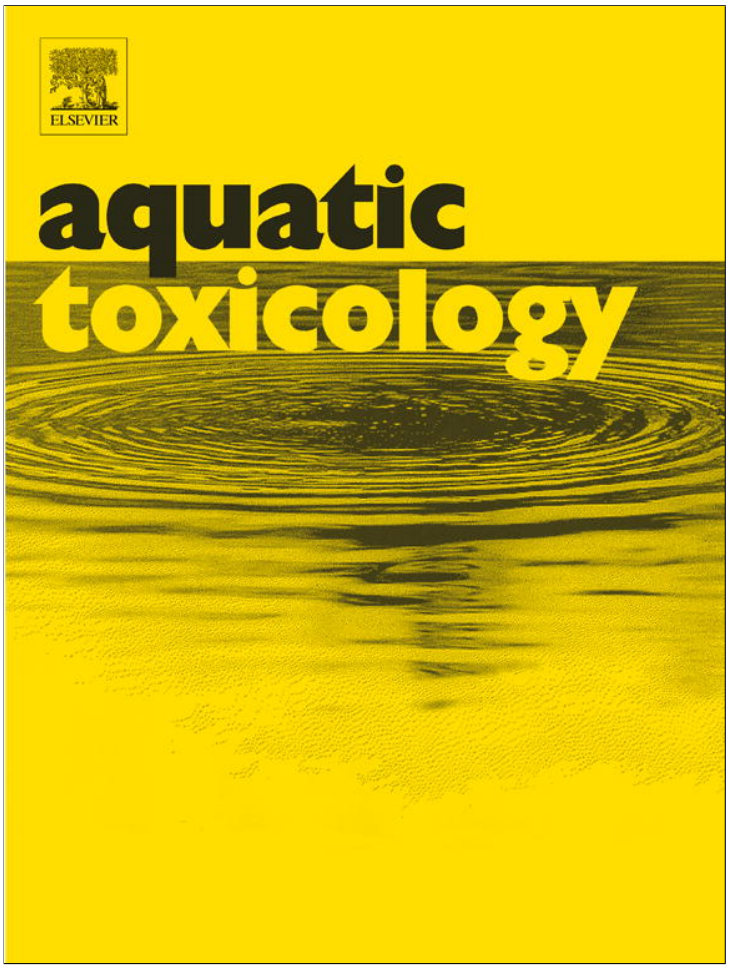


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# The effect of nonylphenol on gene expression in Atlantic salmon smolts

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ABSTRACT

The parr–smolt transformation in Atlantic salmon (*Salmo salar*) is a complex developmental process that culminates in the ability to migrate to and live in seawater. Exposure to environmental contaminants like nonylphenol can disrupt smolt development and may be a contributing factor in salmon population declines. We used GRASP 16K cDNA microarrays to investigate the effects of nonylphenol on gene expression in Atlantic salmon smolts. Nonylphenol exposure reduced gill $Na^+/K^+$-ATPase activity and plasma cortisol and triiodothyronine levels. Transcriptional responses were examined in gill, liver, olfactory rosettes, hypothalamus, and pituitary. Expression of 124 features was significantly altered in the liver of fish exposed to nonylphenol; little to no transcriptional effects were observed in other tissues. mRNA abundance of genes involved in protein biosynthesis, folding, modification, transport and catabolism; nucleosome assembly, cell cycle, cell differentiation, microtubule-based movement, electron transport, and response to stress increased in nonylphenol-treated fish. This study expands our understanding of the effect of nonylphenol on smolting and provides potential targets for development of biomarkers.

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## 1. Introduction

Historically, the North American range of Atlantic salmon (*Salmo salar*) extended southwards along the eastern United States to Long Island and the Housatonic River (Parrish et al., 1998). Now, all natural populations in the Long Island Sound and Central New England regions have been extirpated (Gephard and McMenemy, 2004). Exposure to environmental contaminants may be a contributing factor in these declines. Forest-spraying of pesticides that use nonylphenol as a surfactant are correlated with historical declines in adult return rates of Atlantic salmon in Canada (Fairchild et al., 1999), and its population level effects were hypothesized to be through lower survival of smolts after seawater entry. Nonylphenol has also been shown to inhibit smolt development and salinity tolerance and to delay smolt migration (Lerner et al., 2007; Madsen et al., 2004; McCormick et al., 2005).

Nonylphenol is a major degradation product of nonylphenol ethoxylate, which is a nonionic surfactant widely used in detergents, herbicides, pesticides, cosmetics, plastics, and spermicidal contraceptives. Wastewater treatment removes most nonylphenol ethoxylate and metabolites; however, nonylphenol is still commonly detected in rivers that receive industrial and municipal treatment effluents (Arukwe et al., 2000b; Naylor et al., 1992). Concentrations as high as 325–1000 μg/L have been detected in receiving waters (Liber et al., 1999). Nonylphenol bioaccumulates in organisms and has been detected in amphipods, algae, fish, and other organisms (Ahel et al., 1993; Hecht et al., 2004; Liber et al., 1999).

Both estradiol and nonylphenol inhibit smolt development; treated fish display reduced gill $Na^+/K^+$-ATPase activity and expression, reduced numbers of gill chloride cells, and reduced seawater tolerance (Madsen et al., 1997; McCormick et al., 2005). Nonylphenol is an estrogen mimic; it competes with natural estrogens for binding to the estrogen receptor and induces expression of proteins involved in female egg production (vitellogenin and zona radiata proteins) (Arukwe et al., 2000a; Jobling and Sumpter, 1993; White et al., 1994). However, nonylphenol also appears to regulate some genes independently of the estrogen receptor (Larkin et al., 2002, 2003; Watanabe et al., 2004). Nonylphenol has weak affinity for the progesterone receptor in rats (Laws et al., 2000), activates pregnane X receptor-mediated transcription in cos-7 cells (Masuyama et al., 2000), and is a weak androgen receptor agonist in a heterologous system using a human receptor (Sohoni and Sumpter, 1998). In salmon, both nonylphenol and estradiol affect plasma concentrations of insulin-like growth factor I and thyroid hormones; nonylphenol also affects growth hormone levels (McCormick et al., 2005).

Smolting is an incredibly complex process that involves changes in appearance, behavior, and physiology. While many of the mechanisms that underlie these alterations are not well understood, it is well established that changes in gene expression are involved

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in smolting. We used the GRASP 16K salmonid cDNA microarrays to identify genes whose expression was altered in Atlantic salmon smolts by exposure to nonylphenol. The expression of several genes identified by microarray analyses was verified using qRT-PCR. We then compared the results of this study with a companion study that identified genes differentially expressed in Atlantic salmon smolts compared to parr. We included a phenotypic examination of gill $Na^+/K^+$-ATPase activity, plasma thyroid hormones and cortisol, which have previously shown to be impacted by nonylphenol (McCormick et al., 2005), to verify the impacts of nonylphenol exposure.

## 2. Methods

### 2.1. Fish rearing and treatments

Atlantic salmon of Connecticut River stock obtained as parr from the White River National Fish Hatchery in fall 2005 were reared at the Conte Anadromous Fish Research Center in Turners Falls, MA. Fish were held in 1.5 m diameter fiberglass tanks receiving flow-through (4 L $min^{-1}$) Connecticut River water ($Ca^{2+}$, 9.0 mg $L^{-1}$; $Mg^{2+}$, 1.5 mg $L^{-1}$; $Na^+$, 6.8 mg $L^{-1}$; $K^+$, 1.1 mg $L^{-1}$; $Cl^-$, 11.0 mg $L^{-1}$, pH 6.8–7.8) and maintained under natural photoperiod conditions. Fish were reared under ambient river temperatures through the fall and then at 10 (±1) °C from February through the remainder of the study. Fish were fed to satiation twice daily with commercial feed (Zeigler Bros., Garners, PA, USA). In late January, fish were divided into two groups (parr and smolts) and reared in separate tanks. Fish smaller than 11 cm were considered parr and fish greater than 12 cm were considered smolts. Previous studies have established these size thresholds for smolt development in Atlantic salmon (McCormick et al., 2007).

For the nonylphenol exposure, ten smolts were injected with 150 μg/g body weight branched para-nonylphenol (CAS No. 84852-15-3) dissolved in vegetable oil and ten smolts were injected with vehicle control on day 0 and on day 4 as outlined in (McCormick et al., 2005); fish were sampled on day 7. Food was withheld throughout the treatment period, which occurred from April 17 to April 24.

On day 7, fish were euthanized in 200 mg $L^{-1}$ MS-222 neutralized to pH 7.0; fork length to the nearest millimeter and weight to the nearest 0.1 g were recorded. Blood was drawn from the caudal vein into 1 mL ammonium heparinized syringes and spun at 8000 × *g* for 5 min at 4 °C. Plasma was aliquoted and immediately stored at −80 °C for subsequent measurement of hormones. Four to six gill filaments were severed above the septum, placed in 100 μL of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and immediately frozen at −80 °C. The remaining gill tissue was trimmed from gill arches for expression analysis. The gill, liver, pituitary, hypothalamus and both olfactory rosettes were collected in individual tubes, frozen immediately on dry ice and stored at −80 °C for microarray analyses.

### 2.2. Physiological assays

$Na^+/K^+$-ATPase activity was determined as described in (McCormick, 1993). Briefly, gill tissue was homogenized in 150 μL of SEID (SEI buffer and 0.1% deoxycholic acid) and centrifuged at 5000 × *g* for 30 s. Two sets of duplicate 10 μL samples were run, one set containing assay mixture and the other containing assay mixture and 0.5 mM ouabain. The resulting ouabain-sensitive ATPase activity (expressed as μmoles ADP/mg protein/h) was quantified via a kinetic assay run in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min. Protein concentrations were determined using a BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, IL, USA). Both assays were run on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA).

Plasma cortisol levels were measured by a validated direct competitive enzyme immunoassay as described by Munro and Stabenfeldt (Munro and Stabenfeldt, 1984) and modified by Carey and McCormick (Carey and McCormick, 1998). Assay range as defined by the standard curve was 1–200 ng $mL^{-1}$. The lower detection limit was 0.3 ng $mL^{-1}$. Based on a pooled plasma sample that was included on each plate, the average intra-assay variation was 5.5% ($n$ = 10) and the average inter-assay variation was 8.8% ($n$ = 10). Thyroxine ($T_4$) and 3,5,3′-triiodo-L-thyronine ($T_3$) concentrations were measured by a direct radioimmunoassay described by Dickhoff et al. (1978) and modified by McCormick et al. (1995). The assay range as defined by the standard curve was 1–64 ng $mL^{-1}$ for $T_4$ and 0.5–16 ng $mL^{-1}$ for $T_3$. Intra- and inter-assay coefficients of variation for these assays were 4–11% and 3–5%, respectively.

### 2.3. RNA isolation

Total RNA was extracted from tissue samples frozen at −80 °C using RNeasy kits (Qiagen, Inc., Valencia, CA, USA) according to manufacturer instructions; all samples were treated with RNase-free DNase (Qiagen, Inc., Valencia, CA, USA) to remove genomic DNA. RNA quantity was measured with an ND-1000 UV–Vis Spectrophotometer (Nanodrop Technologies/Thermo-Fisher Scientific, Inc., Wilmington, DE, USA). Quality of the RNA was assessed using a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). One sample used in the microarray hybridizations had an RNA Integrity Number (RIN) (Schroeder et al., 2006) of 8.8; the other 79 samples used in these hybridizations had a RIN number greater than or equal to 9.0. RNA samples were stored at −80 °C.

### 2.4. Microarray hybridization

GRASP 16K cDNA microarrays spotted with 16,006 features were purchased from the Genomics Research on All Salmon consortium (GRASP, http://web.uvic.ca/grasp/). These microarrays have been validated for salmonid species (von Schalburg et al., 2005). Slide pre-processing, sample hybridization, and post-hybridization washing were performed as recommended by GRASP.

The pituitary is a very small organ and only a small amount of total RNA was extracted (1–3 μg total RNA) from each pituitary. Due to the small amount of total RNA extracted, pituitary RNA was amplified in a single round and then labeled using the Ambion Amino Allyl MessageAmpII aRNA amplification kit (Applied Biosystems/Life Technologies Corporation, Carlsbad, CA, USA) according to manufacturer's instructions. Hypothalamus, liver, gill, and olfactory rosette RNA samples were not amplified prior to labeling. These samples were reverse transcribed and labeled using the Superscript Plus Indirect cDNA Labeling System (Invitrogen/Life Technologies Corporation, Carlsbad, CA, USA). All samples were labeled with Alexa Fluor 555 or 647 dyes (Invitrogen/Life Technologies Corporation, Carlsbad, CA, USA). Qiaquick PCR Purification spin columns (Qiagen, Inc., Valencia, CA, USA) were used to clean the cDNA following first strand synthesis and following labeling with Alexa Fluor dyes.

Microarray slides from the same print batch were used for each tissue comparison. Ambion SlideHyb no. 2 (Applied Biosystems/Life Technologies Corporation, Carlsbad, CA, USA) was used for hybridization. The slides were incubated at 50 °C, shaking slowly, for 16–18 h. After hybridization, slides were washed, dried by centrifugation, and then immediately scanned at 10 μm resolution in a ScanArray Gx microarray scanner (Perkin Elmer, Waltham, MA, USA). Laser power and PMT voltage were adjusted using the Line Scan Protocol (ScanArray Microarray Analysis System version

**Table 1**
Primer sequences used for real-time qRT-PCR.

| Gene or best BLAST hit[a] | Accession | Forward primer | Reverse primer |
|---|---|---|---|
| Apolipoprotein A-IV[a,f] | CB505965 | GATCTGAAGGCCCAGCTTACTG | GGTTTGGGCCATTTAGTTGGT |
| Cathepsin D[a] | CB504794 | CGGAGCAACCCCACTCAT | GCATGGTGGGAATCTTATCACA |
| $EF1A_A$ | AF321836 | CCCCTCCAGGACGTTTACAAA[b] | CACACGGCCCACAGGTACA[b] |
| $EF1A_B$ | BG933853 | TGCCCCTCCAGGATGTCTAC[b] | CACGGCCCACAGGTACTG[b] |
| Endoplasmin[a,f] | CA059329 | CCCAACAGCCACCAATCAA | TTGGGCAAGAGCACAAACAG |
| EST: ssallna016029[e] | CA769178 | GGCTGTGCTAGGCTGGAGTT | CGGTTCTGATGGCAGCACTT |
| Ferritin heavy polypeptide 1–1 | NM_001139722[d] | GGGTGCCCCCCAGAAC | CTTCCCCAAAGTGTGTTTGTCA |
| Ferritin middle subunit | NM_001123658 | TGCACAAGATTGCCTCTGACA | AGGTAATGGGTCTCCAGGAAGTC |
| Ferritin lower subunit (fri3) | NM_001141424 | CCCGATGAGCAGCAGGTACT | CCACATACCTGATTGGCAGATG |
| High-affinity copper uptake protein 1[a] | CA042004 | TGAAGATCGGCAGGGAGTTC | TGGAGTTATAGCGCACGTTGA |
| IGF-I | NM_001123623 | CAGTTCACGGCGGTCACATA | GCTCGCAACTCTGGAAGCA |
| Polyubiquitin-C[a] | CA055128 | TCCTCTGCTCTTAACGATGTCTTATC | GGTCAATGGGTGGGATTGG |
| Proteasome subunit beta type 3[a] | BU965861 | TCCCAAGCCATGCTGAATG | AATGACACCCATGCCTGAAAC |
| 40S Ribosomal protein S20[c] | BG936672 | GCAGACCTTATCCGTGGAGCTA[b] | TGGTGATGCGCAGAGTCTTG[b] |
| Stress-associated endoplasmic reticulum protein 1[a,f] | CA044731 | GGATGGGCATGTAGGATGACA | TGGTTGGGCGGATACTGAGT |
| Tubulin alpha chain[a] | CK990304 | GGCTTCAAGGTGGGTATCAACT | GCCAGGTCTCCTCCAGGAA |
| Vitellogenin[a,f] | CB515390 | TCCCAGTGAGGACCACCTACA | CAGAGCGGTTCAGGTTGGA |
| Zona pellucida sperm-binding protein 3[a] | CK991165 | TGTCTGATCGATGCCAAAGTG | TGCGGAACGAGGCATGA |

[a] Best BLAST hit, see methods for BLAST parameters.
[b] Primers designed by Olsvik et al. (Olsvik P.A., Lie K.K., Jordal A.E., Nilsen T.O., Hordvik I., 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol Biol 6,21).
[c] Reference gene for qPCR.
[d] Ferritin heavy polypeptide 1–1 primers match both NM_001139722 (ferritin, heavy polypeptide 1–1) and NM_001123657 (ferritin, heavy polypeptide 1–2).
[e] No BLAST hit with *e*-value <1e−3.
[f] BLAST blastx query coverage less than 50%.

3.0, Perkin Elmer) such that the signal from the Alexa Fluor 555 dye was approximately equal to the signal from the Alexa Fluor 647 dye for each slide and the signal was saturated for approximately 0.3–0.5% of the features on the slide.

### 2.5. Array experimental design

Forty microarray slides were used to compare expression in nonylphenol-injected fish with expression in vehicle-injected fish: eight two-channel arrays each for liver, gill, olfactory rosettes, hypothalamus, and pituitary tissues. RNA was isolated from the tissues of eight nonylphenol-injected fish (six males and two females) and eight vehicle-injected fish (two males and six females) and reverse transcribed separately (samples were not pooled); each slide represented a biological replicate. For each tissue, the eight arrays were balanced for dye: nonylphenol-injected fish were labeled with Alexa Fluor 555 and vehicle-injected fish were labeled with Alexa Fluor 647 on four slides, nonylphenol-injected fish were labeled with Alexa Fluor 647 and vehicle-injected fish were labeled with Alexa Fluor 555 on four slides. Liver, gill, hypothalamus, pituitary, and olfactory rosette tissues were analyzed separately for each experiment, resulting in five distinct analyses.

### 2.6. Microarray analysis

Spot-finding was done with ScanArray Express Microarray Analysis System software version 4.0 using the adaptive circle method. Spot-finding images were manually reviewed and improperly identified spots were manually adjusted.

The TM4 Microarray Software Suite was used for pre-processing, filtering, normalization, and statistical analysis of the microarray data (Saeed et al., 2003). Files containing median spot intensities and median background intensities were imported into the TM4 Microarray Software using Express Converter version 2.1 utility; median background intensities were subtracted from median spot intensities. Converted intensity files were imported into TM4 Midas version 2.20 with RMA and spots where the background-corrected intensity was less than zero for either channel (dye) were removed. The $\log_2$ intensity ratios for each array were normalized using a local Lowess normalization (by subarray or block) and standard deviation regularization for the blocks. Lowess correction was done by block, or pin group, to correct for any differences between spotting pin or spatial differences across the slide. One-class Significance Analysis of Microarrays (SAM; TM4 MeV version 4.2) was used to identify genes with significantly different expression between conditions; the delta value was minimized such that the median false discovery rate (FDR) was less than 1%. Genes with significantly different expression between conditions (nonylphenol versus vehicle control) were also identified by *t*-test (TM4 MeV version 4.2). Three different criteria were used to identify potentially significant differentially expressed genes: $p$-value < 0.001, $p < 0.01$ and fold change $\geq 1.20$, $p < 0.01$ and fold change $\geq 2.0$. To investigate false discovery rate with *t*-test analyses, $q$-values were calculated using the QVALUE software written by Alan Dabney and John Storey (Storey and Tibshirani, 2003).

**Table 2**
Number of significantly differentially expressed features identified by *t*-test with $p < 0.001$ (*t*-test), by *t*-test with $p < 0.01$ and at least two-fold change in expression (*t*-test and 2×), and by SAM analysis (SAM) in nonylphenol-treated fish compared to control fish, by tissue.

| | *t*-Test, $p < 0.001$ | *t*-Test, $p < 0.01$ | | | SAM |
|---|---|---|---|---|---|
| | | Total | ≥1.20-Fold change | ≥2.00-Fold change | |
| Liver | 175 | 678 | 459 | 84 | 124 |
| Gill | 1 | 27 | 7 | 0 | 0 |
| Hypothalamus | 8 | 91 | 23 | 6 | 0 |
| Pituitary | 1 | 47 | 24 | 1 | 0 |
| Rosettes | 7 | 57 | 13 | 2 | 0 |

The translated sequences for features identified as differentially expressed in microarray analyses were compared to the SwissProt database using the blastx program (http://blast.ncbi.nlm.nih.gov). The name of the blast hit having the lowest *e*-value is given as the "best BLAST hit" for each feature (Tables 1, 3 and 4). Features that did not have a BLAST hit with *e*-value less than 1e−3 and features where the region of sequence similarity covered less than 50% of the translated feature sequence are indicated. Note that the best BLAST hit listed in these tables was not always the same as the annotation provided by the GRASP consortium.

### 2.7. Real-time PCR analysis

Real-time PCR was used to validate the expression of select genes identified in microarray analyses (Rajeevan et al., 2001). Gene expression was measured in twenty fish (ten fish exposed to nonylphenol, ten fish exposed to vehicle control), including the sixteen fish used for the microarray hybridizations. Total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Life Technologies Corporation, Carlsbad, CA, USA). Real-time qRT-PCR reactions were done in triplicate on an ABI7900HT using Power SYBR Green Master Mix (Applied Biosystems/Life Technologies Corporation, Carlsbad, CA, USA). Primers used in real-time PCR are listed in Table 1. Mean expression of target gene divided by mean expression of a reference gene was reported. Ribosomal protein S20 was used as a reference gene. Statistical significance of real-time PCR comparisons between control and nonylphenol-treated fish were examined by Mann–Whitney *U*-test.

## 3. Results

### 3.1. Physiological effects of nonylphenol exposure

Gill $Na^+/K^+$-ATPase activity was reduced by approximately 25% after one week exposure to 150 μg/g nonylphenol (Fig. 1, $p = 0.004$). Plasma cortisol was reduced by 60% in nonylphenol treated fish (Fig. 1, $p = 0.019$, Mann–Whitney *U*-test). Plasma $T_4$ was not significantly affected by nonylphenol treatment, whereas plasma $T_3$ was reduced by 20% (Fig. 1, $p = 0.03$).

### 3.2. Differentially expressed genes identified by microarray analyses

GRASP 16K microarrays were used to identify genes in the gill, liver, olfactory rosettes, hypothalamus, and pituitary that were differentially expressed between nonylphenol-treated fish and control fish. The microarray data discussed here have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE33715 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33715). The number of differentially expressed features identified by Significance Analysis of Microarrays (SAM) or by *t*-test at two significance levels ($p < 0.001$ and $p < 0.01$) for all five tissues is listed in Table 2. Table 3 lists the features identified by SAM.

By far the largest effect of nonylphenol on gene expression was seen in the liver (Table 2), with little to no effect seen in gills, olfactory rosettes, hypothalamus, or pituitary. 124 features were significantly different in the livers of nonylphenol treated fish compared to control fish by SAM, with a median false discovery rate less than 1%; all 124 features are more abundant in nonylphenol-treated fish than control fish.

These microarray data were also analyzed using *t*-tests; *q*-values were calculated for the entire dataset to investigate the false discovery rate (Storey and Tibshirani, 2003). Using a $p < 0.01$ significance level for the *t*-test, 678 features were significantly different

**Table 3**
Features significantly (SAM) upregulated in the liver of juvenile Atlantic salmon treated with nonylphenol compared to control fish.

| Best BLAST hit | Accession | Fold change |
|---|---|---|
| 28S ribosomal protein S17[c] | CB504359 | 3.8 |
| 40S Ribosomal protein S13 | CB491393 | 6.8 |
| 60S ribosomal protein L39[c] | CA038333 | 1.2 |
| ADP-ribosylation factor 4 | CB499782 | 1.6 |
| Apolipoprotein A-IV[c] | CB505965 | 2.1 |
| Apolipoprotein A-IV | CB493958 | 1.6 |
| Apolipoprotein C-I | CB491411 | 6.4 |
| ATP synthase lipid-binding protein, mitochondrial | CB493846 | 1.1 |
| Bone morphogenetic protein 4 | CA056395 | 1.3 |
| Calmodulin-A | CA050185 | 1.4 |
| Cytochrome c oxidase assembly factor 5[c] | CB486148 | 2.3 |
| Endoplasmin[c] | CA059329 | 1.5 |
| Endoplasmin[c] | CB492804 | 1.5 |
| EST: omykrblb001037[b] | CB493547 | 1.9 |
| EST: omykrbna502215[b] | CB496826 | 1.4 |
| EST: omykrbna502358[b] | CB497380 | 1.7 |
| EST: omykrbna503004[b] | CB497817 | 2.0 |
| EST: omykrbna503022[b] | CB497857 | 1.8 |
| EST: omykrbna503044[b] | CB497906 | 1.5 |
| EST: omykrbna504040[b] | CB494172 | 1.5 |
| EST: omykrbna509054[b] | CB492399 | 2.5 |
| EST: omykrtcl003081[b] | CB491721 | 7.6 |
| EST: ssallna005085[b] | CA038241 | 1.8 |
| EST: ssalmge504351[b] | CB505701 | 1.4 |
| EST: ssalmge505245[b] | CB503310 | 3.0 |
| EST: ssalnwh501337[b] | CB510155 | 1.6 |
| EST: ssalnwh505093[b] | CB509722 | 4.1 |
| EST: ssalnwh505328[b] | CB509927 | 1.7 |
| EST: ssalob004012[b] | CA036615 | 1.3 |
| EST: ssalob502029[b] | CB508626 | 1.6 |
| EST: ssalob504369[b] | CB508658 | 3.3 |
| EST: ssalplnb501118[b] | CA042416 | 2.7 |
| EST: ssalplnb501213[b] | CA042490 | 3.2 |
| EST: ssalrbg548321[b] | CB514261 | 2.0 |
| EST: ssalrga501057[b] | CA051359 | 1.1 |
| EST: ssalrga508035[b] | CA060454 | 1.8 |
| EST: ssalrga508120[b] | CA060809 | 1.6 |
| EST: ssalrga516287[b] | CA061210 | 3.4 |
| EST: ssalrgb503074[b] | CA054826 | 1.1 |
| EST: ssalrgb515125[b] | CA052877 | 2.5 |
| EST: ssalrgb536133[b] | CA769251 | 1.4 |
| EST: ssalrgb536181[b] | CA056441 | 1.4 |
| EST: ssalrgb536182[b] | CA056442 | 1.3 |
| EST: ssalrgb536204[b] | CA056568 | 1.4 |
| EST: ssalrgb536250[b] | CA056640 | 1.3 |
| EST: ssalrgb536253[b] | CA056644 | 2.0 |
| EST: ssalrgb536254[b] | CA056647 | 2.3 |
| EST: ssalrgb536273[b] | CA056691 | 1.6 |
| EST: ssalrgb536299[b] | CA056739 | 1.5 |
| EST: ssalrgb536317[b] | CA056938 | 1.3 |
| EST: ssalrgb536323[b] | CA056958 | 1.2 |
| EST: ssalrgb536348[b] | CA057016 | 1.3 |
| EST: ssalrgb551343[b] | CB512669 | 2.0 |
| EST: ssalrgb555204[b] | CB516919 | 1.8 |
| EST: ssalrgb555265[b] | CB517012 | 1.1 |
| EST: ssalshc502278[b] | CA040841 | 2.5 |
| EST: ssalshc503347[b] | CA040291 | 1.4 |
| EST: ssalsrkc002051[b] | CA050094 | 2.6 |
| Fatty acid-binding protein, heart | CB489347 | 2.8 |
| Ferritin, middle subunit | CB487639 | 8.1 |
| Ferritin, middle subunit | CB506201 | 6.9 |
| Ferritin, middle subunit | CB505282 | 6.3 |
| Ferritin, middle subunit | CB503780 | 5.6 |
| Ferritin, middle subunit | CN442541 | 5.3 |
| Ferritin, middle subunit[c] | CA042437 | 4.9 |
| Ferritin, middle subunit | CB502663 | 4.7 |
| Ferritin, middle subunit | CB498370 | 4.6 |
| Ferritin, middle subunit | CB507396 | 4.5 |
| Ferritin, middle subunit | CB497884 | 4.4 |
| Ferritin, middle subunit[c] | CA768211 | 4.3 |
| Ferritin, middle subunit | CK991031 | 4.2 |
| Ferritin, middle subunit | CB510731 | 3.7 |
| Ferritin, middle subunit[c] | CA037206[a] | 3.7 |
| Ferritin, middle subunit | CA045790 | 3.3 |

Table 3 (*Continued*)

| Best BLAST hit | Accession | Fold change |
|---|---|---|
| Ferritin, middle subunit | CB508292 | 2.4 |
| Ferritin, middle subunit | CA056696 | 2.3 |
| Ferritin, middle subunit[c] | CK990667 | 2.2 |
| Ferritin, middle subunit | CA056744 | 1.8 |
| Ferritin, middle subunit | CK990310 | 1.6 |
| Ferritin, middle subunit | CA052539 | 1.5 |
| Ferritin, middle subunit | CK990827 | 1.5 |
| Galectin-3[c] | CB514642 | 1.2 |
| Heat shock protein HSP 90-alpha 1 | CB498021 | 1.7 |
| Heat shock protein HSP 90-beta | CA039749 | 1.7 |
| High affinity copper uptake protein 1 | CA042004 | 2.5 |
| Keratinocyte-associated protein 2[c] | CB506143 | 1.3 |
| KIF1-binding protein homolog[c] | CB496780 | 5.4 |
| Mitotic-spindle organizing protein 2[c] | CB515170 | 1.3 |
| Nuclear receptor subfamily 0 group B member 2 | CA037720 | 2.2 |
| PCNA-associated factor | CB490001 | 1.7 |
| Polyubiquitin-B | CA055128 | 1.6 |
| Polyubiquitin-B | CB518072 | 1.1 |
| Proteasome subunit alpha type-1[c] | CB494281 | 1.8 |
| Proteasome subunit alpha type-2 | CB498208 | 2.2 |
| Proteasome subunit alpha type-3 | CB496456 | 2.6 |
| Proteasome subunit alpha type-4 | CB487801 | 3.2 |
| Proteasome subunit alpha type-6 | CA038117 | 2.1 |
| Proteasome subunit beta type-3 | CB493627 | 2.0 |
| Proteasome subunit beta type-3 | BU965861 | 1.4 |
| Protein FAM71B | CB516929 | 1.5 |
| Protein transport protein Sec61 subunit beta[c] | CA044589 | 1.5 |
| Protein transport protein Sec61 subunit gamma[c] | CB497886 | 1.2 |
| Small ubiquitin-related modifier 3[c] | CB490926 | 3.0 |
| Stathmin | CB486047 | 2.2 |
| Stress-associated endoplasmic reticulum protein 1[c] | CA044731 | 1.9 |
| Stress-associated endoplasmic reticulum protein 1[c] | CB512613 | 1.6 |
| Synapse differentiation-inducing gene protein 1-like[c] | CA056593 | 1.5 |
| T-complex protein 1 subunit gamma | CA056693 | 1.4 |
| Thioredoxin[c] | CA041451 | 1.5 |
| TSC22 domain family protein 3[c] | CB509522 | 2.1 |
| Tubulin alpha chain | CB494048 | 3.2 |
| Tubulin alpha chain | CB493545 | 3.0 |
| Tubulin alpha chain[c] | CB511276 | 3.0 |
| Tubulin alpha chain[c] | CB497308 | 2.5 |
| Tubulin alpha chain | CK990304 | 2.4 |
| Tubulin alpha chain[c] | CA047720 | 1.8 |
| Tubulin alpha chain[c] | CA046426 | 1.7 |
| Tubulin alpha chain | CA058890 | 1.6 |
| Tubulin alpha chain | CB511927 | 1.1 |
| Tubulin beta chain | CA056349 | 1.9 |
| Tubulin beta-1 chain[c] | CB511172 | 2.6 |
| Vitellogenin | CA051633 | 4.7 |
| Vitellogenin[c] | CB515390 | 3.7 |
| Zona pellucida sperm-binding protein 3 | CK991165 | 2.3 |
| 28S ribosomal protein S17[c] | CB504359 | 3.8 |
| 40S Ribosomal protein S13 | CB491393 | 6.8 |

[a] Accession number for feature identified in microarray analysis; see Table 1 for accession number of sequence used to design qPCR primers.
[b] No BLAST hit with *e*-value <1e−3.
[c] Query coverage less than 50%.

between nonylphenol-treated and control fish; the largest $q$-value in this set was 0.169. Using a $q$-value threshold of 0.05, 146 genes are significant. The overall proportion of true null hypotheses in this dataset, $\pi_0$, was 0.73.

### 3.3. Validation of microarray results by qRT-PCR

qRT-PCR was used to measure the mRNA abundance of ten genes identified by SAM as differentially expressed in the liver of nonylphenol-treated fish compared to control fish (Table 4). These ten genes include the two genes most frequently identified (ferritin middle subunit and tubulin alpha chain); the gene with the highest observed fold-change (ferritin); and a gene known to be affected by nonylphenol (vitellogenin). These genes were also selected to represent a range of fold change in expression, including several genes with observed fold changes above 2.0 (high affinity copper uptake protein 1 – 2.5×, zona pellucida sperm-binding protein 3 – 2.3, apolipoprotein A-IV – 2.1×) and several genes with observed fold changes below 2.0 (stress-associated endoplasmic reticulum protein 1 – 1.9×, polyubiquitin-B – 1.6×, endoplasmin – 1.5×, proteasome subunit beta type-3 – 1.4×). Eight of the ten genes were significantly differentially expressed between nonylphenol-treated fish and control fish, by qRT-PCR. The abundances of apolipoprotein A-IV and high-affinity copper uptake protein 1 were not significantly different between nonylphenol-treated fish and control fish, by qRT-PCR. Cathepsin D (Genbank accession number CB504794) barely missed the criteria used for *t*-test ($p = 0.001095$, 1.78-fold change) and was not identified by SAM; expression of cathepsin D was significantly different between nonylphenol-treated fish and control fish by qPCR.

Abundance of the ferritin middle subunit (annotated as ferritin heavy chain on the GRASP array) was significantly higher in the liver of nonylphenol-treated fish than in control fish by microarray analysis (22 features identified by SAM; 1.5-fold to 8.1-fold higher in nonylphenol-treated fish). The RNA abundance of all three ferritin subunits was measured by qRT-PCR. Expression of the ferritin middle subunit was 18.9-fold higher in nonylphenol-treated fish ($p < 0.0001$) and the ferritin heavy subunit was 1.7-fold higher in nonylphenol-treated fish ($p = 0.0007$), but the expression of the ferritin light subunit was decreased (fold change 0.36, $p = 0.0355$).

Insulin-like growth factor 1 (IGF-1) was decreased in the liver of nonylphenol-treated fish compared to control fish by microarray analysis (fold change = 0.60, *t*-test, $p < 0.01$). The decrease in IGF-I mRNA abundance in the liver of nonylphenol-treated fish was greater by qRT-PCR (fold change = 0.11, $p = 0.0002$).

Elongation factor genes $EF1A_A$ and $EF1A_B$, 40S ribosomal protein S20, and the EST: ssallna016029 were considered as possible reference genes. Expression of both $EF1A_A$ and $EF1A_B$ was increased slightly, but significantly, in the liver of nonylphenol-treated fish compared to control fish (data not shown). Expression of 40S ribosomal protein S20 and the EST: ssallna016029 was not significantly different between nonylphenol-treated fish and control fish. The 40S ribosomal protein S20 was used for normalization of qRT-PCR assays.

## 4. Discussion

### 4.1. The effect of nonylphenol on gene expression in Atlantic salmon smolts

We used the GRASP 16K microarray to identify transcriptional effects of nonylphenol in the gill, liver, olfactory rosettes, pituitary, and hypothalamus. The liver was the most responsive in terms of number of genes transcriptionally altered by exposure to nonylphenol. The effect of nonylphenol on transcription outside the liver was small. Genes involved in protein biosynthesis, protein folding, and protein transport; proteolysis, protein catabolism, and ubiquitin-dependent protein catabolism; nucleosome assembly, cell cycle, cell differentiation, microtubule-based movement (tubulin subunits), electron transport, and response to stress were increased in the liver of nonylphenol-treated fish. Expression of vitellogenin (Genbank accession numbers CA051633 and CB515390), a commonly used biomarker for exposure to estrogenic compounds, was increased in the liver of nonylphenol-treated fish, as was expression of zona pellucida sperm-binding protein 3 (Genbank accession numbers CK991165 and CB490454).

The largest fold-change in response to nonylphenol and the most commonly identified feature was the middle subunit of ferritin. In teleost fish, a heavy subunit and a middle subunit for ferritin

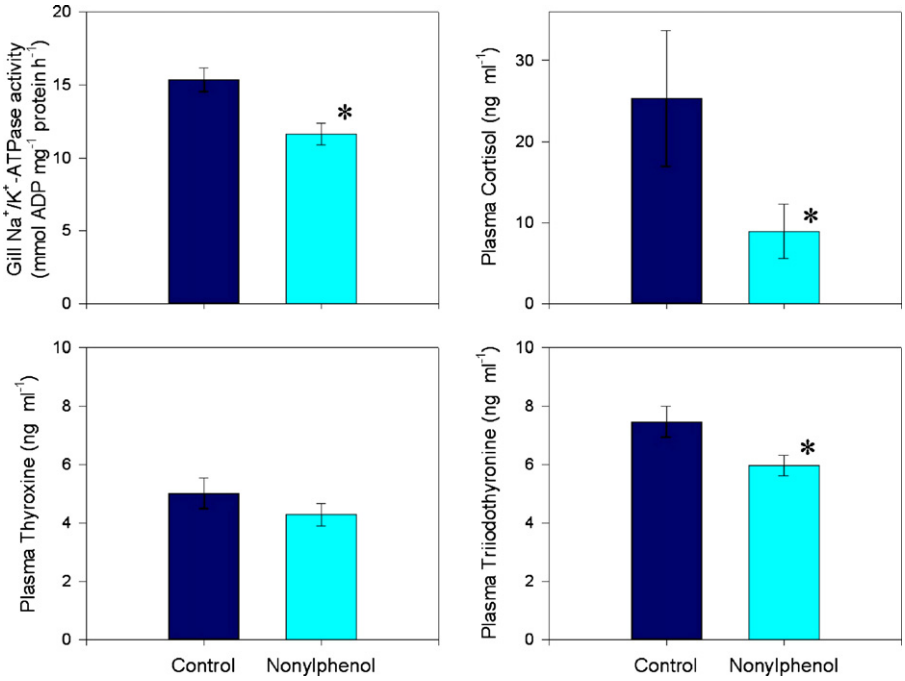


**Fig. 1.** Physiological and endocrine differences between Atlantic salmon smolts treated with vehicle control and nonylphenol (150 g/g) for one week, and sampled on day 7. Values are mean + standard error of 10–12 fish per group. Asterisk indicates a significant difference between parr and smolt ($p < 0.05$, $t$-test).

have been identified (Andersen et al., 1995; Liu et al., 2010; Zhang et al., 2010); a putative cDNA for ferritin lower subunit has also been identified (Leong et al., 2010). Nonylphenol exposure significantly increased expression of the ferritin middle subunit in Atlantic salmon smolts, as determined by our microarray analysis. Expression of both the ferritin heavy and middle subunits was increased by nonylphenol exposure, but expression of the ferritin lower subunit was decreased (qRT-PCR). Expression of the ferritin heavy subunit is not significantly different between parr and smolts (Robertson and McCormick, unpublished results). Ferritin is an iron storage protein that is involved in iron homeostasis and response to infection. Expression of the ferritin heavy subunit increases upon infection in channel catfish (Peatman et al., 2007) and in sea bass (Neves et al., 2009); this increase in ferritin expression may result in the sequestration of available iron, thus starving the pathogen of a required nutrient. The ferritin middle subunit from tongue sole displays antimicrobial activity (Wang et al., 2011). Ferritin also appears to be involved in reproductive development. The ferritin heavy chain is one of the genes most significantly upregulated by estradiol and by nonylphenol in male zebrafish liver (Ruggeri et al., 2008). Ferritin expression also increases in the ovaries of coho salmon during oogenesis (Luckenbach et al., 2008).

In this study, gill $Na^+/K^+$-ATPase activity was reduced by approximately 25% in nonylphenol-treated fish compared to control fish. The salmon gill $Na^+/K^+$-ATPase (NKA) enzyme consists of two subunits ($\alpha$ and $\beta$): the alpha subunit contains the catalytic sites and the beta subunit is involved in protein maturation and insertion into the membrane. Salmon have multiple isoforms for each subunit; two of the NKA-$\alpha$1 isoforms are differentially expressed during smolting and seawater transfer (Nilsen et al., 2007). The GRASP 16K array has six features representing the alpha subunit and four features representing the beta subunit. None of these features were identified as differentially expressed in nonylphenol-treated fish compared to control fish in any tissue.

Due to an unintended imbalance in the sex ratio between nonylphenol-treated fish (two females and eight males) and control fish (eight females and two males), we were unable to assess sex-specific changes in gene expression. Nonylphenol has been shown to increase expression of luteinizing hormone beta in the pituitary of females, but not males, and to increase the expression of the

**Table 4**
Expression of selected genes as measured by qRT-PCR in the livers of Atlantic salmon. Fold change is the mean normalized abundance in nonylphenol-treated fish divided by the mean normalized mRNA abundance in control fish; Mann–Whitney test $p$-value.

| Gene (or best BLAST hit[a]) | Accession | Fold change | $p$-value |
|---|---|---|---|
| Apolipoprotein A-IV[a] | CB505965 | 2.86 | 0.9698 |
| Endoplasmin[a,b] | CA059329 | 3.26 | 0.0029 |
| Ferritin middle subunit | NM_001123658 | 17.38 | <0.0001 |
| High-affinity copper uptake protein 1[a] | CA042004 | 3.97 | 0.2567 |
| Polyubiquitin | CA055128 | 2.88 | <0.0001 |
| Proteasome subunit beta type 3[a] | BU965861 | 4.26 | <0.0001 |
| Stress-associated endoplasmic reticulum protein 1[a] | CA044731 | 3.40 | <0.0001 |
| Tubulin alpha chain[a] | CK990304 | 10.12 | <0.0001 |
| Vitellogenin[a] | CB515390 | 16,796.54 | <0.0001 |
| Zona pellucida sperm-binding protein 3[a] | CK991165 | 31.11 | <0.0001 |
| Cathepsin D[a] | CB504794 | 3.76 | <0.0001 |

[a] Best BLAST hit, see methods for BLAST parameters.
[b] GenBank accession no. CB493461 identified in microarray analyses; GenBank accession no. CA059329 used to design primers for qPCR.

transcription factor Pit-1 in the pituitary of males, but not females (Yadetie and Male, 2002). However, previous results indicate that endocrine disruption of smolt development occurs similarly in both sexes (31).

### 4.2. Nonylphenol treatment affected expression of very few genes known to be regulated by smolting

Robertson and McCormick (unpublished results) used the GRASP 16K cDNA arrays to identify differentially expressed genes in Atlantic salmon smolts compared to parr in the liver, gill, hypothalamus, pituitary, and olfactory rosettes at a single timepoint. The microarray data for this parr–smolt comparison have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE33711 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33711). There was no overlap between the microarray features significantly (SAM) affected by nonylphenol (this study) and microarray features significantly affected in smolts compared to parr (Robertson and McCormick, unpublished results). However using a less-stringent analysis ($t$-test $p < 0.01$, filtered by fold-change threshold of 1.2-fold), ten features were identified as altered both by treatment with nonylphenol and also by smolting. Interestingly, all ten features changed in the opposite direction in nonylphenol-treated fish compared to vehicle than in smolts compared to parr, as was the case with IGF-I. The transcripts for seven genes were more abundant in the livers of smolts compared to parr, but less abundant in the livers of nonylphenol-treated smolts compared to control smolts: complement C3, glutathione S-transferase theta 1, pancreatic progenitor cell differentiation and proliferation factor B (PPDPFB), secreted phosphoprotein 24, and three sequences of unknown function (Genbank accession numbers CB511254, CB510516, and CA058127). The transcripts for three genes were less abundant in the livers of smolts compared to parr, but more abundant in the livers of nonylphenol-treated smolts compared to control smolts: glutathione S-transferase P, PCNA-associated factor, and one sequence of unknown function (Genbank accession number CA041709). The sequence CA041709 has no blastx hit with $e$-value <1e−3 in the non-redundant SwissProt database; however, a tblastx search finds several nucleotide sequences predicted to encode ependymin or ependymin-related proteins with similarity to the translated sequence for CA041709. In general, higher fold changes in expression were seen with the short exposure to nonylphenol compared to the fold changes seen at a single time point in the middle of smolting, a developmental process that takes several months.

Our analysis of changes in gill $Na^+/K^+$-ATPase activity and plasma hormone levels clearly indicated that the nonylphenol treatment we employed had a negative effect on smolt development, so we were surprised by the relatively small number of genes (particularly regulatory genes) related to smolt development that were also affected by nonylphenol. It should be noted, however, that relatively few genes involved in the endocrine control of smolt development were identified in our microarray approach (Robertson and McCormick, unpublished results). This may be due to the fact that changes in hormone gene transcription during smolting occur over a period of weeks and are relatively small in magnitude. In addition, substantial regulation may occur through non-transcriptional or through indirect pathways. Recent studies in coho salmon indicate neither pituitary transcription or circulating levels of thyroid stimulating hormone (TSH) are altered during smolt development (Larsen et al., 2011), indicating that increases in circulating levels of thyroid hormones during smolting are indirect, possibly through sensitivity to TSH. Our inability to detect substantial number of regulatory genes affected by nonylphenol may in part be due to the gradual nature and complexity of smolt development. In addition, only certain aspects of smolt development, such as osmoregulation and migration, may be strongly impacted by estrogenic compounds.

We were able to detect dramatic decreases in IGF-I mRNA abundance in response to nonylphenol treatment. This was predicted based on previous studies indicating that circulating levels of IGF-I decrease in response to nonylphenol (McCormick et al., 1995). The increase in salinity tolerance and gill $Na^+/K^+$-ATPase activity that occurs during smolting is in part supported by increases in plasma levels and local production of IGF-I (McCormick, 2001). Therefore, the decrease in gill $Na^+/K^+$-ATPase activity following nonylphenol exposure in the present study may be driven by decreased IGF-I transcription. Since cortisol is also involved in upregulation of gill $Na^+/K^+$-ATPase (McCormick, 2001), it seems likely that the observed decrease in cortisol is also involved.

The widely used surfactant nonylphenol is an endocrine disrupting compound that has been implicated in historical declines of Atlantic salmon. In addition, nonylphenol is known to inhibit smolting and delay smolt migration. Using a genome-wide approach, we investigated the effect of nonylphenol on gene expression in several tissues of Atlantic salmon smolts; the most dramatic effects were found in the liver. Through comparison with a companion study that identified genes that are differentially expressed in smolts compared to parr we found ten genes whose mRNA abundance was affected in the opposite direction by nonylphenol compared to smolt development, and these are of particular interest for further study.

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