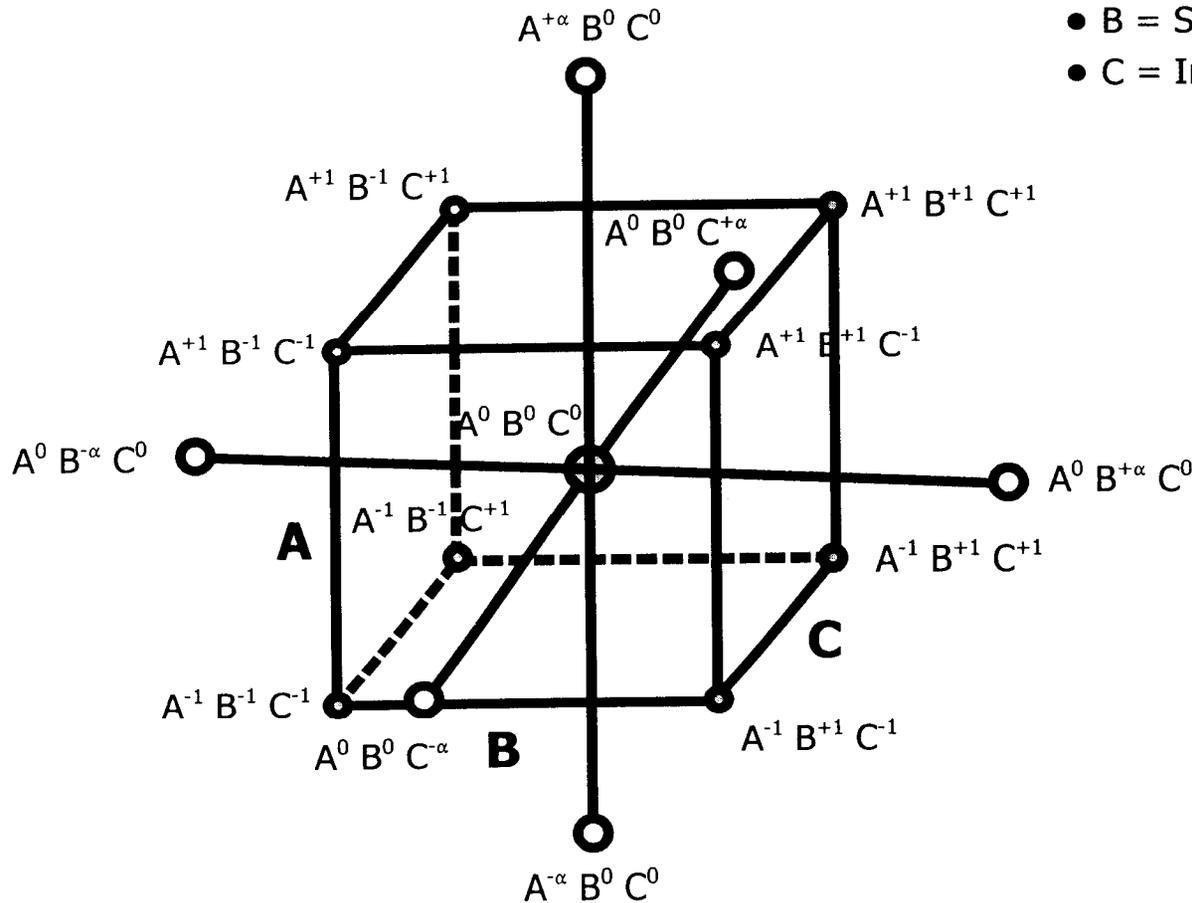
$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \epsilon.$$

Therefore, the 15 experimental points shown in Fig. 1 allow a good estimation of two-way interactions among the three dietary variables (vitamin E + vitamin C, selenium, and iron) plus possible nonlinear response curves for each individual treatment, because each is tested at five levels. A response surface model is fit to the data generated from the experiment using multiple regression analysis. The measured response (e.g., lipid peroxidation) is the dependent (y) variable in the model, and the

levels of the three dietary variables are the independent variables ( $x_1$ ,  $x_2$ , and  $x_3$ ), plus their interaction and squared terms. The combination of independent variables that provides the best fit to the data on the measured response ( $y$ ), based on  $R^2$  values, indicates the shape and dimensions of the response surface.

This design not only allows factorial testing of the effects of high and low levels of dietary variables, but it also allows the determination of optimum levels of dietary variables with regard to one or more measured dependent variables (e.g., oxidative stress assays, growth, food conversion, etc.).

- A = Vitamin E + C =  $-\alpha, -1, 0, +1, +\alpha$
- B = Selenium =  $-\alpha, -1, 0, +1, +\alpha$
- C = Iron =  $-\alpha, -1, 0, +1, +\alpha$



**Corner Points** = Closed Circles at Cube Corners = Factorial Dietary Combinations  
**Axial Points** = Open Circles, distance  $\pm\alpha$  from center point  
**Center Point** = Closed Circle in Cube Center

**Figure 1.** Schematic of experimental design. See Table 2 for the dietary and statistical level of each treatment variable.

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## **Appendix 1**

### Summary of Fish Nutrition and Performance Workshop

Owyhee Plaza Hotel, February 25, 1997.

#### Summary of Discussion

The nutritional needs of juvenile anadromous salmonids do not seem to be receiving an appropriate amount of attention from fish culturists and researchers at the present time. Diets for salmon and steelhead have remained unchanged for a number of years, despite advances in understanding of nutritional needs and improved manufacturing technology. Contracts with feed manufacturers usually specify the composition of feeds for anadromous fish in generic terms, allowing the substitution of alternative ingredients by the manufacturer that may not always be of optimal quality. Feed quality is usually not verified or checked unless overt fish health problems develop that are suspected to have a nutritional basis. The workshop participants felt that conservation agencies should give more attention to these problems.

#### Recommendations

- 1) Responsibility for specifying and verifying feed quality for the state (IDFG) or region (USFWS) should be delegated and centralized within the respective agencies (someone should be in charge).
- 2) A committee should be convened to provide technical assistance on fish nutrition

to the agencies. The committee would:

- a. Develop recommendations for feed specifications based on the current "state of the art" (3 below).
  - b. Recommend guidelines, schedules, and procedures for feed quality assessment.
  - c. Identify areas where current information is inadequate regarding nutritional needs of fish, assessment of feed and fish quality, etc.
- 3) Discussions should be initiated involving agency personnel, the technical advisory committee, and commercial feed manufacturers to determine if the current state of knowledge allows the formulation of improved diets without greatly increasing costs.

#### Brief Summary of Presentations and Discussion

Jim Congleton opened the workshop with a description of health and physiological indices in migrating wild and hatchery chinook salmon and steelhead smolts. Wild and hatchery fish differ in numerous ways; some of the differences may affect viability, and some may have a nutritional basis. Particularly interesting were differences in the antioxidant status of wild and hatchery fish. Tom Welker followed with a discussion of the role of oxidative stress and of antioxidant defenses in fish health.

Rick Barrows provided an overview of recent diet studies at the Bozeman Fish Technology Center. These studies have focused on micronutrients and indicate that

oxidative stress may play an important role in fin erosion in cultured salmonids. Ron Hardy provided some background on salmonid nutritional studies undertaken by the National Marine Fisheries Service.

Descriptions of recent and continuing diet-related studies or problems were given by Ray Jones and Keith Johnson. After lunch, procedures currently in use for specifying and verifying diet composition and quality were discussed: comments and suggestions made during this discussion have been summarized above.

### **List of Participants**

Rick Barrows	US Fish & Wildlf. Serv., Bozeman Fish Tech. Center
Marilyn Blair	US Fish & Wildlf. Serv., Dworshak NFH
Michelle Bouchard	US Fish & Wildlf. Serv., Dworshak NFH
Jim Congleton	USGS, Id Coop. Fish & Wildlf. Research Unit
Ron Hardy	Hagerman Aquaculture Lab., University of Idaho
Dan Herrig	US Fish & Wildlf. Serv., Lower Snake Comp. Plan
Keith Johnson	Eagle Fish Health Lab., Idaho Dept. of Fish & Game
Ray Jones	US Fish & Wildlf. Serv., Dworshak NFH
Joe Krakken	US Fish & Wildlf. Serv., Lower Snake Comp. Plan
Jay Pravecek	Eagle Fish Health Lab., Idaho Dept. of Fish & Game
Tom Welker	Dept. Fish & Wildlf., University of Idaho