Preliminary examination of oxidative stress in juvenile spring Chinook salmon *Oncorhynchus tshawytscha* of wild origin sampled from transport barges

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Migrating juvenile wild Chinook salmon *Oncorhynchus tshawytscha*, collected and loaded onto transport barges at Lower Granite Dam on the Snake River, were sampled from barges at John Day Dam, 348 km downstream, at 5 day intervals beginning in late April and ending in late May. An increase in lipid peroxidation and decrease in vitamin E in liver were observed from early to late in the barge transportation season. These changes seemed unrelated to changes in plasma cortisol or corresponding glucose levels, which declined from early to late in the season, or the concentration of n-3 highly unsaturated fatty acid (HUFA) concentrations in tissue but may be related to water temperature, which increased during the transport season, or other changes associated with the parr–smolt transformation.

Key words: cortisol; dam passage; HUFA; lipid peroxidation; vitamin E.

Construction of hydroelectric dams on the Snake and Columbia Rivers and their tributaries has greatly altered the riverine ecosystem (National Research Council, 1996) and produced conditions deemed unfavourable for emigrating juvenile salmonids (*e.g.* increased travel time, elevated water temperature, exposure to predators, passage through hydroelectric turbines and gas supersaturation). To reduce exposure to unfavourable conditions in the Snake–Columbia River hydrosystem, juvenile salmonids are collected at Lower Granite, Little Goose and Lower Monumental Dams on the Snake River and McNary Dam on the Columbia River, loaded onto trucks or barges, and transported and released at a site that is 235 km upstream of the mouth of the Columbia River (Ward *et al.*, 1997). Although transportation reduces exposure to the hydrosystem, it may adversely affect emigrants by potentially increasing horizontal transmission of pathogens (Maule *et al.*, 1988) or by elevating stress levels (Maule *et al.*, 1988; Schreck *et al.*, 1989; Congleton *et al.*, 2000). In addition, migrating smolts are undergoing physiological changes as a result.

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of the parr–smolt transformation, many of which have been linked to oxidative stress in fishes. These physiological alterations, along with fluctuations in the hydrosystem conditions (e.g. temperature, known to affect oxidative stress), can vary during the migration season, suggesting that susceptibility of emigrants to oxidative stress may have a temporal component as well.

Reactive oxygen species, the causative agents of oxidative stress, are formed continuously in animals as a consequence of normal aerobic cellular metabolism. A variety of antioxidant defences, including enzymes and small-molecular-mass molecules, have evolved in vertebrates to protect against reactive oxygen species. These defences, however, are not completely efficient, and oxidative stress can occur, resulting in damage to most types of biological molecules (Martínez-Cayuela, 1995). Oxidative stress has generated much interest in relation to human health but less so for fish health, despite evidence that oxidative stress may play a role in the pathology of fish disease and that fish tissues are particularly susceptible to oxidative damage (Love, 1981).

Several factors may predispose migrating salmonid smolts to oxidative stress. Juvenile salmonids undergoing the parr–smolt transformation experience significant physiological changes in preparation for transition to marine life (Hoar, 1988). The cell membranes of juvenile salmonids are characterized by an increase in n-3 highly unsaturated fatty acids (HUFA) during the parr–smolt transformation (Henderson & Tocher, 1987), which are more susceptible to peroxidative damage than are monounsaturated and saturated lipids (Love, 1981). Vitamin E (tocopherol) is the primary antioxidant protecting membrane lipids from peroxidation and is directly influenced by the vitamin E:HUFA ratio in cell membranes (Bell et al., 2000). As tissue HUFA content increases, an increased level of vitamin E is required to counter peroxidative damage (Bell et al., 2000); consequently, vitamin E levels can be inversely correlated with peroxidation of membrane lipids in migrating Chinook salmon Oncorhynchus tshawytscha (Walbaum) smolts (Welker & Congleton, 2005) and may be a useful indicator of oxidative stress susceptibility.

Levels of some hormones (e.g. cortisol and thyroxine), known to increase oxidative tissue damage, have been observed to increase naturally during smoltification (Hoar, 1988; Cowley et al., 1994) and as the smolt migration season progresses in spring O. tshawytscha (Beckman et al., 2000; Congleton et al., 2000). Passage through dams on the Snake–Columbia River hydrosystem (Mesa, 1994) and barge transport around the dams (Congleton et al., 2000) can cause stress and increase stress hormone levels in O. tshawytscha smolts. Furthermore, the responsiveness of the cortisol response to stressors is elevated during smoltification (Barton et al., 1985; Young, 1986), and the response can be more pronounced in wild fishes (Congleton et al., 2000). A heightened stress response, including elevated cortisol levels, increases peroxidative tissue damage in juvenile O. tshawytscha (Welker & Congleton, 2003, 2004). Similarly, whole-carcass concentrations of the antioxidant ascorbic acid and antioxidant enzyme cofactor selenium were shown to decrease during barge transport of O. tshawytscha smolts (Halver et al., 2004).

Previous research has suggested that oxidative stress may be important in migrating juvenile salmonids, but the focus has been primarily on concentrations of antioxidant trace metals (Felton et al., 1994; Halver et al., 2004) and not on the end results of oxidative stress, such as peroxidative tissue damage. Welker & Congleton (2005), however, showed that peroxidative tissue damage and vitamin E
increases and decreases, respectively, in juvenile anadromous salmonids as they migrate through the Snake–Columbia River hydrosystem. Antioxidants are often not, however, reliable markers of oxidative stress in fishes (McFarland et al., 1999). Polar lipid peroxidation is one of the primary markers for oxidative stress and is biologically significant, because accumulation of lipid hydroperoxides in cellular membranes alters membrane fluidity and can disrupt membrane function (Martínez-Cayuela, 1995). The primary objective of this preliminary study was to examine lipid peroxidation, HUFA and vitamin E levels in barged wild O. tshawytscha juveniles to determine oxidative stress status and to evaluate changes in these variables during the barging season.

In the spring of 2000, juvenile wild O. tshawytscha that had been loaded into fish transport barges at Lower Granite Dam (LGD: 46° 40′ N; 117° 26′ W) on the Snake River were collected from barges at John Day Dam (JDD:45° 43′ N; 120° 41′ W), 348 km downstream. Fish were sampled on 24 and 29 April and 4, 9, 14, 19 and 24 May (the typical emigration for juvenile spring O. tshawytscha in the lower Snake River begins mid-April and ends in early June). Kidney and liver samples were collected from 16 fish, euthanized with a lethal dose of buffered (240 mg l\(^{-1}\) NaHCO\(_3\)) tricaine methanesulphonate (MS-222; 200 mg l\(^{-1}\)), on each sampling date and used in the analyses below (kidney samples were not assayed for vitamin E).

Fish were captured by lift net (Matthews et al., 1986) and immediately transferred to a 200 mg l\(^{-1}\) solution of MS-222, an anaesthetic concentration that is quickly lethal and that is known to prevent postcapture changes in plasma cortisol (Barton et al., 1985). Blood samples were taken from fish by puncturing the caudal vasculature with a 20 gauge needle and aspirating a sample (0.2–0.75 ml) into a heparinized syringe. Samples were centrifuged for 5 min at 1200 g, and the plasma was immediately frozen on dry ice. After bleeding, kidney and liver samples used in lipid peroxidation and vitamin E analyses were excized and frozen on dry ice. Polar lipids were extracted by homogenizing frozen tissue samples for 30 s in 10 parts ice cold methanol (wt/vol) and 0.01% butylated hydroxytoluene to prevent \textit{in vitro} peroxidation. This procedure rapidly isolated polar lipids with minimal contamination by neutral lipids (primarily triglyceride) as evaluated by thin-layer chromatographic separation using a dual solvent system of chloroform: methanol: acetic acid: H\(_2\)O (65:25:8:4) and hexane: diethyl ether (4:1; Turunen, 1988). Extracted samples were assayed individually for lipid peroxidation (LPO) using the ferrous oxidation-xylenol orange (FOX) method to quantify polar lipid peroxides (Shantha & Decker, 1994; Burat & Bozkurt, 1996) with slight modifications. Methanol rather than water was used as the assay solvent system, and lipid peroxidation was expressed as \(\mu\)g hydrogen peroxide equivalents per g polar lipid by replacing the \(\text{Fe}^{3+}\) calibration curve with one utilizing hydrogen peroxide. The lipid content of tissue polar lipid extracts was determined by the sulpho-phospho-vanillin reaction (Frings et al., 1972; Van Handel, 1985). Lipid concentrations were estimated from a menhaden \textit{Brevoortia tyrannus} (Latrobe) fish oil (Sigma-Aldrich; sigmaaldrich.com) calibration curve.

Vitamin E in liver was determined by the method of Desai (1984). Lipophilic fractions, extracted with absolute ethanol, were saponified with 60% (w/v in water) KOH and 1% (w/v in ethanol) ascorbic acid at 70°C. After phase separation by the addition of equal parts of hexane and water, the hexane phase was evaporated under nitrogen
and redissolved in absolute ethanol. To this solution, ferric chloride, orthophosphoric acid and bathophenanthroline reagents were added. Vitamin E present in the lipid residue reduces ferric ions to ferrous ions and forms a pink-coloured complex with bathophenanthroline—orthophosphoric acid with a peak absorption of 550 nm (the method was validated by HPLC in the laboratory at the Department of Food Science and Human Nutrition, Washington State University, U.S.A).

Polar lipid extracts were also used in liver fatty acid composition analyses to determine n-3 HUFA concentrations (sum of 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3 expressed as mass %). Data obtained from fatty acid analysis were also used to calculate the vitamin E: HUFA ratio for each sampling date. The liver lipid extracts from six samples for each sampling date were evaporated under N₂ and stored at −80°C until fatty acid methyl ester (FAME) analyses were performed. Liver FAME analysis was performed on a gas–liquid chromatograph (Hewlett Packard 6890 Series with auto injector; www.home.agilent.com) fitted with a flame ionization detector. Fatty acid profiles were determined by split injection (20:1) onto a 0.2 μM film capillary column coated with CP-Sil 88 (100 m × 0.25 mm, Chrompack; www.chrompack.com) using a programmed temperature gradient method as described by St.-Hilaire et al. (2007). Individual fatty acids were identified by comparison of retention times to those of pure standards (butter, Matreya Inc.; www.matreya.com: menhaden oil CAS 8002-50-4 and Supelco 37 FAME mixture No. 407885 Supelco Inc.; www.sigmaaldrich.com). A response correction factor for each fatty acid methyl ester was used to convert peak area percentage to mass %. Correction factors were determined by analysing butter oil of known fatty acid profile with certified values (BCR632; European Community Bureau of Reference; http://irmm.jrc.ec.europa.eu/html/homepage.htm).

Plasma cortisol concentrations were determined by radioimmunoassay (Foster & Dunn, 1974) modified for use with salmonid plasma (Redding et al., 1984). Plasma glucose was measured by an autoanalyser (Dimension AR-IMT, DuPont; www.dupont.com).

The effects of sampling date on oxidative stress and cortisol and glucose responses were tested with a univariate general linear model (GLM) approach to ANOVA. If the effect of sampling date on measured physiological variables was statistically significant, differences between individual sampling dates were determined by examining their contrasts with the associated confidence interval (Bonferroni correction for multiple comparisons: $\alpha = \frac{1}{c}$, where $c =$ number of comparisons or groups and $\alpha = 0.05$). A significance level of $\alpha = 0.05$ was used for all statistical tests. The individual fish was the experimental unit in this study.

Peroxidation of kidney ($P < 0.001$) and liver ($P < 0.05$) polar lipids increased significantly from early to late in the barge transportation season in fish sampled from barges at JDD (Fig. 1). Lipid peroxidation values for liver and kidney in juveniles sampled on 19 and 24 May were significantly higher compared with fish sampled on 24 and 29 April. Conversely, liver vitamin E declined in migrating juveniles from early to late in the season and was significantly lower ($P < 0.05$) by the last sampling date compared with early in the study (Fig. 1). The relationship between lipid peroxidation and vitamin E in liver was negatively correlated and statistically significant (Pearson’s $r = -0.37$; $P < 0.01$). The HUFA content in liver cell membranes remained unchanged during the study, but the vitamin E:HUFA ratio decreased significantly during the transportation period ($P < 0.05$; Fig. 2).
Fig. 1. Mean ± s.e. liver (●) and kidney (▼) lipid peroxidation (LPO) and liver vitamin E (○) concentrations for juvenile wild *Oncorhynchus tshawytscha* sampled from barges at John Day Dam during the 2000 emigration. Liver or kidney lipid peroxidation values, significantly different between sampling dates, are denoted with different lower case letters (*P* < 0·05; Bonferroni correction for multiple comparisons).

Fig. 2. Mean ± s.e. highly unsaturated fatty acid (HUFA; ●) concentrations and vitamin E : HUFA ratio (○) in liver for juvenile wild *Oncorhynchus tshawytscha* sampled from barges at John Day Dam during the 2000 emigration. Vitamin E:HUFA ratios significantly different between sampling dates are denoted with different lower case letters (*P* < 0·05; Bonferroni correction for multiple comparisons).

Sampling date had a significant effect on plasma cortisol (*P* < 0·01) and glucose (*P* < 0·001) concentrations. Cortisol and glucose concentrations were highest during the early portion of the barge transportation period and then declined significantly, reaching their lowest values on 14 May for glucose and 19 May for cortisol (Fig. 3). Values for both variables remained unchanged thereafter.
Lipid peroxidation was inversely related to vitamin E content in liver of barged wild juveniles: lipid peroxidation increased and vitamin E decreased from early to late in the barge transportation season. Low levels of vitamin E in migrating juvenile *O. tshawytscha* have been correlated with increases in lipid peroxidation (Welker & Congleton, 2005). In the present study, vitamin E levels declined during the transportation period. Other antioxidants, which were not examined but are important components in the antioxidant defence system of salmonids, may have also declined during the transportation period and contributed to the increase in oxidative stress. Halver *et al.* (2004) found that hatchery-reared *O. tshawytscha* smolts suffered significant loss of carcass selenium, a cofactor for the antioxidant enzyme glutathione peroxidase, after 30 h barge transport. Selenium-dependent glutathione peroxidase is part of cellular antioxidant defence system that works synergistically with vitamins E and C in fishes (Hilton, 1989). Deficiencies of selenium are known to cause decreased activity of glutathione peroxidase in salmonids (Poston *et al.*, 1976), while dietary supplementation can produce increased activity (Felton *et al.*, 1996). Halver *et al.* (2004) also observed a significant decline in liver vitamin C after barge transport from Lower Granite Dam to Bonneville Dam late but not early in the transportation season. The findings of Halver *et al.* (2004) support the present observations of a temporal increase in oxidative stress, but stress or oxidative stress levels were not measured in fish prior to transport from LGD to JDD. Future research should include pre-transportation sampling. Levels of antioxidants in migrating juvenile salmonids have been little studied, and further research is needed to determine the relationship between oxidative stress and antioxidant capacity of migrating fishes.

Lipid peroxidation in kidney was also significantly higher in late compared with early in the transportation season. In mammals, lipid peroxidation has been linked to loss of renal function (Nath & Salahudeen, 1990) and failure (Paller *et al.*, 1984;
Baliga et al., 1999). Given the important role the kidney plays in the stress response (Bonga, 1997), immune system (Agbede et al., 2000), and osmoregulation and water balance (Bond, 1996), high levels of oxidative stress coupled with a low antioxidant capacity could limit the ability of kidney to function properly. Salmonid smolts fed diets fortified with vitamins E and C, and selenium had 100% survival after transfer to sea water compared with only 30–60% survival of smolts fed an unfortified diet (Halver, 1986), suggesting that protecting smolt cell membranes from peroxidative damage is important for survival during transition to sea water. Lipid peroxidation of cellular membranes in fishes can alter the ability of cells to maintain ionic balance (Wilhelm, 1996). Smolts experiencing high levels of oxidative stress may be less capable of making the transition from fresh to sea water, but it is unknown whether the levels of oxidative damage observed in this study were sufficient to reduce kidney or liver function.

Neither tissue lipid peroxidation nor vitamin E appeared to be correlated to changes in plasma cortisol or corresponding glucose levels. This is contrary to previous reports in O. tshawytscha (Welker & Congleton, 2003, 2004), where increases in plasma cortisol following exposure to physiological stress were correlated to peroxidative tissue damage. Juvenile O. tshawytscha exposed to a low-water stressor exhibit increased lipid peroxidation, which correlated strongly with increased levels of cortisol (Welker & Congleton, 2003, 2004). Levels of the antioxidant vitamin C are also known to decline in O. tshawytscha under stress (Felton et al., 1998). Even though oxidative stress increased as plasma cortisol levels declined during the transportation season, plasma cortisol and glucose levels were high throughout this study. It is possible that the detrimental effects of cortisol or other hormones involved in the smoltification process on oxidative stress are not evident until later in the season after vitamin E and other antioxidant defences have been depleted.

An alternative explanation for the increase in lipid peroxidation later in the migration would be an increase in n-3 HUFA, which are highly susceptible to peroxidation (Bell & Cowey, 1985) and typically increase during smoltification (Sheridan et al., 1985). Long-chain n-3 HUFA levels in liver, however, remained unchanged throughout the study period. The liver vitamin E: HUFA ratio that declined during the transportation season was solely due to loss of vitamin E, since HUFA levels did not change. Other physiological changes associated with the parr–smolt transformation, such as changes in levels of hormone like thyroxine (Hoar, 1988; Cowley et al., 1994), have been observed to naturally increase during smoltification and as the smolt migration season progresses in spring O. tshawytscha (Beckman et al., 2000; Congleton et al., 2000) and may have contributed to oxidative stress. Elevated water temperatures are also known to increase oxidative stress in fishes (Parihar & Dubey, 1995; Chien & Hwang, 2001). Forebay water temperatures for the Lower Granite, Little Goose, Lower Monumental and Little Goose Dams on the Snake River and McNary and John Day Dams on the Columbia River (in order, upstream to downstream) are shown in Fig. 4. Water temperature increased from a minimum of 9.8°C at the Lower Granite Reservoir tailrace on 24 April to a maximum of 14.8°C at the John Day Reservoir forebay on 24 May, an increase of c. 5°C during the course of the study. The average for the dams was 10.3°C on 24 April and 14.3°C on 24 May with an overall average of 11.9°C for the study period. The importance of rising water temperature in the Snake–Columbia River hydrosystem on oxidative stress is speculative, and the influences of in-river and transport barge water quality variables...
Fig. 4. Forebay water temperatures for dams [Lower Granite (●), Little Goose (○), Lower Monumental (▼), Ice Harbor (▲), McNary (■) and John Day (□)] on Snake and Columbia Rivers during the study period. Fish were sampled at Lower Granite and then barged, upstream to downstream, through Little Goose, Lower Monumental and Ice Harbor on the Snake River and McNary and John Day Dams on the Columbia River.

on oxidative stress require further investigation. The increase in water temperature during the study period was modest, and the degree of temperature increase needed to stimulate oxidative stress is unknown in salmonids. Excluding changes in environmental temperature, water quality may not be a probable explanation for changes in oxidative stress variables. Water quality is closely monitored and tightly controlled on transport barges. River water circulates through the barges in a single pass, and there is a closed-circuit re-circulation system that can shut off water intake in case of contamination in the river. The barge water pumping system also de-gasses the water in areas where gas supersaturation is a problem (J. Bailey, pers. comm.).

It is difficult to conclude whether barge transport influenced oxidative stress in juvenile wild *O. tshawytscha* in this study without pre-transportation, baseline fish with which to compare. Peroxidative damage to membrane lipids increased from early to late in the barge transportation season. These changes seem unrelated to changes in cortisol levels or corresponding glucose concentrations but may be related to water temperature that increases significantly from early to late in the transportation season or possibly to physiological processes related to smoltification. These data should be used as a starting point for future studies to determine the underlying causes for the temporal increase in peroxidative tissue damage and whether the damage is biologically significant, possibly affecting transition to sea water and survival of migrating smolts. Examination of juvenile *O. tshawytscha* before and after barge transport, the influence of river and barge conditions, comparison of barged to in-river wild and hatchery-reared migrants, changes associated with the parr–smolt transformation and post-transport survival should be included in future oxidative stress research.

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References


