Title

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Permalink

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Journal

Science of The Total Environment, 484

ISSN

00489697

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Publication Date

2014-06-01

DOI

10.1016/j.scitotenv.2013.09.097

Peer reviewed

Science of the Total Environment 484 (2014) 365–378

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Assessing reproductive and endocrine parameters in male largescale suckers (*Catostomus macrocheilus*) along a contaminant gradient in the lower Columbia River, USA

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HIGHLIGHTS

- Reproductive and endocrine parameters of male largescale suckers from the lower Columbia River were studied.
- Sperm biomarkers differed significantly among sites along a contaminant gradient.
- Correlations were found between congeners and T4, T3, VTG and sperm biomarkers.
- Sub-lethal effects of xenobiotics were least apparent at the reference site.
- Hypothalamic–pituitary–thyroid axis involvement was indicated.

ARTICLE INFO

Article history:
Received 1 June 2013
Received in revised form 27 September 2013
Accepted 28 September 2013
Available online 31 October 2013

Editor: Damia Barcelo

Keywords:
DNA fragmentation
Sperm
Motility
Apoptosis
Thyroid hormones
Flow cytometry

ABSTRACT

Persistent organochlorine pollutants such as polychlorinated biphenyls (PCBs), dichlorodiphenyldichloroethylene (*p,p′*-DDE), and polybrominated diphenyl ethers (PBDEs) are stable, bioaccumulative, and widely found in the environment, wildlife, and the human population. To explore the hypothesis that reproduction in male fish is associated with environmental exposures in the lower Columbia River (LCR), reproductive and endocrine parameters were studied in male resident, non-anadromous largescale sucker (*Catostomus macrocheilus*) (LSS) in the same habitats as anadromous salmonids having conservation status. Testes, thyroid tissue and plasma collected in 2010 from Longview (LV), Columbia City (CC), and Skamania (SK; reference) were studied. Sperm morphologies and thyrocyte heights were measured by light microscopy, sperm motilities by computer-assisted sperm motion analysis, sperm adenosine triphosphate (ATP) with luciferase, and plasma vitellogenin (VTG), thyroxine (T4), and triiodothyronine (T3) by immunoassay. Sperm apoptosis, viability, mitochondrial membrane potential, nuclear DNA fragmentation, and reproductive stage were measured by flow cytometry. Sperm quality parameters (except counts) and VTG were significantly different among sites, with correlations between VTG and 7 sperm parameters. Thyrocyte heights, T4, T3, gonadosomatic index and Fulton's condition factor differed among sites, but not significantly. Sperm quality was significantly lower and VTG higher where liver contaminants and water estrogen equivalents were highest (LV site). Total PCBs (specifically PCB-138, -146, -151, -170, -174, -177, -180, -183, -187, -194, and -206) and total PBDEs (specifically BDE-47, -100, -153, and -154) were negatively correlated with sperm motility. PCB-206 and BDE-154 were positively correlated with DNA fragmentation, and pentachloroanisole and VTG were positively correlated

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0048-9697/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.scitotenv.2013.09.097

with sperm apoptosis and negatively correlated with ATP. BDE-99 was positively correlated with sperm counts and motility; T4 was negatively correlated with counts and positively correlated with motility, thus indicating possible androgenic mechanisms and thyroid endocrine disruption. Male LSS proved to be an informative model for studying reproductive and endocrine biomarkers in the LCR.

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1. Introduction

The Columbia River and its tributaries form a predominant watershed in the Pacific Northwest. From headwaters in the Canadian Rockies, the Columbia flows across the State of Washington and along the border between Washington and Oregon to its mouth at the Pacific Ocean. The Columbia River is the link between landscapes and habitats for many species, and is a primary migration route for anadromous fish from the entire basin. Historically the Columbia produced more chinook (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), and steelhead trout (*Oncorhynchus mykiss*) than any other river in the world, yet wild runs of salmonids have diminished, both in numbers and diversity due to overfishing, passage barriers, and land use changes. Despite an extensive hatchery system in the Pacific Northwest, natural populations of anadromous salmonid fishes continue to decline (Paquet et al., 2011). Several Columbia River basin fish stocks are listed as threatened or endangered under the federal Endangered Species Act of 1973 (P.L. 93–205, 87 Stat 884), on global lists, and through the states of Washington and Oregon (AFWA, 2010) (Appendix A). Additionally, these salmonids are a significant source of nutrient cycling primarily through post-spawning mortality.

Ecosystem contaminants, being health concerns for people, fish, and wildlife, are well-documented in the description of regionally consistent trends of decreasing Columbia River water quality over 50 years (Fuhrer et al., 1996), an evaluation of wastewater-treatment-plant effluent and storm water runoff inputs (Morace, 2012), as well as other written communications on toxics in the basin (USEPA, 2009; Tetra Tech, Inc., 1996). Four persistent hydrophobic contaminant groups are widely present, all of which are endocrine disrupting compounds (EDC) that biomagnify. These include mercury, dichlorodiphenyltrichloroethane (DDT) and its breakdown products, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ether (PBDE) flame retardants (USEPA, 2009). Consequently, regional conservation strategies have included initiatives to reduce toxics and to understand their interactions within the food web in the lower Columbia River (LCR).

Without the confounding factors inherent in an anadromous life history, the widely prevalent resident largescale sucker (*Catostomus macrocheilus*) (LSS) more directly reflects contaminant uptake from sites along the LCR for investigating the potential effects of EDC on males. For this study, rather than investigating the threatened anadromous salmonids of concern directly (Appendix A), LSS was selected as a surrogate to understand the potential biological effects of persistent organic pollutants on male reproductive and endocrine parameters.

Salmonids are exposed to persistent organic pollutants in freshwater, estuarine, and coastal environments (Missildine et al., 2005). In the North Pacific Ocean, adults can bioaccumulate persistent organic pollutants which are then biotransported into freshwater by the migrating fish, their roe, and later their degrading carcasses. During the migration, organohalogens are transferred from fat to muscle (Cullon et al., 2009; Svedsen et al., 2007), which can then be consumed by foraging biota (Ewald et al., 1998) including bald eagles (*Haliaeetus leucocephalus*) (Buck et al., 2005), great blue herons (*Ardea herodias*) (Thomas and Anthony, 1999), and osprey (*Pandion haliaetus*) (Henny et al., 2011). Along the LCR, spatial differences exist in the concentrations of persistent organic pollutants as shown by PBDE concentrations in osprey eggs (Henny et al., 2011). A temporal difference was noted from 1992 to 2000, over which time total PBDE concentrations increased 12-fold in the salmonid mountain whitefish (*Prosopium williamsoni*), which was an order of magnitude higher than in LSS. This illustrates the influence of feeding habits on biomagnification (Rayne et al., 2003), with LSS foraging on insect larvae and periphyton (Dauble, 1986).

Sperm biomarker assays reflect reproductive capacity and measure cellular and molecular mechanisms of action which may be altered following organismal exposures to environmental contaminants (Jenkins, 2011b). In addition to affecting individuals at any life stage, many environmental EDCs can modify the germ line, resulting in transgenerational impacts to male fertility, as seen with vinclozin, methoxychlor (Anway et al. 2005), BDE-99 (Kuriyama et al., 2005), and dioxin (Theobald and Peterson, 1997). Reproductive dysfunction in male fish due to EDC can reduce sperm numbers (Haubruge et al., 2000; Patiño et al., 2003), sperm motility (Jenkins et al., 2009; Lahnsteiner et al., 2006), and fertility (Lahnsteiner et al., 2006). Secondary sex characteristics (Angus et al., 2001; Bayley et al., 2002; Jenkins et al., 2009) and thyroid hormone-responsive genes (Nourizadeh-Lillabadi et al., 2009) also can be influenced. Human sperm motility and serum thyroxine levels have been shown to be negatively related to specific PBDE congeners (Abdelouahab et al., 2011), and some studies showed that PBDEs act as EDC via alterations in the hypothalamic–pituitary–thyroid axis (Yang et al., 2011).

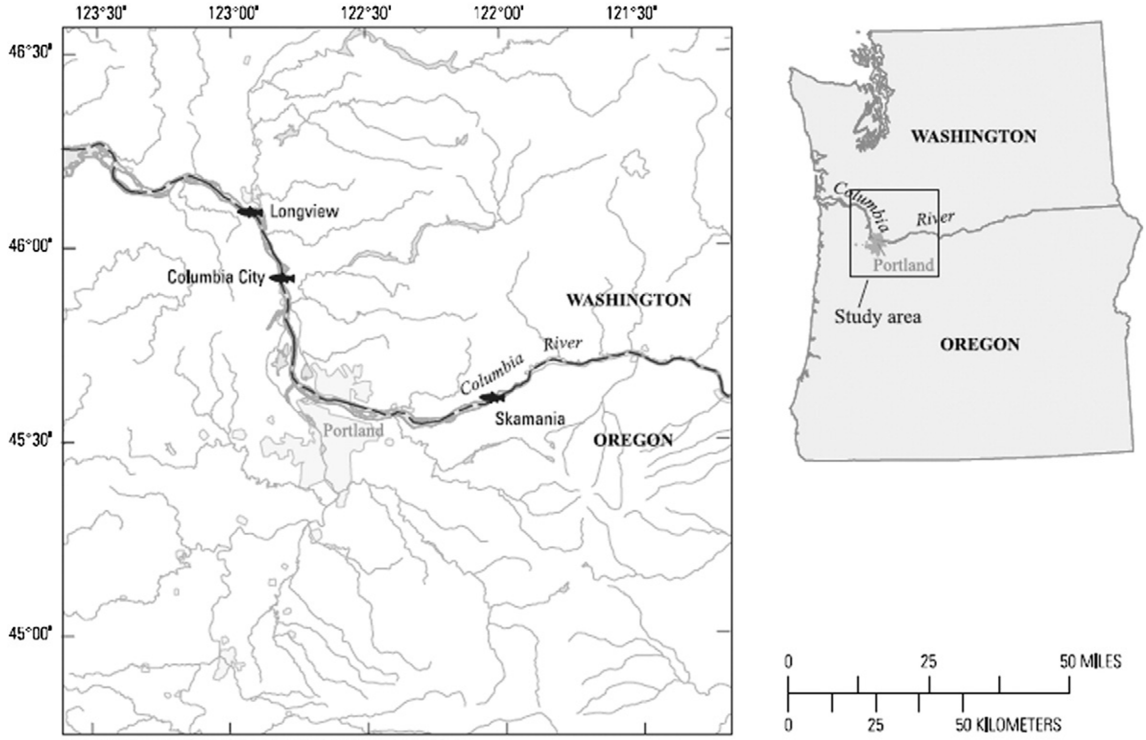
Biomarker results in fish from populations along pollution gradients can be separated from other environmental parameters (e.g., temperature) by the differential responses in males and the possible associations with co-occurring contaminants. Undetectable at the organism level, biomarkers are measures of biological responses to environmental chemicals that are made at the sub-individual level which indicate a deviation from the normal status (van der Oost et al., 2003). In this study on the LCR, we investigated whether LSS sperm quality differed at Skamania (SK), Columbia City (CC), and Longview (LV) (Fig. 1), with SK having the lowest and LV the highest concentrations of liver contaminants (Nilsen et al., 2014-in this issue) and estrogenicity. To estimate estrogenicity, estradiol equivalents were derived from passive sampling devices deployed at each site for one month period prior to fish collection (Alvarez et al., 2014–this issue). Endpoints included the following sperm indices: morphology, counts, motility, apoptosis, viability, mitochondrial membrane potential (MMP), adenosine-5′-triphosphate (ATP) content, and DNA fragmentation, as well as the proportion of testicular haploid cells as a measure of spermatogenic stage. Plasma vitellogenin (VTG), thyroxine (T4), triiodothyronine (T3), and thyrocyte heights were measured, and fish condition factor and gonadosomatic index (GSI) were calculated.

2. Materials and methods

2.1. Sampling

2.1.1. Locations

Contaminant input at CC and LV is primarily from urban and industrial effluents in the Portland–Vancouver region. The furthest upstream site (SK) was considered the reference location because it is less disturbed (Nilsen et al., 2014-in this issue). Fulton's condition factor (body weight/total length^3) and GSI ([gonad mass/total body mass] × 100) were calculated (Torres et al., 2014-in this issue), and fish were bled. Testes were removed on site, as well as lower jaws containing thyroid follicles for



Base from U.S. Geological Survey digital data, various scales
Universal Transverse Mercator, zone 10
North American Datum of 1983

Fig. 1. Three sampling sites for largescale sucker (*Catostomus macrocheilus*) on the lower Columbia River, where the contaminant gradient from high to low was Longview, Columbia City, Skamania (reference location).

use in biomarker assays. Livers were removed and used for contaminant analysis from individual fish (see Nilsen et al., 2014-in this issue).

2.1.2. Sample shipping and handling

Milt from whole testes shipped to the analytical laboratory, rather than milt collected on site, proved preferable for use (Appendix B), therefore in 2009 and 2010 sperm biomarkers were generated using milt from shipped testes (Section 2.3). Because most samples in 2009 were infested with bacteria (Fig. 2b), and samples from CC arrived after 48 h rather than within the standard 24 hour period, sperm quality data for the 2009 season were compromised (data not shown), precluding data interpretation for all parameters except sperm counts. To address that bacterial contamination, various antibiotic treatments were tested for potential cytotoxic impacts on sperm quality as well as presumptive effectiveness (Appendix B). Thus the optimal shipping buffer for testes in 2010 was Hanks' Balanced Salt Solution (HBSS), pH 7.5 (Glenn, 1998) at 320 mOsm/kg containing 100 μg/l streptomycin (Appendix B).

2.2. Thyroid hormones, thyroid histology, and vitellogenin analyses

2.2.1. Thyroid hormones

Blood that had been drawn by heart puncture with heparinized syringes was kept on ice and transported within 4 h to the Western Fisheries Research Center (Columbia River Research Laboratory, Cook, WA) for further centrifugation and plasma extraction. Plasma was then aliquoted for thyroid hormone and vitellogenin analyses.

Specific immunoassays were used to measure plasma T4 and T3 in duplicate using Coat-A-Count RIA solid phase radioimmunoassay kits (Cat. No. TKT45 and TKT35, respectively; Siemens Medical Diagnostics, Deerfield, IL, USA). Sensitivity of the T4 assay was 2.5 ng/ml; inter- and intra-assay variability (calculated as coefficient of variation) were 3.8% and 9.1%, respectively, and at 1000 ng/ml, T3 has 2% cross reactivity in the T4 RIA (manufacturer's data). Sensitivity of the T3 assay was 0.7 ng/ml; inter- and intra-assay variabilities were 7.6% and 5.8%, respectively, and at 100 ng/ml, T4 has 0.38% cross reactivity in the T3 RIA. Plasma samples were diluted two-fold using phosphate buffered saline before T3 analysis.

2.2.2. Thyroid histology

Lower jaws were treated with a decalcifying agent (Cal-Ex, Cat. No. 6381-92-6, Fisher Scientific, Pittsburgh, PA, USA) and Bouin's fixative (Cat. No. 1120-16, Ricca Chemical, Arlington, TX, USA) prior to trimming, dehydration, and embedding in paraffin. Serial sections were cut to 6 μm thickness and stained with hematoxylin and eosin. Digital images of thyroid follicles were taken with an Olympus® digital camera (DP10; Tokyo, Japan) attached to a compound microscope. All measurements were conducted digitally using Image-Pro® Express Software (Media Cybernetics, Silver Spring, MD, USA) as previously described by Mukhi et al. (2005). Briefly, five follicles per fish were chosen for all analyses according to their histological integrity and quality, and appropriate angle of cut. Thyrocyte height (index of hypertrophy) was determined at four specific locations around each follicle (12, 3, 6, 9 o'clock). The average height was determined for each follicle, and the average value of the 5 follicles was used as the individual fish value.

2.2.3. Vitellogenin

Plasma samples on dry ice were shipped to the Department of Physiological Science at the University of Florida for VTG analyses. A standard curve was generated using homologous, purified VTG from LSS females. Following antibody validation, the concentration of VTG was evaluated using enzyme-linked immunosorbent assay (Denslow et al., 1999). The detection limit for the assays was 0.001 mg/ml.

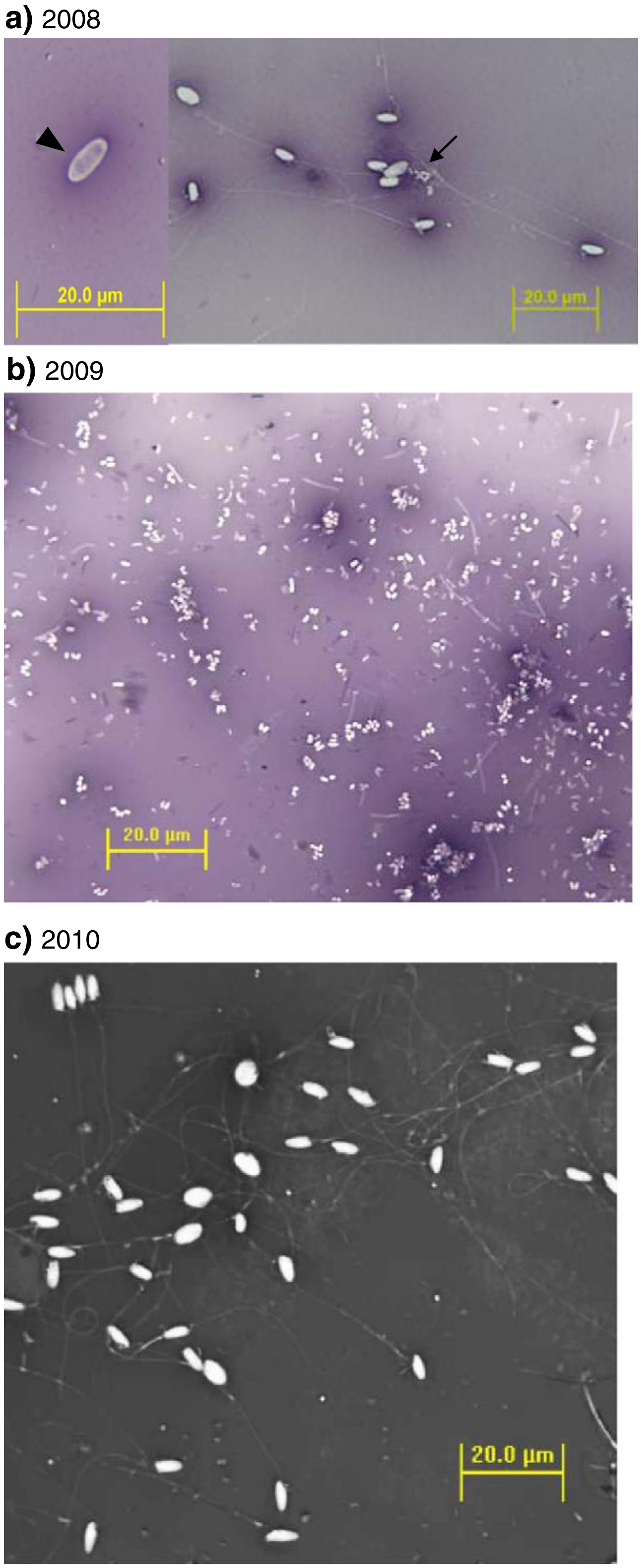


Fig. 2. Eosin–nigrosin staining of largescale sucker sperm imaged using light microscopy at 600×. Testes had been shipped without antibiotics in 320 mOsm/kg Hanks' balanced salt solution (HBSS) in 2008 (a) and 2009 (b), and with 100 µg/l streptomycin in HBSS in 2010 (c). Bacterial contamination (arrow) of shipped samples was minimal in 2008 (a), overwhelming in 2009 (b), and in 2010, the bacterial prevalence was low. A few enterococci and an apparent microsporidial parasite (arrowhead), the only type occasionally observed, are noted in (a). Some cocci are present in (c), while (b) indicates no spermatozoa and only bacteria in the shipped sample (fish LV10). Average sperm head widths were 2.56 µm (SE 0.049), head lengths were 5.13 µm (SE 0.066), and tail lengths were 47.83 µm (SE 1.16).

2.3. Sperm quality parameters

2.3.1. Morphology of spermatozoa

A 2.5 µl aliquot of milt diluted 1:100 in HBSS was mixed with 2.5 µl eosin–nigrosin dye (Lane Manufacturing, Inc., Denver, CO, USA) and gently smeared onto a slide. Morphologies were scored by using a light microscope (Olympus BX41; Olympus America, Inc., Center Valley, PA, USA) at 1000× magnification, counting more than 500 cells per slide, with duplicate slides per individual. Morphologies were assessed in accordance with World Health Organization protocols (WHO, 1987), whereby the two most commonly observed abnormalities were scored in relation to normal forms. The average head lengths and widths, and tail lengths were calculated from 50 normal spermatozoa per animal ($n = 3$ LSS) (Fig. 2c).

2.3.2. Sperm counts

Aliquots of 1 µl of milt were diluted in 99 µl of 4% paraformaldehyde and stored at 4 °C until enumeration by flow cytometry (Jenkins et al., 2011) with a FACSCalibur® (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Duplicate counts of 20K events were performed and recounted if duplicates were greater than 15% different. All flow cytometry data were analyzed with CellQuest software (BDIS) unless otherwise noted.

2.3.3. Sperm motility

Motility was assessed with computer-assisted sperm motion analysis (CASA) by using a 1:100 dilution of milt in HBSS. Milt was first activated by adding deionized water (18 mOsm/kg), then 2 µl was placed into a chambered slide (Leja 20 SC20-010040-B, Leja Products, Nieuw-Vennep, The Netherlands). Sperm motility was viewed by using phase contrast microscopy (Olympus) at 200× magnification and data were collected within 30 s. One visual field per sample was filmed at 60 frames/s, the replay edited to eliminate errors in cellular identification and movement, and then analyzed with software (SpermVision, Version 3.0, Minitube of America, Verona, WI, USA). Software settings included: area of cell identification 14–80 µm^2; immotile at average orientation of head (AOC) <9.5 µm; locally motile at distance straight line (DSL) <6 µm.; hyperactive at velocity curved line (VCL) >80 µm/s and linearity (LIN) (as velocity straight line [VSL]/velocity average path) <0.65 and amplitude of lateral head displacement (ALH) >6.5 µm; linear straightness (STR) (as VSL/VCL) >0.9 and LIN > 0.5; non-linear movement as STR < 0.9 and LIN < 0.5; and, curvilinear motion as distance average path (DAP)/radius ≥ 3 and LIN < 0.5. Total- and progressive motility data were statistically analyzed.

2.3.4. Apoptosis and viability

Using a 100 µl aliquot of milt diluted 1:20 in cold binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4), spermatozoa were stained with annexin V (A13199, fluorescein conjugate) (Life Technologies, Grand Island, NY, USA) (5 µl) that binds to phosphatidylserine residues, and counterstained with propidium iodide (PI) (Life Technologies) (1.5 µg/ml) (2.5 µl) which permeates compromised membranes. After incubation in the dark at 4 °C for 15 min, 400 µl cold binding buffer was added, then duplicate samples and controls (10K events each) were analyzed by flow cytometry. Quadrant analysis allowed separation of events into live non-apoptotic, live apoptotic, dead apoptotic, and dead necrotic cell populations. Cell viability was determined by a lack of PI staining (Fig. 3b).

2.3.5. Mitochondrial membrane potential

Rhodamine 123 (Life Technologies) and PI (Sigma-Aldrich, St. Louis, MO, USA) were used to stain spermatozoa in duplicate samples of 10 K events each (Jenkins et al., 2011). Rhodamine is a cell-permeant, cationic dye sequestered by active mitochondria and is used to assess mitochondrial membrane potentional. Samples were analyzed by flow cytometry.

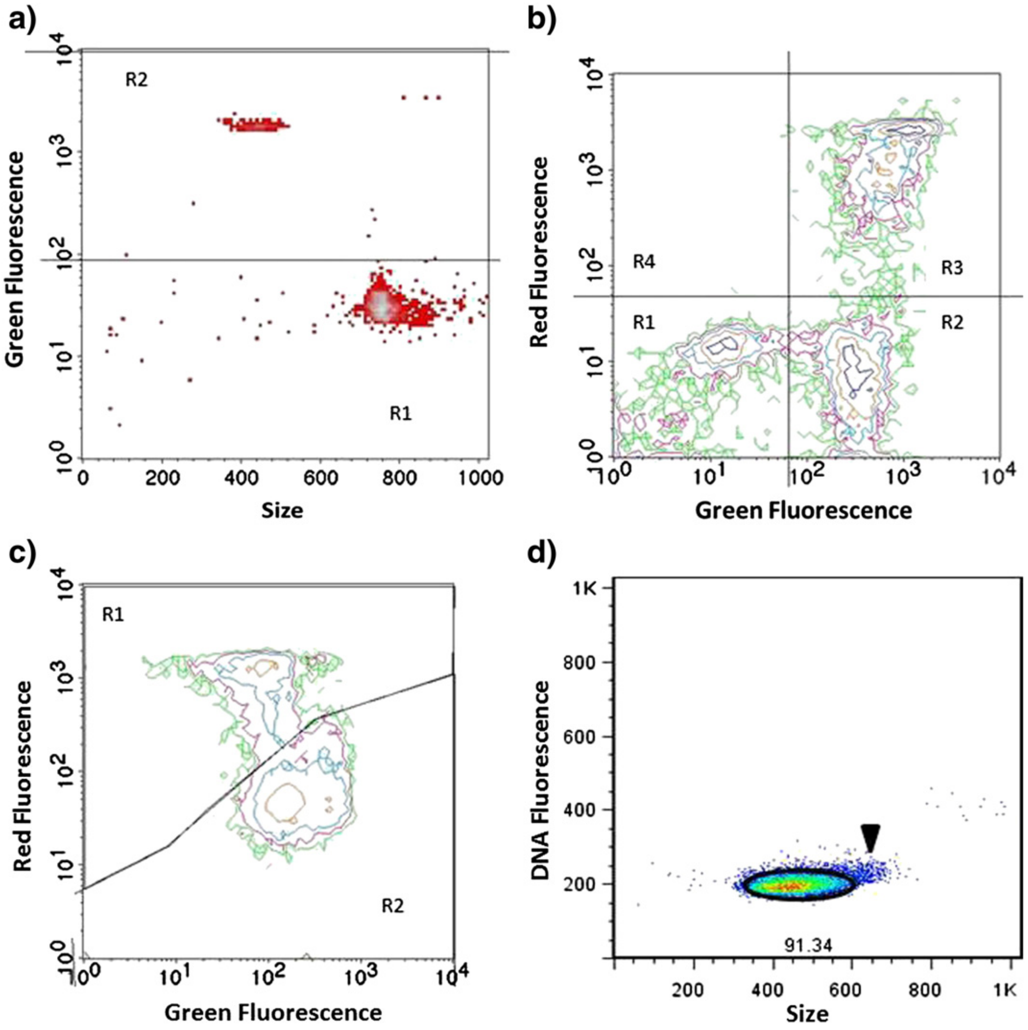


Fig. 3. Flow cytometry-generated plots showing largescale sucker sperm from 2010 fish with (a) sperm counts, where Region 1 (R1) shows a known number of fluorescent beads and R2 contains sperm. (b) By using an annexin V/propidium iodide (PI) assay, four different subpopulations of sperm were observed: fully viable sperm cells, not labeled with annexin V-FITC or PI (R1); a population of live apoptotic spermatozoa, labeled with annexin V-FITC but not with PI (R2); a population of dead apoptotic cells stained with both stains (R3); and dead, necrotic cells stained with PI but not with annexin V (R4). (c) Spermatozoa stained with Rhodamine-123 that display high mitochondrial membrane potential are shown in R2, and non-functional spermatozoa are shown in R1. (d) Nuclei outside the main population (NOMP) have fragmented DNA (arrowhead).

2.3.6. ATP content

The quantity of ATP per 1×10^6 spermatozoa was determined by using an ATP-binding luciferase assay (ViaLight Plus Kit, Lonza Group, Inc. Allendale, NJ, USA) with a TECAN GENios plate reader (Mannedorf, Switzerland). Standard curves were generated daily by using an ATP standard (ViaLight) (1:5, 1:10, 1:20, 1:40 dilutions) processed the same way as the field samples, resulting in $r^2 = 0.9102$ (LV), $r^2 = 0.9975$ (SK), and $r^2 = 0.9945$ (CC). For each field sample, 50 µl aliquots of spermatozoa (counted in duplicate with a Countess Automated Cell Counter, Life Technologies) were placed in a white 96-well plate, 50 µl lysis solution was added to wells, and the plates were incubated at 24 °C for 10 min. Then luciferase (100 µl) was added, plates were incubated in the dark for 2 min, and luminescence intensities (relative light units; RLUs) were measured, then used to calculate ATP concentration per number of cells.

2.3.7. DNA fragmentation

Milt was diluted to 2×10^6 cells/ml in HBSS, stained with PI, and analyzed by flow cytometry. Nuclear fragmentation was calculated as percentage of nuclei outside the main population (NOMP) using FlowJo software (FlowJo Flow Cytometry Analysis Software, Ashland, OR, USA) (Jenkins, 2011b; Jenkins et al., 2011).

2.3.8. Spermatogenic staging

To assess the relative numbers of cells in early to late spermatogenic stages of maturation (Blazer et al., 2013; Kaufman and Nagler, 1987), a piece of testis was cut and weighed, then minced for 1 min in HBSS. The suspension was fixed in 4% paraformaldehyde (1:1 by volume) and stored at 4 °C. For analysis, the suspension was diluted to 2×10^6/ml, filtered with 30 µm nylon mesh, stained with PI at 37 °C for 1 min and then at 24 °C for an additional 14 min. Data were generated by flow cytometry, collecting 10K events in duplicate per sample. Gating was used to exclude doublets, and the percentage of haploid nuclei out of the total haploid plus over-haploid events was determined (Jenkins, 2011b; Jenkins and Draugelis-Dale, 2006).

2.4. Statistical analyses

Not every testis from the field contained spermatozoa because some individuals had spawned prior to collection (Torres et al., 2014-in this issue). Sample sizes in 2010 were 7 to 16 per site for each biomarker.

Due to technical problems, $n = 6$ for NOMP at SK. Biomarker data were analyzed using parametric and nonparametric analysis of variance (ANOVA), with multiple comparisons (Tukey's Studentized range test). DNA fragmentation (NOMP), and testicular haploid staging were expressed as percentages (proportions). Based on the binomial distribution of proportions, when deviation from normality is great (<30% and >70%), the square root (sqrt) of the proportion is arcsine transformed to achieve approximate normality (Zar, 2010); NOMP and haploid staging were arcsine (sqrt) transformed accordingly. For other responses, when homogeneity of variance and normality of residuals were violated, transformations were attempted to meet assumptions for parametric ANOVAs. When all transformations failed, data were ranked for nonparametric analyses. Sperm counts and ATP data were log transformed. VTG and T4 data were ranked for nonparametric analyses, while T3 and thyrocyte heights did not require transformations. Statistical analyses were performed using SAS (SAS Institute, 1999) with level of significance $\alpha = 0.05$.

Relationships between biological parameters and hepatic contaminant concentrations were evaluated with nonparametric Spearman's rank correlation. Likewise, relationships between blood plasma and histology data with sperm biomarkers were evaluated with parametric Pearson correlations or nonparametric Spearman's rank correlation, where applicable. Groupings (totals identified with Σ) included Σ-legacy pesticides (chlordanes, dachtal, hexachlorobenzene, nonachlors, pentachloroanisole, dieldrin) with and without DDTs, Σ current-use pesticides (chlorpyrifos, trifluralin, oxyfluorfen), ΣPCBs (PCB-110, -118, -138, -146, -149, -151, -170, -174, -177, -180, -183, -187, -194, -206); ΣBDEs (BDE-47, -99, -100, -153, -154); personal care product triclosan; and Σ DDT (DDD and DDE). Significant correlations with $\alpha = 0.10$ are reported in Tables 3–6.

3. Results and discussion

3.1. Fish and sites

Most of the salmonids in the LCR are migratory, and interspecies differences exist in chemical sensitivities among freshwater fish (Teather and Parrott, 2006). Thus studying the widely prevalent LSS as an important component of the food web can provide insight into potential alterations of teleostean endocrine systems and reproductive capabilities due to exposure to environmental contaminants. Catostomids are not of direct economic or recreational benefit to humans and are not typically a focus of conservation efforts until they are imperiled, like the razorback sucker (*Xyrauchen texanus*) in the Colorado River. However the white sucker, *Catostomus commersoni*, has been the subject of several investigations concerning compromised aquatic conditions. In studies involving bleached kraft mill effluent, sperm motility (McMaster et al., 1992), plasma testosterone glucuronide and other reproductive characteristics differed among sites, indicating that reproduction was affected through the pituitary–gonadal axis (Van der Kraak et al., 1992).

Establishing evidence linking environmental exposure to EDC with reproductive effects can be procedurally challenging. Methodological complications can include a lack of unexposed control groups, the potential interactive effects of multiple chemicals, and remote site locations far from laboratories. In this LCR study, Skamania served as a suitable reference site based on LSS movement data (Dauble, 1986), because mixing between the LSS populations was unlikely there, as this site is more than 60 km from Columbia City. Some Columbia City LSS may have moved the 25 km downstream to mix with Longview fish, but mixing of Longview fish upstream was unlikely. Secondly the analytical chemistry results establishing concentrations of individual contaminants in livers (Nilsen et al, 2014-in this issue) and estrogenicity equivalents in water (Alvarez et al., 2014–this issue) confirmed the gradient of fish exposure and environmental contamination as LV > CC > SK. The PBDE concentrations were higher than the PCB concentrations in LSS livers at each site, with LV having the highest levels of PBDE, organochlorines (not including DDTs), and current-use pesticides (Nilsen et al, 2014-in this issue). Finally, transporting testes from the field to the laboratory required adding antibiotics to minimize bacterial contamination, thereby maintaining sperm cell quality to yield reliable data. By first addressing these procedural constraints, our results suggested that the sperm quality of LSS in the LCR was differentially impacted by contaminants, the pituitary–gonadal axis was involved, and fish at the reference site SK were least affected.

Table 1

Counts of largescale sucker (*Catostomus macrocheilus*) spermatozoa per ml of milt.[a]

Site	2009			2010		
	Count	SE	N	Count	SE	N
Longview	1.2×10^{10}	1.8×10^{9}	16	7.6×10^{9}	5.5×10^{8}	11
Columbia City	1.1×10^{10}	1.3×10^{8}	13	5.1×10^{9}	5.9×10^{8}	8
Skamania	1.5×10^{10}	1.8×10^{9}	16	6.5×10^{9}	4.2×10^{8}	12

[a] No significant differences were noted among sites within each year.

Biological responses at higher hierarchical levels within an organism are always preceded by earlier changes in biological processes (van der Oost et al., 2003). To investigate potential effects of contaminants on male LSS reproductive parameters, biomarkers at the molecular, cellular, and hormonal levels were examined. The general health of male LSS and the occurrence of parasites were different among sites, with higher health status and lower parasite incidence at the reference site SK (Torres et al., 2014-in this issue). Fish condition factor (Torres et al., 2014-in this issue), often used to assess the overall condition and stoutness of a fish, was not different for LSS among sites ($P = 0.3095$), likely because sites were differentially influenced by nutrients, chemical mixtures, and hydrology. In an experiment in which adult fathead minnows (*Pimephales promelas*) were fed daily for 25 d with BDE-47, the condition factor of only the males was significantly reduced ($P < 0.011$) compared with controls (Muirhead et al., 2006). Contrary to that finding, long-term continuous exposure to BDE-209 was related to an increase in the condition factor in adult male zebrafish (*Danio rerio*), suggesting a potential link with obesity-related genes (He et al., 2011). In this LCR study, trans-chlordane, an extremely persistent organochlorine insecticide banned in 1988 (Keith, 1997), was the only compound found to associate with condition factor ($\rho = -0.34556$; $P = 0.0527$).

The GSI, an index of the gonad mass in proportion to the total body mass, was also not different among sites in this study (Torres et al., 2014-in this issue), yet we noted that GSI, as well as sperm motility, was negatively correlated with seven PCB congeners and ΣPCBs (Table 3). Similarly, in a study at the Puget Sound, Washington on the relationship between anthropogenic chemical exposure and reproductive parameters in male English sole (*Parophrys vetulus*), GSI was negatively correlated with ΣPCBs (Sol et al., 2008). In contrast, chlorpyrifos (an organophosphate insecticide) and current-use pesticides were positively correlated with GSI in this study with LSS (Table 3). Male medaka (*Oryzias latipes*) GSI was not different between BDE-47-treated fish and controls, although this congener was reproductively toxic in males as noted by a cessation of spawning and a lower percentage of mature spermatozoa (Muirhead et al., 2006). Similarly, BDE-47 was not related to GSI, yet it was negatively correlated with sperm motility in LSS (Table 3). The reduced GSI in male zebrafish exposed to BDE-209 indicated anti-androgenic effects by this congener (He et al., 2011). Because results indicate various modes of action on gonads and other reproductive parameters are apparent among exposures with individual PBDE and PCB congeners, heterogeneous reproductive and endocrine mechanisms are certainly transpiring where fish are exposed to complex chemical mixtures.

Freshwater ecosystems are recipients of compounds that move hydrologically downhill; hence food web approaches are useful in assessing risks posed there by contaminants (Baird et al., 2001) especially with compounds as ubiquitous as PBDE. The most prominent PBDE congeners found in the environment, humans, birds of prey and

Table 2
Average percent (SE) sperm mitochondrial membrane potential, viability, total apoptotic sperm, live apoptotic sperm, fragmented DNA, abnormal sperm morphology, haploid testicular cells, total and progressive motilities and ATP levels measured in samples from largescale suckers from sites along the lower Columbia River in 2010.

	Longview	Columbia City	Skamania
Mitochondrial membrane potential	56.6^{b} (4.7)	90.1^{a} (1.7)	82.1^{a} (1.8)
Viability	82.4^{b} (2.2)	93.1^{a} (1.5)	94.8^{a} (16)
Total apoptotic	44.7^{a} (4.9)	24.8^{b} (4.0)	14.1^{b} (3.9)
Live apoptotic	27.5^{a} (3.1)	17.3^{a,b} (3.0)	9.0^{b} (2.3)
DNA fragmentation	10.3^{a} (0.8)	5.3^{b} (0.7)	7.1^{b} (2)
Abnormal sperm morphology	10.5^{a} (0.8)	5.3^{b} (0.7)	7.1^{b} (2)
Haploid cells	96.6^{a,b} (0.8)	96.4^{b} (0.6)	98.3^{a} (0.4)
Total motility	15.5^{a} (3.9)	14.6^{a} (3.5)	26.8^{a} (4.1)
Progressive motility	6.6^{a} (3.3)	5.7^{a} (2.7)	12.5^{a} (2.7)
mM ATP/10^{6} cells	0.00085^{b} (0.00048)	0.00247^{a} (0.00077)	0.00511^{a} (0.00104)

Significant differences among sites per biomarker are noted with different superscripts.

other wildlife are BDE-47, BDE-99, and BDE-100 (Eriksson et al., 2001; Chen and Hale, 2010; Nilsen et al., 2014-in this issue), with some inter-regional differences. At sites furthest downstream on the LCR, sperm quality values in LSS were the lowest. Along a similar stretch of the LCR, the lowest bald eagle nest productivities were found at the river's mouth, where the highest concentrations of organochlorines in eggs occurred (Buck et al., 2005).

Most PBDE are hydrophobic, lipophilic, and have half-lives on the order of 1–20 years or more. They are similar in molecular structure to PCBs and thyroid hormones (Hu et al., 2007; Lema et al., 2008), and they adsorb tightly to the organic fraction of bioavailable suspended particulates. For osprey and other biota, waste-water treatment plants along the LCR were likely a source of PBDEs (Henny et al., 2011), which are not degraded substantially or otherwise removed by processing in such plants (Rayne and Ikonomou, 2005). Although newer alternatives for flame retardants are replacing PBDEs, the decaBDEs (such as BDE-209) are still industrially produced and, along with their metabolites, will continue to bioaccumulate (Wenning et al., 2011). In this study with LSS, neuroendocrine hypothalamo-pituitary-thyroid (HPT) axis involvement was strongly indicated with PCB-206, other PCB congeners, pesticides and metabolites (Table 6). Congeners BDE-47 and BDE-99, as well as ΣBDE and *p,p′*-DDE, were negatively associated with human T4 levels (Abdelouahab et al., 2011), implying involvement of the HPT axis. Also in human males, levels of decaBDEs were shown to be inversely correlated with serum testosterone (Johnson et al., 2013).

3.2. VTG and thyroid parameters

Thyroid hormones are important for early development and somatic growth in juveniles and adults of some teleost species (Yamano, 2005; Carr and Patiño, 2011), but PBDE effects on thyroid function have been inconsistent for this group of vertebrates (Torres et al., 2013). Plasma T4 levels in male LSS ranged from undetectable (LOD, 2.5) to 10.4 ng/ml, and plasma T3 from undetectable (LOD, 0.7) to 9.1 ng/ml. Total LSS T4 and T3 levels were both numerically highest at the most contaminated site, but because the values were not significantly different among sites (Table 4), location-associated influences on circulating thyroid hormone levels were not apparent. Three PCB congeners were negatively correlated with T4, yet both T4 and T3 were positively correlated with pentachloroanisole and dieldrin (Table 6). Some studies have

Table 3
Spearman rank correlation coefficients ρ (rho) between biological parameters and liver contaminants from largescale suckers, 2010.[a]

	GSI	Abnormal morphology	Counts	Total motility	Progressive motility	Live apoptotic	Total apoptotic	Mitochondrial membrane potential	ATP	Viability	NOMP
PCB-138			*−0.33897*	−0.36904							
PCB-146	−0.40458			*−0.30549*	*−0.34782*						
PCB-149	−0.52536										
PCB-151				*−0.31119*	−0.37021						
PCB-170				−0.38823	−0.44939						
PCB-174	*−0.29677*			−0.41256	−0.47017						
PCB-177	*−0.33134*			*−0.31640*	−0.41428						
PCB-180				−0.35179	−0.42641						
PCB-183	*−0.31100*			−0.37047	−0.43557						
PCB-187	*−0.34236*			*−0.34715*	−0.43859						
PCB-194				−0.38628	−0.46131						
PCB-206	*−0.32924*	−0.49026		−0.39758	−0.49619			*0.31658*			0.37562
ΣPCB	*−0.29615*			*−0.34772*	−0.39589						
Σ Triclosan		*−0.44426*									
BDE-99			0.39449	0.40261							
BDE-100				*−0.33159*	−0.43615						
BDE-47				*−0.31573*	−0.43043						
BDE-153					−0.38418						
BDE-154				−0.40970	−0.51794						*0.31256*
ΣBDE				*−0.31748*	−0.42928						
PCA[b]						*0.30553*			−0.46323		
Dacthal				*0.31101*							
Chlorpyrifos	0.39693	0.49364									
Current pesticides	0.40254	0.49418				0.35043	*0.31483*				
HCB[c]					*−0.30063*						
Legacy pesticides		0.55789									
p′p′-DDE		0.59149									
p′p′-DDD									*0.34725*		
DDD + DDE[d]		0.56667									

[a] Values in italics were significant at α = 0.10, and all others were significant at α = 0.05; only significant relationships are shown. Haploid testicular cells did not show any correlations with contaminants.

[b] Pentachloroanisole.

[c] Hexachlorobenzene.

[d] Σ DDT.

Table 4
Average concentration (SE) of vitellogenin (VTG) and thyroid hormones (ng/ml), and thyrocyte height (μm) from largescale suckers at sites along the lower Columbia River in 2010.

Site	VTG	Thyroxine (T4)	Triiodothyronine (T3)	Thyrocyte height
Longview	0.021[a] (0.004)	3.008 (0.934)	4.356 (0.421)	7.410 (0.590)
Columbia City	0.005[b] (0.004)	2.033 (1.329)	3.747 (0.668)	8.189 (0.996)
Skamania	0.000[b] (0.000)	2.533 (0.821)	3.622 (0.461)	8.630 (0.571)

Significant differences among sites per endpoint are noted with different superscripts.

shown PCBs and certain pesticides may alter 5′-deiodinase activity in fish, leading to increased T3 and reduced T4 (Brar et al. 2010; Brown et al. 2004). As previously suggested, plasma levels of T4 and T3 may be inconsistent biomarkers of thyroid disruption in teleosts (Carr and Patiño, 2011). Since histological evaluations of the thyroid follicle may provide more reliable biomarkers (Carr and Patiño, 2011), the thyroid follicles in LSS were studied, and were found to be distributed ventrally in the pharynx, forming clusters around the ventral aorta, with a few of the follicles being isolated. Not encapsulated or surrounded by connective tissue, the follicle clusters were comprised of cells of various widths. Measures of thyrocyte height, an index of hypertrophy, did not yield statistical site differences, but values were smallest at LV (Table 4).

Although the thyroid endocrine system plays a role in gonadal development and reproduction of teleosts (Cyr and Eales, 1996), the effects of thyroid endocrine disruption on reproductive fitness have been difficult to document (Brown et al., 2004; Carr and Patiño, 2011). Recent studies with zebrafish have indicated a relationship between the status of the thyroid endocrine system and gonadal sex differentiation, pubertal development, and reproduction (Mukhi et al., 2007; Mukhi and Patiño, 2007; Sharma and Patiño, 2013). Specifically, Torres et al. (2013) showed that although thyroid condition and pubertal development were not affected by concentrations of BDE-47, growth during the juvenile-to-adult transition was affected, especially in males. In contrast, dietary BDE-47 did impair thyroid function and certain parameters of the reproductive development of fathead minnows (Lema et al. 2008), underscoring the disparity in endocrine impacts of PBDE congeners among species. In two fish species resident to the San Francisco Bay area, changes in the thyroid endocrine system were related to contaminant exposures, with decreased T4 levels inversely proportional to PCBs (no PBDE data) (Brar et al., 2010). Comparable results were shown in this LCR study with LSS; plasma T4 levels were negatively associated with PCB-146, -177, and -206 (Table 6). Based on studies of the effects of PBDEs on fish thyroid and reproductive parameters, Torres et al. (2013) concluded that only those congeners that affect the thyroid system are also able to affect the reproductive system in teleosts, and they act in a species-specific manner. In this study, no PBDE congeners were correlated with thyroid parameters or morphometric indices of reproductive status (Torres et al., 2014-in this issue), but this was not the case for functional indices of sperm quality (see below).

Up to seven correlations (both positive and negative) were noted between contaminants and LSS T4 and T3 levels, whereas only dacthal was correlated, positively, with thyrocyte height (Table 6). Several correlations were also noted between thyroid- and sperm quality parameters (Table 5). For example, T4 was negatively correlated with PCB-206 ($\rho = -0.44089$; Table 6) and with sperm counts ($\rho = -0.32043$; Table 5). Looking further, PCB-206 was negatively correlated with total and progressive sperm motility ($\rho = -0.39758$; $\rho = -0.49619$) and positively with NOMP ($\rho = 0.37562$) (Table 3). T3 was positively correlated with pentachloroanisole ($\rho = 0.36832$; Table 6) and negatively correlated with sperm ATP ($r = -0.46794$; Table 5). Looking further, pentachloroanisole was also negatively correlated with sperm ATP ($r = -0.46323$), and positively correlated with live sperm apoptosis ($r = 0.30553$) (Table 3). Negative correlations were also seen between VTG and ATP, and between VTG and MMP ($\rho = -0.43617$ and $\rho = -0.58437$, respectively; Table 5). Higher levels of sperm ATP indicate good sperm quality and low levels of VTG in males do not point to EDC exposure.

Plasma VTG is a phospholipid protein produced in the liver under the control of 17β-estradiol; it is a precursor of egg yolk and is normally detected in female oviparous vertebrates (Goodbred et al., 2007; Patiño and Sullivan, 2002). Endocrine disruption in males via exposure to xenoestrogens is indicated by an induction of VTG. In this study, VTG levels were significantly higher at the most contaminated site LV (LV > CC = SK; $P < 0.0001$) (Table 4). This observation is consistent with levels of estrogenic equivalents along the gradient in the LCR (Alvarez et al., 2014–this issue). VTG was positively correlated with BDE-47, BDE-153, and ΣPBDE, as well as pentachloroanisole, current-use pesticides, and hexachlorobenzene (Table 6). Of all the plasma biomarkers, VTG showed the most correlations with sperm quality parameters (Table 5).

3.3. Sperm quality parameters

Evidence in support of anti-androgenic modes of action by PBDEs included a delay in puberty in male rats with decreased growth of androgen-dependent tissues following exposure to a PBDE mixture; these effects were attributed to the compounds acting as androgen receptor antagonists (Stoker et al., 2005). In fathead minnows, histological analyses of testes from individuals treated with BDE-47 revealed a more than 50% reduction in mature sperm, and egg-laying in treated breeding pairs stopped after 10 days (Muirhead et al., 2006). Male minnows exposed to BDE-47 had fewer mature spermatozoa and more primary

Table 5
Nonparametric Spearman's rank correlation coefficients ρ (rho) and parametric Pearson r between largescale sucker sperm quality parameters and vitellogenin (VTG), thyroxine (T4), triiodothyronine (T3), and thyrocyte height.

	Abnormal morphology	Counts	Total motility	Progressive motility	Live cell apoptosis	Total apoptosis	Mitochondrial membrane potential	ATP	Viability
VTG[a]	*0.45034*[b]			−0.45476	0.56031	0.52479	−0.58437	−0.43617	−0.47705
T4[a]		*−0.32043*	*0.33434*						
T3[c]								−0.46794	
Height[c]					*−0.29283*[d]			*0.42168*	

[a] Nonparametric Spearman's rank correlation coefficients ρ (rho).
[b] Values in italics were significant at $\alpha = 0.10$, and all others were significant at $\alpha = 0.05$.
[c] Parametric Pearson r.
[d] $P = 0.1099$.

Table 6
Spearman's rank correlation coefficients ρ (rho) between largescale sucker vitellogenin (VTG), thyroid hormones, and thyrocyte height with contaminants in livers from largescale suckers[a], 2010.

	VTG	Thryoxine (T4)	Triiodothyronine (T3)	Thyroid cell height
PCB-146		*−0.34614*		
PCB-177		*−0.35175*		
PCB-206		−0.44089		
BDE-47	0.38726			
BDE-153	0.40707			
ΣBDE	*0.35098*			
PCA[b]	0.54050	*0.30486*	0.36832	
Dieldrin		*0.34332*	0.37124	
Dacthal				*0.31362*
Chlorpyrifos		*0.31795*		
Current pesticides	0.53714	*0.34079*		
HCB[c]	0.42095			

[a] Values in italics were significant at $\alpha = 0.10$, and all others were significant at $\alpha = 0.05$.
[b] Pentachloroanisole.
[c] Hexachlorobenzene.

spermatocytes and spermatids compared with control males (Lema et al., 2008).

Spermatogenesis involves mitosis, meiosis, and cellular differentiation in the production of mature, haploid sperm. For LSS at LCR, the percentage of testicular cells in the haploid stage was significantly different ($P = 0.0275$) among sites (Table 2), with the highest percentage of mature sperm forms being found at the reference location (SK ≥ LV ≥ CC), indicating that LSS were in the best reproductive condition there. Analysis of suspensions of testicular cells allows the identification of different spermatogenic cell types on the basis of their DNA ploidy/stainability level (Jenkins, 2011b). In this flow cytometric method all cells from the testicular tissue are analyzed, including spermatocytes, spermatozoa, spermatids, somatic cells, Leydig cells, and Sertoli cells. Genotoxic effects can increase the number of diploid spermatids due to a failure of meitotic chromosomes to segregate (Hacker-Klom et al., 1986). These LSS spermatogenic staging results were similar to those seen with western mosquitofish (*Gambusia affinis*) in an environment polluted with insecticides and organochlorines, at which the ploidy values showed fewer mature sperm forms at contaminated sites (Jenkins and Draugelis-Dale, 2006; Jenkins et al., 2009).

In LSS, sperm motility was the parameter most often correlated with contaminants (all of the PCB congeners and most of the PBDE congeners were negatively correlated; Table 3). The forward-moving, progressive LSS sperm motility was more negatively affected by PCBs and PBDEs than was total motility, as indicated by more and higher correlation coefficients (Table 3). This type of forward, progressive motion is especially relevant for external fertilization in aquatic habitats, and distance traveled has been shown to be important in human sperm fertilizing abilities (Hirano et al., 2001). Across individuals, total motility ($P = 0.0517$) ranged from 0% to 53.8% and progressive motility ($P = 0.0767$) ranged from 0 to 41%. The LSS at the SK reference site had the highest motility averages (Table 2). The LSS at CC, showing the lowest motilities, may have experienced depressed keto-testosterone production related to triclosan (a personal-care antimicrobial product with anti-androgenic activity resulting in depressed testosterone production in rat Leydig cells; Kumar et al., 2008) which occurred in considerably higher concentrations at CC than at the other sites (Nilsen et al., 2014-in this issue). Human sperm motility is especially vulnerable to PCBs (Rignell-Hydbom et al., 2005), and has been negatively correlated with environmental exposure to BDE-47, BDE-100, and ΣBDE (Abdelouahab et al., 2011).

Although studying andrology parameters from fish exposed throughout their lifetime is a comprehensive, integrating strategy for investigating the potential effects of EDC, direct exposures to spermatozoa are also relevant because fertilization occurs in the aquatic environment. For example, automated sperm morphology analysis detected the altered morphologies of goldfish (*Carassius auratus*) sperm after cell exposure to mercuric chloride (Van Look and Kime, 2003). Likewise, the CASA results of cadmium-exposed spermatozoa of African catfish (*Clarias gariepinus*) showed that progressive motility was subsequently reduced (Kime, 1996). Similar motility reductions were shown with exposures of nonylphenol to Japanese medaka spermatozoa (Hara et al., 2007) and synthetic pyrethroids to Sprague–Dawley rat spermatozoa (Song et al., 2008). A study involving four xenoestrogens that bind to steroid receptors on the sperm cell membranes of Atlantic croaker (*Micropogonias undulates*) resulted in decreased motility (Thomas et al., 1998). Motility is one of the most important parameters to consider in evaluating the fertilizing ability of sperm (Hirano et al. 2001), and CASA allows a precise quantification of sperm motility patterns (Betancourt et al., 2006).

As with most freshwater fish, the LSS spermatozoa are immotile in milt until activated after release. Motility depends on endogenous ATP to transport chemical energy, and reduced motility may be associated with mitochondrial damage (O'Connell et al., 2002). Carp (*Cyprinus carpio*) sperm motility quits when 50 to 80% of ATP is exhausted via hydrolysis (Billard et al., 1995). For LSS, MMP was positively correlated with PCB-206 ($P \leq 0.10$; Table 3), the congener most often found correlated with sperm quality and endocrine parameters (Tables 3 and 6). The MMP, ranging from 35.2% to 95.6%, was significantly lower at LV than at CC and SK ($P < 0.0001$) (Table 2; Fig. 3c). Likewise significantly lower ATP values were found at LV than at CC and SK ($P < 0.0008$) with SK values over 6 times higher (Table 2), with only pentachloroanisole correlated (negatively) (Table 3). Pentachloroanisole, the main degradation product of both pentacholorphenol and the herbicide/fungicide pentachloronitrobenzene, is a stable congener that bioaccumulates and transports atmospherically (Hoff et al., 1992). It was also positively correlated with VTG and T3 ($P < 0.05$; Table 6). Other pesticides — malathion, diazinon, atrazine, and fenoxaprop-ethyl — have been shown to directly affect bovine sperm motility at the level of the mitochondrial respiratory chain (Betancourt et al., 2006). Sperm motility and MMP were decreased after zebrafish were exposed long-term to BDE-209 (He et al., 2011). A reduction in sperm MMP and an increase in intracellular H_2O_2 were seen in adult male mice that had neonatal exposure to BDE-209, indicating a toxic mechanism of oxidative stress (Tseng et al., 2006).

Xenobiotics and biotransformation reactions play important roles in the mechanistic aspects of oxidative damage, of which DNA is a target (Rempel et al., 2009; Zharkov, 2013). Sperm DNA integrity is essential for the accurate transmission of genetic information, and sperm chromatin abnormalities or DNA damage may result in male infertility (Agarwal and Said, 2003; Jenkins, 2011b; Rempel et al., 2009). In this study, site differences were noted in DNA fragmentation ($P = 0.0016$), where LV > SK = CC (Table 2; Fig. 3d), indicating less intact sperm chromatin at the most contaminated site. In sperm, fertilization capability is reduced by the resultant single- and double-strand DNA breaks (Fraga et al., 1996; Jenkins, 2011b). Although the mean concentrations of liver ΣBDEs and ΣPCBs were generally not statistically different between SK and CC, some individual congener concentrations were higher at CC (Nilsen et al., 2014-in this issue).

The lowest amount of DNA fragmentation was found in LSS sperm from CC, the site where more nutrient resources may have been present, reflected by higher condition factors found there (Torres et al., 2014-in this issue). In fish, the concentration of ascorbic acid in the seminal plasma (milt) is regulated by dietary levels of vitamin C, and low levels have been associated with damage to germ cells and high percentages of lethal mutations in rainbow trout embryos (Ciereszko et al. 1999). Dietary intake of vitamin C is necessary for teleosts because they cannot synthesize it, and it is a critically important antioxidant in the male reproductive tract (Ciereszko et al., 1999). In humans, low ascorbic acid content in seminal fluid has been associated with increased oxidative damage to sperm

DNA (Fraga et al., 1996). Because LSS at LV had higher levels of PBDEs in their livers (Nilsen et al., 2014-in this issue), the fish likely harbored more oxidants, which can deplete their tissues and milt of antioxidants. Again, LSS at LV, which had the lowest condition factors (Torres et al., 2014-in this issue), showed significantly higher levels of sperm DNA fragmentation than fish from the other sites.

No significant differences in LSS sperm count were noted among sites for both 2009 and 2010 (Table 1; Fig. 3a). The bacterial contamination that inflated 2009 values (Table 1) was arrested by using streptomycin in the shipping buffer in 2010 (Appendix B). Similarly, sperm counts were not affected in mice and humans that were exposed to PBDE (Tseng et al., 2006; Abdelouahab et al., 2011). In our study with LSS, BDE-99 was positively correlated with sperm counts and with total motility (Table 3), with T4 being negatively correlated with sperm counts and positively correlated with total motility (Table 5). Sperm production is regulated by thyroid and sex hormones, and these results suggest that BDE-99 is disrupting thyroid homeostasis, perhaps by mimicking T4 function by virtue of its structural similarity (Yang et al., 2011).

Significant differences among sites were noted in percent viable, membrane-intact LSS sperm (Table 2; Fig. 3b), where SK = CC > LV ($P = 0.0020$), and the range over all individuals was 66.2%–98.3%. The assertion that the integrity of genomic DNA is also critical to cell survival (Zharkov, 2013) is supported by these results showing the lowest cell viability and the highest DNA fragmentation at the most contaminated site. Because sperm viability is indispensable for fertilization and males with low values have been intensively selected against (Malo et al., 2005), this parameter is typically not a sensitive biomarker for endocrine disruption studies therefore these results with LSS are definitive for differential xenobiotic influences among sites. The viability of human hepatoma cells was lower with time and increasing PBDE concentration, reactive oxygen species were generated, and apoptosis was induced when cells were cultured in the presence of BDE-209 (Hu et al., 2007). Membrane permeability may occur at the end of the apoptotic process if cells have not already been removed by phagocytes. Because phagocytes within LSS testes would display Sertoli-like cell function, future research might target this cell type.

Apoptosis is an evolutionarily conserved process of programmed cell death during which distinct biochemical and ultrastructural changes occur (Hikim and Swerdloff, 1999). Germ cell apoptosis during normal spermatogenesis plays an important role in sperm production (Anzar et al., 2002). Sperm apoptosis is regulated hormonally, therefore with gonadotropin and testosterone restriction in adult rats, apoptotic processes increased (Hikim and Swerdloff, 1999). Because the process of apoptosis is associated with morphological and biochemical changes, including chromatin aggregation, cytoplasmic condensation, nuclear fragmentation, and alternation of plasma membrane asymmetry, various flow cytometry methods can identify apoptotic features. In this study phosphatidylserine, normally present on the inner cytoplasmic leaflet of the plasma membrane of healthy cells, was identified as one of the early events in the apoptosis process. Kinetically, MMP disturbance occurs next, followed by chromatin fragmentation.

In this LCR study, significant differences were noted among sites in both percent live apoptotic ($P = 0.0032$) and percent total apoptotic ($P = 0.0027$) spermatozoa, with the highest values at LV (Table 2; Fig. 3b). Live, apoptotic cell populations is a sensitive sperm quality biomarker, and these apoptosis results indicated that not only are more germ cells undergoing programmed cell death at the most contaminated site, but the LSS spermatogenic process differs among sites as shown by the highest percentages of haploid testicular cells at the reference site ($P = 0.0275$; Table 2). Likewise, male medaka exposed to the estrogenic alkylphenol, 4-nonylphenol, showed a six-fold greater extent of apoptosis in histological sections of testes (Weber et al., 2002). Decreases in MMP may be considered a later marker for apoptosis, and the MMP results from this study parallel the apoptosis results in that lower quality was indicated at the most contaminated site (Table 2). The contaminants that correlated (positively) with apoptosis were pentachloroanisole ($\rho = 0.30553$) and the current-use pesticides ($\rho = 0.35043$; Table 3). Both live cell apoptosis and total apoptosis were correlated with VTG ($r = 0.56031$ and $r = 0.52479$, respectively) (Table 5).

Sperm morphology profiles are of better quality in non-apoptotic sperm fractions of humans (Aziz et al., 2007); this also was observed with LSS sperm abnormalities (Table 2). For LSS, significantly higher numbers of abnormal sperm forms were noted at LV ($P = 0.0032$), with no difference noted between SK and CC (LV > SK = CC; Table 2). The two most commonly observed abnormalities were macrocephalic and microcephalic heads, and the percentages of morphological abnormalities ranged from 7.4% to 30.4% over all individuals. Current-use and legacy pesticides, chlorpyrifos, $p'p'$-DDE and Σ DDT were all positively correlated with abnormal sperm morphology, yet PCB-206 was negatively correlated (Table 3). Reductions in the percentages of normal sperm morphology have been related to high sperm chromatin fragmentation and lower motility (Spano et al., 1999). Consistent with this study with LSS showing no correlation between any BDE congeners and abnormal sperm morphologies, BDE-99 and BDE-209 administered to rodents did not result in abnormal morphologies (Kuriyama et al., 2005; Tseng et al., 2006).

4. Conclusions

Male largescale suckers from three sites (Longview, Columbia City, and Skamania) along the lower Columbia River were evaluated for potential effects of exposure to contaminants in their aquatic environment. Sperm biomarker results that significantly differed among sites included sperm morphology, viability, mitochondrial membrane potential, live apoptotic cells, total apoptotic cells, ATP content, DNA fragmentation (NOMP), and percent haploid testicular cells. Sperm motilities showed similar trends among sites. Vitellogenin was also significantly different among sites, and was correlated with numerous sperm biomarkers. Several correlations were found between specific contaminant congeners and T4, T3, and VTG, as well as sperm motilities and other sperm biomarkers. Overall, these results indicated that sub-lethal effects of xenobiotics in the LCR are being mediated along several points in endocrine and reproductive axes in male largescale suckers, and the least affected fish were at the reference site Skamania. Because biomagnification of hydrophobic contaminants in mountain whitefish in the upper Columbia River can be an order of magnitude higher than that in LSS (Rayne et al., 2003), the reproductive significance to salmonids in the LCR could be even greater than what is suggested by the results of this study with largescale suckers.

Acknowledgments

Funding for this study was provided by USGS Regional and Rex-flex funds. The authors thank N. Trahan of Science Publishing Network, USGS for the map, and L. Johnson, NOAA Fisheries Service, Northwest Fisheries Science Center, Seattle, WA for technical information on local fish. M. Holder and T. Smoak are thanked for work on Appendix A. N. Denslow is thanked for the VTG analysis. D. McKenzie of the Dept. of Biological Sciences and S. Jaques of the Endocrine Diagnostic Laboratory of the Texas Veterinary Medical Diagnostic Laboratory at Texas A & M University are thanked for their help on thyroid hormone analysis. The Texas Cooperative Fish and Wildlife Research Unit are jointly supported by the U.S. Geological Survey, Texas Tech University, Texas Parks and Wildlife Department, U.S. Fish and Wildlife Service and The Wildlife Management Institute. E. Furlong (USGS) and two anonymous journal reviewers are thanked for critical reviews. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. government.

Appendix A. Conservation status of fish species in the lower Columbia River

Conservation status of fish species in the Lower Columbia River.

Common name	Scientific name	State ranking[a]		Abundance rankings by NatureServe[b]			ESA[c]	
		OR	WA	Global	OR	WA	OR	WA
Chinook salmon	*Oncorhynchus tshawytscha*	SC, 1	C	G5	S4	S3S4	T	E
Chum salmon	*O. keta*	SS, 1	C	G5	S3	S3	T	T
Coho salmon	*O. kisutch*	LE, 1	None	G4	S3	S3	T	T
Sockeye salmon	*O. nerka*	NL	C	G5	S4	S2S3	E	E
Steelhead	*O. mykiss*	SS, 1	C	G5	S5	S5	T	T
Cutthroat trout	*O. clarkii* ssp. Henshawi	LT, 2	NL	G4	S4	S4	NL	NL
Bull trout	*Salvelinus confluentus*	SS, 1	C	G4	S3	S3	T	T
Green sturgeon	*Acipenser medirostris*	None, 4	None	G3	S3	S2	T	T
Oregon chub	*Oregonichthys crameri*	LE, 1	NL	GU[d]	S2	NL	T	T
Umpqua chub	*Oregonichthys kalawatseti*	SS, 1	NL	G2G3	S2SD	NL	NL	NL
Pacific lamprey	*Entosphenus tridentatus*	SS, 4	M	G4	S3	S3S4	NL	NL
Eulachon	*Thaleichthys pacificus*	NL	C	G5	S4	S4	T	T
Western brook lamprey	*Lampetra richardsoni*	SS, none	NL	G4G5	S4	S3S4	NL	NL

[a] State status by alphabetical characters and Oregon Natural Heritage Information by; SC = sensitive species/critical category; SS = sensitive species; C = candidate, LE = endangered; LT = threatened; NL = not listed; M = monitored; 1 = threatened with extinction or presumed to be extinct throughout the entire range; 2 = imperiled because of rarity or because other factors demonstrably make it very vulnerable; 3 = rare, uncommon or threatened, but not immediately imperiled; 4 = not rare and apparently secure, but with cause for long-term concern; 5 = demonstrably widespread, abundant, and secure.

[b] NatureServe/National Heritage ranks. 5 = widespread, abundant, and secure; 4 = apparently secure; 3 = vulnerable; 2 = imperiled because of factors making it vulnerable to extinction or extirpation; 1 = critically imperiled; U = unrankable; NL = not listed.

[c] Listing under the federal U.S. Endangered Species Act. T = threatened; E = endangered; NL = not listed.

[d] Formerly G2, needs reassessment.

Appendix B. Preliminary experiments, validations, and antibiotics selection

Validation assays and antibiotics selection

Handling of tissues and cell preparation

In 2008, experiments were performed to determine whether shipped milt or gonads were better for obtaining sperm quality data. Milt osmolality was measured by using a vapor pressure osmometer (Wescor Corp., Logan, UT, USA). Milt collected directly into a hyperosmotic extender can counteract potential damage caused by unavoidable and variable urine contamination (Jenkins et al., 2011). Shipments were made using either HBSS at ~495 or ~320 mOsm/kg. Stain concentrations (1–5 μl) for flow cytometric assays were tested. Based on cessation of motility, heat inactivation for LSS spermatozoa (2×10^8 cells/ml) occurred at 68 °C for 10 min. Using both dead and live spermatozoa, viability and mitochondrial membrane potential (MMP) assays were then validated (using 318 mOsm/kg buffer) (Jenkins and Draugelis-Dale, 2006).

Antibiotics selection

Bacteria reduce motility and alter morphology of fish sperm (Jenkins and Tiersch, 1997). In 2009, the majority of LSS testes shipped overnight were so inundated with bacteria that sperm quality results, beyond counts, were unattainable, therefore bacteriostatic and/or bactericidal additives were a necessity. Bacterial rods, cocci-bacilli, and cocci were present, thus both Gram positive and -negative bacteria were likely present. Although antibiotics can control bacterial growth in animal semen used for artificial breeding and for gamete transfers, harmful cytotoxic effects can occur (Jenkins, 2011a). Non-cytotoxic antibiotics (Sigma) were tested for use in the 2010 field season by using koi carp (*Cyprinus carpio*) sperm. Spermatozoa with a starting motility of 100% were collected and equal aliquots added to 1 ml HBSS containing one of the following concentrations of antibiotics: penicillin (1477 units/mg) (Pen) at 100 and 200 μg/ml; streptomycin (720 units/mg) (Strep) at 100 and 200 μg/ml; penicillin/streptomycin (5000 units of penicillin and 5 mg streptomycin) (PS) at 1:25 and 1:50 dilutions; and an HBSS control. Samples were maintained at 4 °C in 3 ml tubes with loose caps for 6 days. MMP and DNA fragmentation (see below) were evaluated at days 2 and 6, and sperm motilities were assessed visually (Jenkins et al., 2011) at day 6.

Apoptosis

An apoptosis assay was established by using spermatozoa from koi carp because LSS were not expressing milt at that time. Apoptosis was induced by incubation at 37 °C for 24 h, from which sperm were subsampled from the incubation tube throughout the time period, stained with fluorescent Annexin V conjugate and counterstained with propidium iodide (PI) (Life Technologies, Grand Island, NY, USA) (Fig. B1c). Controls included known dead (heat-inactivated) and live cells maintained at 4 °C, as well as a panel of stains.

Shipping buffers

The range of LSS milt osmolalities found in preliminary experiments was 267 to 298 mOsm/kg. The motility of sperm shipped in 494 mOsm/kg HBSS ($n = 3$ fish) was 69% (SE 9.9), but in 318 mOsm/kg ($n = 4$ fish) it was 91.1% (SE 3.5). Likewise, viability and MMP were 90.7% (SE 0.03) and 90.8% (SE 0.03) respectively in the 494 mOsm/kg buffer, but 97.0% (SE 0.01) and 97.0% (SE 0.01) respectively in the 318 mOsm/kg buffer. Validation assays ($n = 3$ fish) for viability and MMP using shipped milt or sperm from shipped testes (Jenkins and Draugelis-Dale, 2006) generated R^2 values ranging from 0.9476 to 0.9929. Therefore the 320 mOsm/kg HBSS was selected for shipment and testes were chosen over milt because extracted milt from shipped testes yielded better sperm quality.

Statistics

To test for differences in day and treatment, and their interaction, analyses of variance (ANOVA) for motility, MMP, and DNA fragmentation was performed, followed by Tukey's studentized range test. A t-test was used to find differences within a treatment between days 2 and 6. All statistical analyses were performed using SAS (SAS Institute, 1999) with $\alpha = 0.05$.

Results

Analysis of arcsine (sqrt) transformed MMP data showed no significant differences among antibiotic treatments, but higher values were

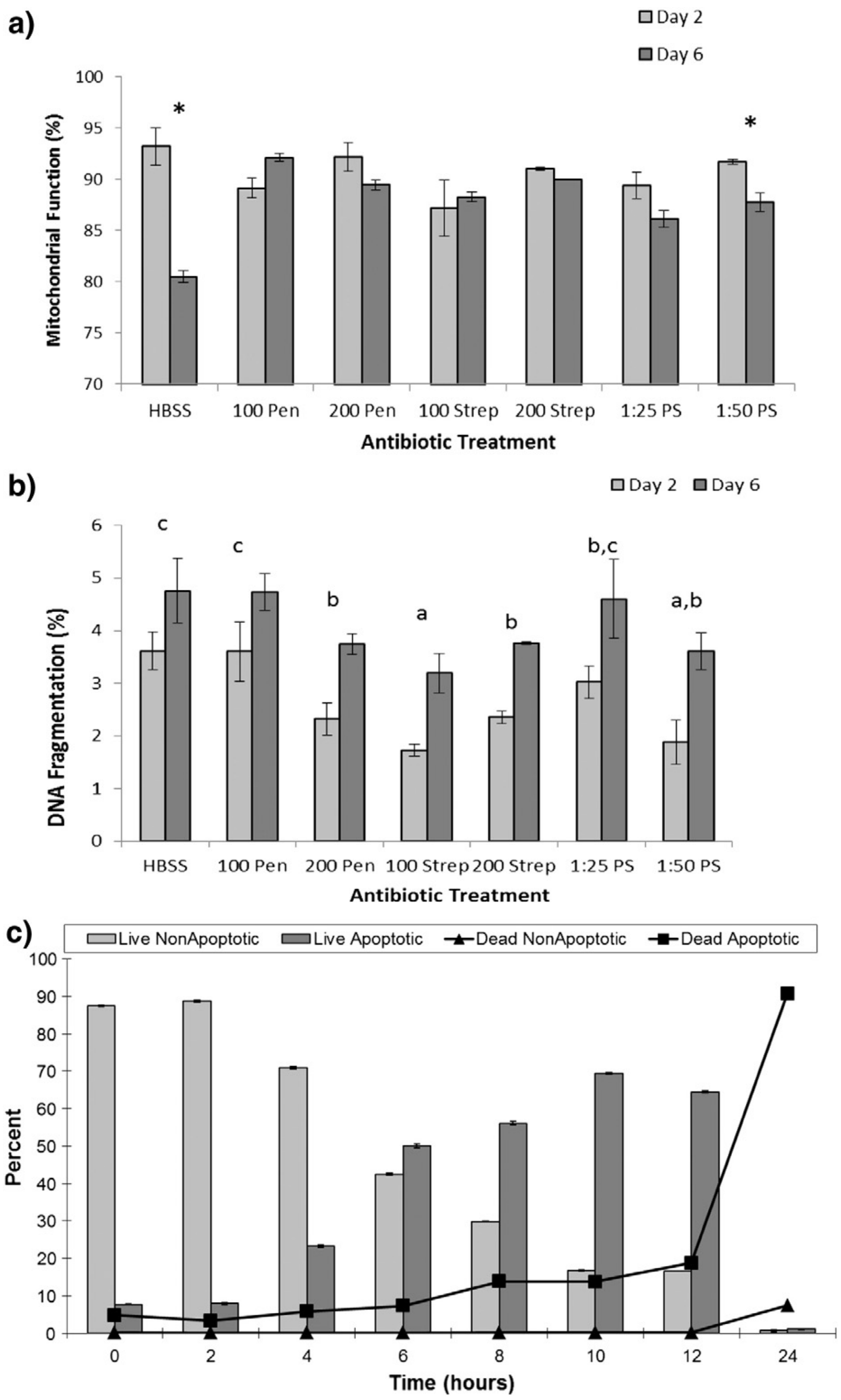


Fig. B1. (a and b) Mitochondrial membrane potential (MMP) and DNA fragmentation assays were performed with largescale sucker (*Catostomus macrocheilus*) spermatozoa stored in antibiotic treatments for 6 days to find the optimal shipping conditions. (c) An apoptosis assay for phosphatidylserine detection was established by heat-induction at 37 °C over 24 h using spermatozoa from koi carp (*Cyprinus carpio*). Data were collected using flow cytometry, and the results indicated that the optimum shipping buffer was Hanks' balanced salt solution containing 100 μg/l streptomycin. (a) Overall, the MMP was higher on day 2 ($P = 0.0251$) among all treatments; significant differences within treatments are shown (asterisks). No difference was noted among treatments. (b) DNA fragmentation (nuclei outside the main population; NOMP) was higher on day 6 than day 2 ($P < 0.0001$) among all treatments. NOMP was significantly different among treatments, indicated by superscripts ($P = 0.0014$). One superscript per pair of bars indicates that the letter was the same for both days in that treatment response. (c) Over the course of the apoptosis induction, the proportions of the four sperm subpopulations reflected a progression through the apoptotic process, so that by 24 h nearly all cells were dead and necrotic.

noted on day 2 ($P = 0.0251$). Within treatments, significant differences were noted between days with HBSS and 1:50 PS (Fig. B1a). Overall, DNA fragmentation was higher on day 6 ($P < 0.0001$) than on day 2, but treatments differed ($P = 0.0014$) with HBSS = 100 μg/ml Pen > 1:25 PS > 200 μg/ml Strep = 200 μg/ml Pen > 1:50 PS > 100 μg/ml Strep (Fig. B1b). No differences were noted in motility on day 6 among treatments, with values low and ranging from 0 to 10%. Overall, MMP among treatment groups was similar, but the DNA fragmentation results indicated that the least nuclear DNA damage occurred in the 100 μg/ml Strep treatment (Fig. B1b), which is effective against both Gram positive and negative bacteria. Thus, this antibiotic treatment was included in the shipping buffers for the 2010 field season.

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