

Supplemental Materials

Estrogenicity Analysis Methods and E2-EQ Results Table

Isolating and Sequencing of partial Johnny Darter Vtg3 cDNA

Dissected fish livers were preserved on dry ice and sent to the GLTED laboratory for vitellogenin mRNA (Vtg) analysis. Based on a search of NCBI [64] and UniProt [65] a vitellogenin protein or mRNA sequence was not available for Johnny Darters. Consequently, multiple sets of PCR primers were designed using a vitellogenin 3 (Vtg3) gene sequence available for a closely related species, the Orangethroat Darter *Etheostoma spectabile* [66]. Primers were developed based on mRNA sequence XM_032526230.1, with exon boundaries identified. Primers were designed using the Integrated DNA Technologies RealTime PCR tool with the maximum amplicon size set to 500 bp, minimum to 250 bp, and optimum at 450 bp. Forward and reverse primers from different sets were then selected to produce primer pairs with an amplicon length of approximately 1000 pb. A total of five primer pairs, spanning exon boundaries were designed (Table S1; List S1).

Table S1. Primers designed for Orangethroat Darters, Vtg3.

Primer ID	Start position	Tm (°C)	Amplicon Length	Sequence 5'→3'
Fp1116_otd_vtg_15-9_fw1	20	61.7	1160	GCTGCCTCGTTGGTCTAG
Fp1117_otd_vtg_15-9_rv1	1179	61.7	1160	ATTAGGAGTCACATCACCAGC
Fp1118_otd_vtg_9-3_fw1	693	61.7	966	TCTCATTACGAAGGCTCATGG
Fp1119_otd_vtg_9-3_rv1	1658	62	966	ATCATGCGAATCTCAGTGGG
Fp1120_otd_vtg_7-10_fw1	1304	61.9	1072	TGGTGCTTTTCGTATGGCTC
Fp1121_otd_vtg_7-10_rv1	2375	62	1072	TGGAAGTAGCGAACCTCAAAG
Fp1122_otd_vtg_10-1_fw1	2103	62	1122	TCTTAGCGCCAGTGACTTTC
Fp1123_otd_vtg_10-1_rv1	3224	62.2	1122	TCATAAAGGTCGCTGTGATGG
Fp1124_otd_vtg_1-17_fw1	2933	61.6	1064	GTGTGGAGTCTGAGTTAAGGAG
Fp1125_otd_vtg_1-17_rv1	3996	62.1	1064	GTGAGATTTTGTTGCAGCG

List S1. XM_032526230.1 Orangethroat Darter vitellogenin 3 sequence with primer pairs

- “nnnnn” indicates intron

- otd_vtg_15-9
- otd_vtg_9-3
- otd_vtg_7-10
- otd_vtg_10-1
- otd_vtg_1-17

ATGCGAGGGCTCCTCCTATGCTGCCTCGTTGGTCTAGCCAnnnnn

CGAGTCAAAGTGTCAAGTTATGnnnnn

AGCCCCACCTGATGCCGAATAAACCTATGAGTATAAATATGAGGGAGAGGTCAATTTTGGACT
CGGCAT

GCTAAACCTTGCTGAGTCTGGTGCAAGAATCACGTGCAAGATCAAGATCAGTGGGGTGTCTGGCG
CAAACA

TTCATCCTTCAGnnnnn

GTTTCAGATTTGGCCTTCAAGGAGTTCAATGGCTTCCAGGGGAAAGACAGCTTTATTGCCTCCCC
AAAGC

TCACCCAGCGTATTGCTTCCCAGCTTGTCAAACCTTTCATGTTTGACTACACTGGTGGACACGTTG
GTAA

CATCCGAGCCTCTGCTGAGATTTCTGACACTATTGTCAACATTGTGAGAGGGATTCTGGGATTGTT
CCAA

GTCACTGTGAAGACCACACAGACGATCTATGAGCTTGAAGAGnnnnn

GCTGGCATCCATGGCATTGTCCGAGTAACTATGCTACTGAAGTAAACATGGAGACAAAGGACA
TGACCA

TCACTCAGGTTGTGGATATTGGTAACTGCAGGGAGAAAGTAGCCATGTACAAGGAATGGCTAC
CGCTGT

GTTTGACAAAGTCTCCAAACAGnnnnn

AGGGGTGAATCTGTCATTTC AACAGTGAGATATATTTACACAGTCAAGCCAACAGCAGAGGGAG
GTCTCA

TTACGAAGGCTCATGGCCTGGAGCAACAGCACTTCAGTCCCTTCAACGTGAAGGGCGGCAGTTTC
CAGAT

GCTAGCGATnnnnn

GAAGGAAATGGTGCTGCTCGGAGTGAGTGACACACCGAGAGCCGTCGTGTCTGAGCCAATGGAA
AGCAAG

GGCAACCTTGTTTACCATTTTGTCAATGCAGATGCTAATTTACCTATTTTGATGCAGAGACTGGAC
AACC

CAGTACCAAAGnnnnn

GCTATTGAGTTGATCAAGCGTCTGGCAGAGGTTAATCGTTACAAGATTGACAGTGCAACAACAG
AGGACA

CGATAAAGCTGTATCAACTCCTACGAGCAATTCCCTATGAAAAATTAGAAGTTATGTGGAAGCA
ATTTGC

AGCAAATGATGAGCAAAGnnnnn

ACGTTGGTTTTTGGACATGATTGTTGAGGTAAACGATGCCAGAATCCTGAAGTTCTTCCAAATGA
GGTC

CAGGCTGGTGATGTGACTCCTAATGAAGGTCTGCAAGCCATTTTGCTGGTGATGAACCATCTCCA
GCCTA

TTCCTGATCTGGTTGAGATGGCTAAAnnnnn

ATGTTTTTAAGCATGCCCTTTTGTAATCCAATATCTATCTTTGGCATGCTGTGGTGCTTTCGTATG
GCT

CTCTGGTGTACAAGCACTGCACCTATTATACACCTTGTCTGTAGCTGTTGTTCAAnnnnn

CCACTACTGAACATGGCTGTGGAAAGTCACAGGAATGGCTCCACGGCAGACATGGTCATCGCAC
TGAAAG

CTCTGGGGAATGCAGGTCATCCAGGCAGCATTAAAACCATCATGCGCTTCCTCCCCGGAGTTGGT
GCCAG

CCCTGTGAATCTGCCACCTCGTGTGCTGAGTGCCGCTGTGCAGTCTATGAGACTTATAGCTGCCAG
AGAC

CCTCACAGTnnnnn

GTCCAAGACATCACCATGAGTCTGTTCTGCAAAAGAACCTTCCCACTGAGATTCGCATGATGGC
CTTCA

TGATATTGTTTGACACTCAGCCATCAATGGCTCTGATTTCCACAGTGACTGTACATCTACAGGAA
GAAAA

AGACCTCCATGTTGTTAGCTTTGCATACTCCTACTTTAAAAGTGTCGCCAGATCCAGAACTCCAA
ACAAC

CACTTCCTnnnnn

CTCAACTGCCTGCAACGTTGCCATAAAAATCCTGGCCCCTAAATTTGGCCGTCTCAGCTATCATTACAGC

AAAGCAACACGCATGGACTGGTTTAATGnnnnn

ATGATTACCTAATTGGTACAGCAGCAGAAGTCTTCATGCTGCGAAATGCAACAAACATCTTTCCC
ACTGA

AATCATGATGAAAGGAAAATTTTATTTCAATTGGAAGCATTCTGCAGCTATTGGAGnnnnn

TTGGGTATCCATGCTGAGGGGCTTAAGGAACTGCTTGGCGCTGGCATCCCTAGTTTTAAAGGAGATCTTA

GCGCCAGTGACTTTCAGGCTGTTCTCAATGTGnnnnn

CTTAAAACTGGGAAGTTCTGCCCAATGATAAGCCTGTCCTCTCTGCCTATACACGGGCCTTTGG
ACAAG

AGTGGTTCTTTGCTGATATTACCAAAGACACCATTCGGAACATGTTTCAGGnnnnn

GCTTTCAGCCCTACAGGGAAAGAGAGCCCTCTGTGGTCTGCGATTGGGAATTTACAGAAGGGAG
TGTCAT

GGCATTGGACAAAGCCTTACTTGATCTTTGAGGTTGCTACTTCCAAGCTACTACCCTAGGCCTCC
CACT

GGAGATAAGCAAATATTATCAATCCATCAATGGGATCACTGTAAATGnnnnn

CCAAGGCTGCAGTAAATCCAACACTGACTGATCGTCTTGGACAACCTGCTGACTTCTGACATTTCA
CTGGA

GACTGATGGTTTTATTGGnnnnn

TTACACTAAGGATTTTTGGGTTTTCTATGGGATCAACACAGAGCTTTTCCAGTGTGGTTCTGAGTTT
AAG

AGCAAAACACCTATTTCCATCCCTTGGAATTTTGCTGCCAAGATCAATGTCGGAGAGAGGAAGTT
TGAAC

TCGACTTCCCTCCATGCAAAAAAGAGTTTGAACTTTTTTCAGTCAGnnnnn

CTCCAATGTGTACGCAGTCTCCAGGAACCTTGAAGAACCAGCTTTGACCAAAATGACTCCAATTA
TGGCC

ACCACTACTGACTCCAATGATGAAGCTGTCCGCAAGGGGCCAACAGCTGCGGGGCCTGAGTCAG
ATCAGnnnnn

ATACTGACACCAAACGTCTGGCATCCAAGATCCAAGATGTGTGCTGAGAGTAAGATTTATGGAG
CTGGTC

TGTCTGTGGAGTCTGAGTTAAGGAGAGAGTATTATCATGAGGAATACCCCCTTTACTATTTCCTGG
GATA

TACCAACATGGCATTGAAAGTAGTCCCAGnnnnn

TTCAGGCAATCAAAGCTGTTGACAAAATCCACTTTGAGGTTAATGCCGGCCCGAGCAAACATCC
AGTCAG

CACACTGCAACTGCTAAAGACTCTGAGGAGGCTTTCCAAGnnnnn

GAAGCCACCCAGGGAGTACGTCTGTCCTCTGATTCAGCCTCAAGCGACAGCGGATCTCATCACA
GCCATC

ACAGCGACCTTTATGAAnnnnn

AGCTTGAACCTAACACCTGAAGCTATGTTGAATATTAAAGCCTTGGCCATGAGTGGCAACCAGA
AGCCAG

AGGGTTACGATGCAGCCTTCTTCCACACAGCCGAGGCAAACATTCAGAATGCCCAGATGATTGT
GTCCCA

GGTCGGGGAAGACACCAATTGGAAGATGTGCGTTGACACCAATGTGAATGCCCATGTTGAGGCA
AAGnnnnn

GCACACATTGGATGGGGAGCTGAATGTCAGTCCTATGAAATGTCAGTGGAATCTGCAACTGCCC
ATCAGC

CTGGCTCCAAGCCAACACTCAAGGTCACAGTACACTGGACCAAGATACCAGGGACCATCGCAG
AGTTGGG

CACAAGnnnnn

AATTGAAAGCTACATTCCAGGCGTGGCTTTTTTTCTTGGCTTCTACGAGACACATGAGAGAAATG
CCAAG

CAGGAGATTTCTGCAACAGTAGCTGCCGCCTCAGCAGACAGTGTGCGATGTGAAGATTAAATTCCC
AGAGnnnnn

CTTACAGTCTACCGCCAGGCTTTTCCAGTTTCACTGCTGACTGGCAGTTTTTCAGGAGTTTCAACAC
TACA

GAAACACAACAATCGGCAGCTTTGGACGCGCATAAAAACATnnnnn

TGGATTGTGTTGACTGATTTCCATGCAAAACAGATGAGCAAAAACAAGCAAAGAGCTGTGCCAG
TTTTGC

TTGCTTTTTTAAATATGTTTTGTGTTGAAAGACAACAAAGCCCAGATGGGATCCAGATGTGGCTTT
TCAC

AGTGATAA **TGCTGCAACCAAAATCTCAC** CACTGTGATGAAAGTGAGGATCATAAAAGTTGCATC
AGTAAT

CCATCAAATGTCACCCTTTTGAAAATAACCTGTAAAGGTCCAGAATTATTCATCATAAAGTGTGT
TAATA

TTTGAACACATCTTTTGATGAAATATTGTGTAAAGTCATGCTTTTGTCCCAAAATATTGTATCATC
ATAA

TGCATGCATTCATCAAAATATGTTTCAACAATCTGTGTTTTTGCTCCAAACCTGTTAATATAAATC
CTTT

GCTAATCCAAA

Total RNA was extracted from Johnny Darter liver samples (n=12 females; n=77 males) using Qiagen RNEasy Mini RNA purification kits (Qiagen 74106). RNA quality and quantity was evaluated using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Complementary DNA was prepared from n=8 female and n=2 male Johnny Darter liver RNA samples using a High-Capacity cDNA reverse transcription kit (Applied Biosystems 4368812). Complementary DNA from six females was pooled for use in an initial set of PCR reactions. Touchdown PCR was conducted using pooled female liver Johnny Darter cDNA as a template and the five Orangethroat Darter primer pairs (Table S1) with Jumpstart Taq Polymerase (Sigma D9307). PCR products were visualized on a 3% agarose gel and amplicon sizes were verified against a 100 bp DNA ladder (Sigma D3687). Bands of the expected approximate amplicon size were obtained for all five primer sets. Bands were excised and DNA was extracted from the bands using a Nucleotrap kit (Machery-Nagel, Düren, Germany; Ref 750584). Extracted Johnny Darter PCR products were then sent to the University of Minnesota for Sanger Sequencing. Many of the partial sequences received did not have sufficient data to use. However, sequences from segments produced by the first two primer pairs (otd_Vtg_15-9 and otd_Vtg_9-3; List S1) contained acceptable base pair data. These sequences were aligned and assembled based on overlap into a contiguous 1108 bp segment. This segment then had 30 bp trimmed off each end to produce a final 1048 portion of the putative Johnny Darter vitellogenin 3 sequences (List S2). A BLASTx search was performed using the partial Johnny Darter Vtg3 sequence to confirm orthology with Vtg3 of other related fish species (List S2). Johnny Darter specific primers (Table S2) were designed using the partial Johnny Darter Vtg3 sequence (List S2) using the Integrated DNA Technologies RealTime PCR tool.

List S2. Aligned partial Johnny Darter vitellogenin 3 sequence

Aligned sequence: 1048 bp

ATGAGGGAGTGGTCAATCTTTGGACTCGGCATGCTAAACCTCGCTGAGTCTGGTGCAACAATCAC
GTGCAAGATCAAGATCAGTGGGTTGTCGACACAAACATTCATCCTTCAGGTTTCAGATTTTGCCTT
CAAGGAGTTCAATGGCTTCCAGGGGAAAGACAGCTTTATTGCCTCCCCAAAGCTCACCCAGCGT
ATTGCTTCCCAGCTTGTCAAACCTTTCATGTTTGACTACATTGGTGGACACGTTGGTAACATCCGA
GCTTCTGCTGAGATTTCTGACACTATTGTCAACATTGTGAGAGGGATTCTGGGATTGTTCCAAGTC
ACTGTGAAGACCACACAGAAGATCTATGAGCTTGAAGAGGCCGGCATCCATGGCATTGTGCCAA
GTA ACTATGCTACTGAAGTAAACATGGAACAAAGGACATGACCATCACTCAGGTTGTAGATGT
CAGTAACTGCAGGGAGAAAGTAGCCATGTACAAGGGAATGGCTACCGCTGTGTTTGACCAAGTC
TCCAAACAGAGAGGTGAATCTGTCAATTTCACAGTGAGATATATTTACACAGTCAAGCCAACAG
CAGAGGGAGGTCTCATTACGAAGGCTCATGGCCTGGAGCAACAGCACTTCAGTCCCTTCAACGT

GAAGGGCGGCAGTTTCCAGATGCTAGCGATGAAGGAAATGGTGCTGCTCGGAGTGAGTGACACA
CAGAGAGCAGTCGTGTCTGGGCCAATGGAAAGCAAAGGCAACCTTGTTTACCATTTTGTCAATGC
AGATGCTAATGTACCGATTGTGATGCAGAGAATGGACAACCCAGTACCAAAGGCTATTGAGTTG
TTCAAGCATCTGGCAGAGGTTAATCGTTACAAGATTGACAGTGCAACAACAGAGGACACGATAA
AGCTGTATCAACTCCTGCGAGCATTTCCTATGAAGAACTAGAAGTTATGTGGAAGCAATTTGCA
GCAAATGATGAGCAAAGACGTTGGTTTTAGACATGATTGTTGAGGTAAACGATGCCAGAATCCT
GAAGTTCTTCAAAA

Johnny Darters' male liver Vtg qPCR

Male liver total RNA samples were diluted to 10 ng/μl for use in real-time quantitative PCR reactions. A Johnny Darter specific qPCR amplicon from the PCR reaction with Fp1116_otd_vtg_15-9_fw1 and Fp1117_otd_vtg_15-9_rv1 (Table S1) was used as a vtg3 standard. The amplicon was diluted 10⁶-fold for use as a standard in the qPCR analyses (seven additional dilutions in a 10-fold series). For qPCR, each 20 μl reaction contained 20 ng total RNA template, 2 μl of appropriate dilutions of vtg3 cDNA standard, or 2 μl RNase-free water (no template control) combined with 215 nM of gene-specific forward and reverse primers (Table S2) and Power SYBR Green RNA-to-CT 1-step master mix (Applied Biosystems 4389986). Samples were incubated at 48°C for 30 min and 95°C for 10 min. 40 cycles of qPCR amplification were then performed at 95°C for 15 sec, 60°C for 60 sec, and 95°C for 15 sec. After amplification, a melting curve was generated to evaluate specificity. All 77 samples were analyzed on a single 96 well plate, and the analyses were performed in duplicate (two separate plates). Amplification efficiencies in the two analyses were 110 and 132%. Data from each analysis were normalized to the mean across all samples tested on each plate and then averaged to generate the final relative number of copies.

Table S2. Johnny Darter specific qPCR primers designed based on partial Vtg3 sequence (List S3).

Primer ID	Start position	Tm (°C)	Amplicon Length	Sequence 5'→3'
fp1184_FHM_darterqPCR2_FW	138	59.96	73	GTTCAATGGCTTCCAGGGGA
fp1185_FHM_darterqPCR2_RV	210	60.04	73	AAGCTGGGAAGCAATACGCT

List S3. The initial partial Johnny Darter vitellogenin 3 sequence received from Sanger Sequencing

EPA2252_D01_009.seq: 809 bp (produced from otd_Vtg_15-9)

CCTGCTCCGATAAAACCTATGAGTACAAATATGAGGGAGTGGTCAATCTTTGGACTCGGCATGCT
AAACCTCGCTGAGTCTGGTGCAACAATCACGTGCAAGATCAAGATCAGTGGGTTGTCGACACAA
ACATTCATCCTTCAGGTTTCAGATTTTGCCTTCAAGGAGTTCAATGGCTTCCAGGGGAAAGACAG
CTTTATTGCCTCCCCAAAGCTCACCCAGCGTATTGCTTCCCAGCTTGTCAAACCTTTCATGTTTGAC
TACATTGGTGGACACGTTGGTAACATCCGAGCTTCTGCTGAGATTTCTGACACTATTGTCAACATT
GTGAGAGGGATTCTGGGATTGTTCCAAGTCACTGTGAAGACCACACAGAAGATCTATGAGCTTG
AAGAGGCCGGCATCCATGGCATTGTGTTCCAAGTAAGTATGCTACTGAAGTAAACATGGAAACAAA
GGACATGACCATCACTCAGGTTGTAGATGTCAGTAACTGCAGGGAGAAAGTAGCCATGTACAAG
GGAATGGCTACCGCTGTGTTTGACCAAGTCTCCAAACAGAGAGGTGAATCTGTCAATTCAACAGT
GAGATATATTTACACAGTCAAGCCAACAGCAGAGGGAGGTCTCATTACGAAGGCTCATGGCCTG

GAGCAACAGCACTTCAGTCCCTTCAACGTGAAGGGCGGCAGTTTCCAGATGCTAGCGATGAAGG
AAATGGTGTCTGCTCGGAGTGAGTGACACACAGAGAGCAGTCGTGTCTGGGCCAATGGAAAGCA
AAGGCAACCTTGTTTACCATTTTTGTCAATGCAG

EPA2253_E01_007.seq: 476 pb (produced from otd_Vtg_9-3)

GGCGGCAGTTTCCAGATGCTAGCGATGAGGAATGGTGCTGCTCGGAGTGAGTGACACACAGAGA
GCAGTCGTGTCTGGGCCAATGGAAAGCAAGGCGACCTTGTTTACCATTTTTGTCAATGCAGATGCT
AATGTACCGATTGTGATGCAGAGAATGGACAACCCAGTACCAAAGGCTATTGAGTTGATCAAGC
ATCTGGCAGAGGTTAATCGTTACAAGATTGACAGTGCAACAACAGAGGACACGATAAAACTGTA
TCAACTCCTGCGAGCAATTCCTATGAAGAACTAGAAGTTATGTGGAAGCAAATTTGCAGCAAA
TGATGAGCAAAGACGTTTGGTTTTTAGACATGATTGTTGAGGTAAACGATGCCCAGAATCCTGAA
GTTCTTTCAAATGAGGTTCCAGGCTGGTGATGTGACTGCTAATGAAGCTCTGCAAGCCCCATTTTT
GCTGGTGATGAACCATCTCCAGCC

EPA2253_C12_092.seq: 440 bp (produced from otd_Vtg_9-3)

CCTTCACGTGAAGGGCGGCAGTTTCCAGATGCTAGCGATGAAGGAAATGGTGCTGCTCGGAGTG
AGTGACACACAGAGAGCAGTCGTGTCTGGGCCAATGGAAAGCAAAGGCAACCTTGTTTACCATT
TTGTCAATGCAGATGCTAATGTACCGATTGTGATGCAGAGAATGGACAACCCAGTACCAAAGGC
TATTGAGTTGTTCAAGCATCTGGCAGAGGTTAATCGTTACAAGATTGACAGTGCAACAACAGAG
GACACGATAAAGCTGTATCAACTCCTGCGAGCATTTCCTATGAAGAACTAGAAGTTATGTGGA
AGCAATTTGCAGCAAATGATGAGCAAAGACGTTGGTTTTTAGACATGATTGTTGAGGTAAACGAT
GCCAGAATCCTGAAGTTCTTCAAATGAGGTTCCAGGCTGGTGATGTGACTGCTA

EPA2254_F01_005.seq: 31 bp (produced from otd_Vtg_7-10)

TTGTCCTGTAGCTGTTGTTTCAGCCACTACTG

EPA2254_D12_090.seq: 159 bp (produced from otd_Vtg_7-10)

TCCTGTAGCTGTTGTTTCAGCCACTACTGGACATGGCTGTGGAAAGTCAGAGGAATGGCTCCACGG
CAGACATGGTCATCGCACTGAAAGCTCTGGGAAATGCAGGTCATCCAGGCAGCATTAATAACCAT
CATGCGCTTCCTCCCCGGAGTTGCTGCCAG

EPA2255_G01_003.seq: 117 bp (produced from otd_Vtg_10-1)

GAGTTCTGCCTAATGATAAGCCTGTCCTCTCTGCCTATACACGGGCCCTTTGGACAAGAGTGTTCT
TTGCTGATATTACCAAAGACACCATTTCGGAACATATTCAGGGCTTTCAGTC

EPA2255_E12_088.seq: 0 bp (produced from otd_Vtg_10-1)

NNNNN

EPA2256_H01_001.seq: 0 bp (produced from otd_Vtg_1-17)

NNNNN

EPA2256_F12_086.seq: 0 bp (produced from otd_Vtg_1-17)

NNNNN

EPA2257_A02_016.seq: 1 bp (produced from otd_Vtg_1-17)

C

EPA2257_G12_084.seq: 1 bp (produced from otd_Vtg_1-17)

NNNNN

In vitro estrogenic activity

Total estrogenic activity of water samples collected in association with Johnny Darter field collections was evaluated using the T47-KBluc in vitro bioassay [67]. Water samples (500 mL from each site per collection date) were filtered (0.7 μ m) and extracted by solid phase extraction using 200 mg sorbent Oasis Hydrophilic-Lipophilic-Balanced (HLB) glass cartridges (Waters, Milford, MA) and concentrated to 1000X in dimethyl sulfoxide (DMSO). Extracted samples at 2X and 0.5X concentrations relative to the original water sample were evaluated compared to a 17 β -estradiol (E2) standard curve ranging from 108 to 0.106 ng E2/L (2-fold dilutions series; triplicate wells). Samples were evaluated in

triplicate within a 96-well plate, with DMSO concentrations ≤ 0.4 % per well. All plates included triplicate media controls and solvent controls (0.4% DMSO), which were used to calculate the significant response threshold within each assay (mean + three standard deviations of the control responses). Sample responses were analyzed by non-linear regression (log-agonist vs. response) against the E2 standard curve using GraphPad Prism (v7.04) to express total estrogenic activity of the sample in terms of 17 β -estradiol equivalents (E2-EQ). Results are reported in Table S3.

Table S3. 17 β -estradiol equivalents (E2-EQs, ng/L) displayed as mean (\pm SD) at each site during each sampling event. Mean \pm SD derived using n= 3–6 technical replicates per sample. The U–SV* water sample on week 12/13 was taken independently of fish sampling because the site was not established for fish collection until week 14. N/A = Not applicable (did not sample).

Site	(Weeks 12 and 13) March 2021	(Week 16) April 2021	(Week 32) August 2021	(Week 41) October 2021
FU-BT	2.96 (0.62)	3.50 (0.50)	N/A	0.93 (0.09)
U-BT	3.13 (0.21)	2.97 (0.39)	N/A	3.67 (0.28)
D-BT	6.07 (0.95)	4.27 (0.36)	N/A	6.75 (1.35)
FD-BT	3.31 (0.49)	3.43 (0.92)	N/A	4.06 (1.12)
CP	5.52 (2.16)	8.51 (2.86)	1.13 (0.12)	N/A
FU-SV	1.32 (0.77)	0.92 (0.11)	1.25 (0.18)	N/A
U-SV	N/A	1.51 (0.37)	1.42 (0.22)	N/A
U-SV*	6.23 (1.31)	N/A	N/A	N/A
D-SV	49.31 (22.93)	32.03 (16.37)	6.83 (0.88)	N/A
FD-SV	10.18 (3.27)	31.48 (15.70)	2.10 (0.36)	N/A

Fish Collection Summary Tables and Male Gonad Staging and Results Figure

Summary Tables of *E. nigrum* Sampled from the Big Thompson River, St. Vrain Creek, and Cache la Poudre River in 2020 and 2021.

Table S4. The number of individuals of Johnny Darter *Etheostoma nigrum* sampled in 2020 and 2021.

	2020			2021		
River	Big Thompson River	St. Vrain Creek	Poudre River	Big Thompson River	St. Vrain Creek	Poudre River
Females	85	74	15	265	243	64
Males	72	31	6	183	144	31
Unknown Sex	6	12	10	15	23	25
Total	163	117	31	463	410	120

Table S5. Johnny Darter *Etheostoma nigrum* sampling summary for 2020 and 2021 at sites on the Big Thompson River (BT). Upstream and Downstream are in reference to the sampling site's location to the WWTP effluent input into the river.

	2020					2021				
Sampling Site	Upstream		Downstream			Upstream		Downstream		
	WB	BB (U)	LWE (D)	C9	BTPSWA (FD)	FP (FU)	BB (U)	LWE (D)	BTPSWA (FD)	
Females	4	30	18	3	30	75	47	81		62
Males	7	23	22	1	19	36	30	60		57
Unknown Sex	1	3	0	0	2	2	0	6		5
Total	12	56	40	4	51	113	77	147		124

Table S6. Johnny Darter *Etheostoma nigrum* sampling summary for 2020 and 2021 at sites on St. Vrain Creek (SV). Upstream and Downstream are in reference to the sampling site's location to the WWTP effluent input into the river.

Sampling Site	2020					2021				
	Upstream	Downstream				Upstream			Downstream	
	GPP		GW		SNA	GPP	IW		GW	SNA
	(FU)	LWE	(D)	ECL	(FD)	(FU)	(U)	USLWE	(D)	(FD)
Females	21	0	9	29	12	75	53	3	60	55
Males	10	1	6	4	10	44	31	1	36	33
Unknown										
Sex	6	0	0	1	5	3	2	0	2	16
Total	37	1	15	34	30	122	86	4	98	104

Table S7. Johnny Darter *Etheostoma nigrum* sampling summary for 2020 and 2021 at sites on the Cache la Poudre River (CP).

Sampling Site	2020		2021
	TB	C5 (CP)	C5 (CP)
Females	4	30	64
Males	7	23	31
Unknown Sex	1	3	25
Total	12	56	120

Male Gonad histology

Table S8. Description of gonad developmental staging according to OECD used to stage male Johnny Darter *Etheostoma nigrum* collected in our study. Table adapted from Johnson et al. 2009 [47].

Stage	
Juvenile	Gonad consists of spermatogonia exclusively; it may be difficult or impossible to confirm the sex of these individuals
0	<i>Undeveloped:</i> entirely immature phases (spermatogonia to spermatids) with no spermatozoa
1	<i>Early spermatogenic:</i> immature phases predominate, but spermatozoa may also be observed; the germinal epithelium is thicker thinner than it is during Stage 2
2	<i>Mid-spermatogenic:</i> spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions; the germinal epithelium is thinner than Stage 1, but thicker than Stage 3
3	<i>Late spermatogenic:</i> all stages may be observed, however, mature sperm predominate; the germinal epithelium is thinner than it is during Stage 2
4	<i>Spent:</i> loose connective tissue with some remnant sperm

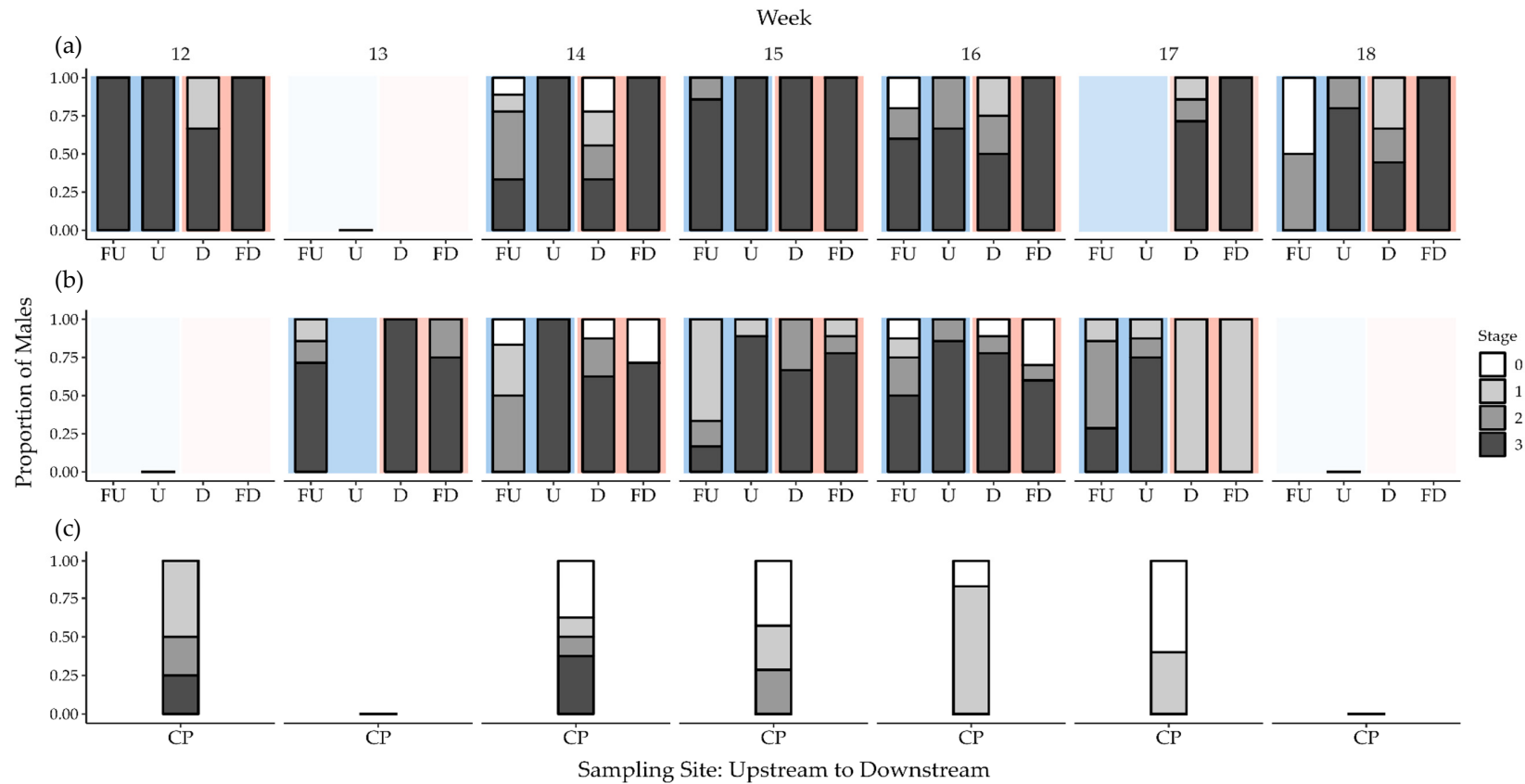


Figure S1. Proportions of males in each reproductive stage sampled each week during the spawning season in 2021 at the (a) the BT and (b) SV far upstream (FU), upstream (U), downstream (D), and far downstream (FD) sites, and the (c) CP site. Stages were assigned using OECD guidelines [47]. Red highlighted columns indicate sites subject to warmer overwinter water temperatures (downstream of the WWTP) and blue highlighted columns indicate sites subject to cooler overwinter water temperatures (upstream of the WWTP) on the SV and BT. The SV was not sampled during week 12, BT and CP were not sampled during week 13, and only BT was sampled during week 18 due to high flows elsewhere. The U-SV site was not established until week 14. No males were sampled at FU or U-BT

Detailed Pesticide and Personal Care Products (PPCPs) and Nutrients (NNPs) results

Ninety-six of 193 PPCPs and 3 of 3 NNPs (nitrate, nitrite, and orthophosphate) were detected at least once (Table S9; Figure S2). In the pre-spawn and spawn samples, 89 unique chemicals were detected, though the pre-spawn samples contained 10 unique PPCPs the spawn samples did not and the spawn samples also contained 10 unique PPCPs the pre-spawn did not. The post-spawn samples detected only 64 PPCPs/NNPs, and no PPCPs detected were unique to the post-spawn samples.

Table S9. Average concentrations of NNPs and PPCPs at Cache la Poudre (CP), Big Thompson (BT), and St. Vrain (SV) sampling sites during the pre-spawn and spawn sample collections in March and April of 2021. NNPs are in µg/L and PPCPs are in ng/L.

Sampling Site		Far Upstream			Upstream		Downstream		Far Downstream	
		CP	SV	BT	SV	BT	SV	BT	SV	BT
River Analyte										
NNPs	Nitrate as N	1017.5			1017.45	258.5	2100	5430	1509	2810
	Nitrite as N	21.35		419	22.6	6.7	13.8	465.05	14.45	152.3
	Orthophosphate as P	28.55	16.55	26.6	24.95	15.95	923.5	1252.8	479.45	455.3
	(+/-)11-nor-9-carboxy-delta-THC							69.4		
PPCPs	10,11-dihydro-10-hydroxycarbamazepine	391	11				920	2060	676	614
	2,4,5-TP					13		21.8		16.8
	2,4-D			301	14.8	247	18.2	247	39.6	288
	3-Hydroxycarbofuran						19	62.3	79.4	24
	6-Acetylmorphine							17.4		
	Acebutolol							55.7		13.8
	Acetaminophen					15.3		13.9	21.3	11.1
	Amitriptyline							22		
	Amitriptyline (+/-)-E-10-hydroxylated							13.95		
	Atenolol	62.6					29	460.5	29.7	144.5
	Atrazine					12.9			14.9	
	Bentazon									25.25
	Benzoylcegonine	12.1						42.7		16.6
	Bromacil							61.3		
	Bupropion	39.5					126.5	429	83.6	87.95
	Caffeine	149	64.3	103	242.35	333.2	102.15	51.8	791	97.3
	Carbamazepine	48.8					105.8	191.5	100	57.45
	Carbamazepine 10,11 epoxide	19.2					70.35	156	50.4	44.1
	Carisoprodol						17.1			

Celecoxib							102		29.1
Codeine	16.7					15.45	90.3	16.4	29.45
Cotinine			10.4		16.7	10.5	13.6	10.2	13.55
DEET	20.7	137	33.8	46.5	28.7	30.45	16.4	42.4	22.9
Desmethylcitalopram	16.7					40.1	137	24.6	37.4
Desmethylvenlafaxine	376					844.5	2695	690	801.5
Dextromethorphan						14.85	47.75		14.2
Dextropropion	96.2					184	555	136	164.5
Diclofenac						100.95	411	118	136.5
Diclofenac 4-hydroxy							85.05		
Diltiazem							19		
Diphenhydramine	10.5					17.75	292		81.95
Diuron						25.8	65.75		27.15
EDDP	11.1					55.9	98.8	36.2	31.15
Escitalopram	22.1					68.65	269.5	30.2	70.65
Famotidine	104					114.5	564	91.2	232
Fluconazole	38					81.15	294.5	44.9	86.65
Furosemide	32.4					225	1260	156	162.5
Gabapentin	723		13.2	27.3	20.6	56.1	210	77.1	305
Gemfibrozil	69.8					22.5	574.5		268.5
Hydrochlorothiazide	163					636.5	2125	504	545.5
Hydrocodone							23.55		
Hydromorphone							30.35		13.8
Hydroxybupropion	244					436.5	862	315	433
Ibuprofen							18		22.1
Imidacloprid	38.4						38.5		
Lamotrigine	658		11.7		10.5	1402.5	5125	1240	2310
Lidocaine	152					408.5	1205	318	420
Lorazepam							17.4		
MCPD			58		42.4		47.8		44.4
MDMA							11.2		
Meprobamate	27.8					28.65	82.25	47	31.35
Metaxalone						10.3	18.95		
Metformin	553		38.6		20.5	214.5	335	192	328.5
Methadone						10.7	14.7		
Methamphetamine	16.5					19.7	18.8		19.3
Methylphenidate							10.8		
Metolachlor ESA	252			21.1		66.9	236	44.5	102
Metoprolol	85.6					172.5	683	130	199.5
Modafinil	12.2					22.65	54.15	18	18.95
Modafinil acid	56.1					110	223		
_____ Monoethylglycinexylidide						15	58.15	14	25.75

Morphine	18.6		53.25	395.5	31.9	83.7
Naproxen	79.4	37.1		75.9	108	75.3
Norfentanyl				11.85		
Norquetiapine			18.65	58.3	13.4	19.75
Omeprazole				13.3		
Oxazepam			13	29.2		11
Oxcarbazepine	17.3		318	407	108	73.85
Oxycodone			12.3	73.5		16.6
Oxymorphone			20.45	78.25	11.9	17.3
Phenobarbital	13.8		34.35	69.95	28.8	22.4
Phentermine	26.4			143.5		45
Phenytoin			27	51.15		
Pravastatin				83.35		50.7
Pregabalin	60.8		10.5	215.1	14.1	147.55
Primidone			49.25	205.5	70.3	85.35
Propranolol	17.5		27.6	120.5	15.8	31.75
Quetiapine				15.7		
Ritalinic acid	39			140		36.15
Sotalol	41.7		105.35	244	86	106
Sulfamethoxazole	109		59.35	225	62.7	135
Sumatriptan	10.5		17.8	88.45	14.1	21.65
Tebuconazole				58.6		
Temazepam			18.3	49.25		12.9
Thiabendazole		18.9	20.6	40.9	13.3	14.15
Tramadol	93.2		177	583	150	231.5
Trazodone				44.45		11.4
Triamterene	18.2		34.55	114.5	19.6	42.15
Triclopyr		27.7	33.75			29.7
Triclosan				11.3		
Trimethoprim	58.1		113.35	401.5	80.2	160.5
Valsartan	68		34.65	820	22.4	249
Venlafaxine	88.1		236	741.5	163	249
Zolpidem phenyl-4-carboxylic acid	16.7		36.55	110.2	28	40.2

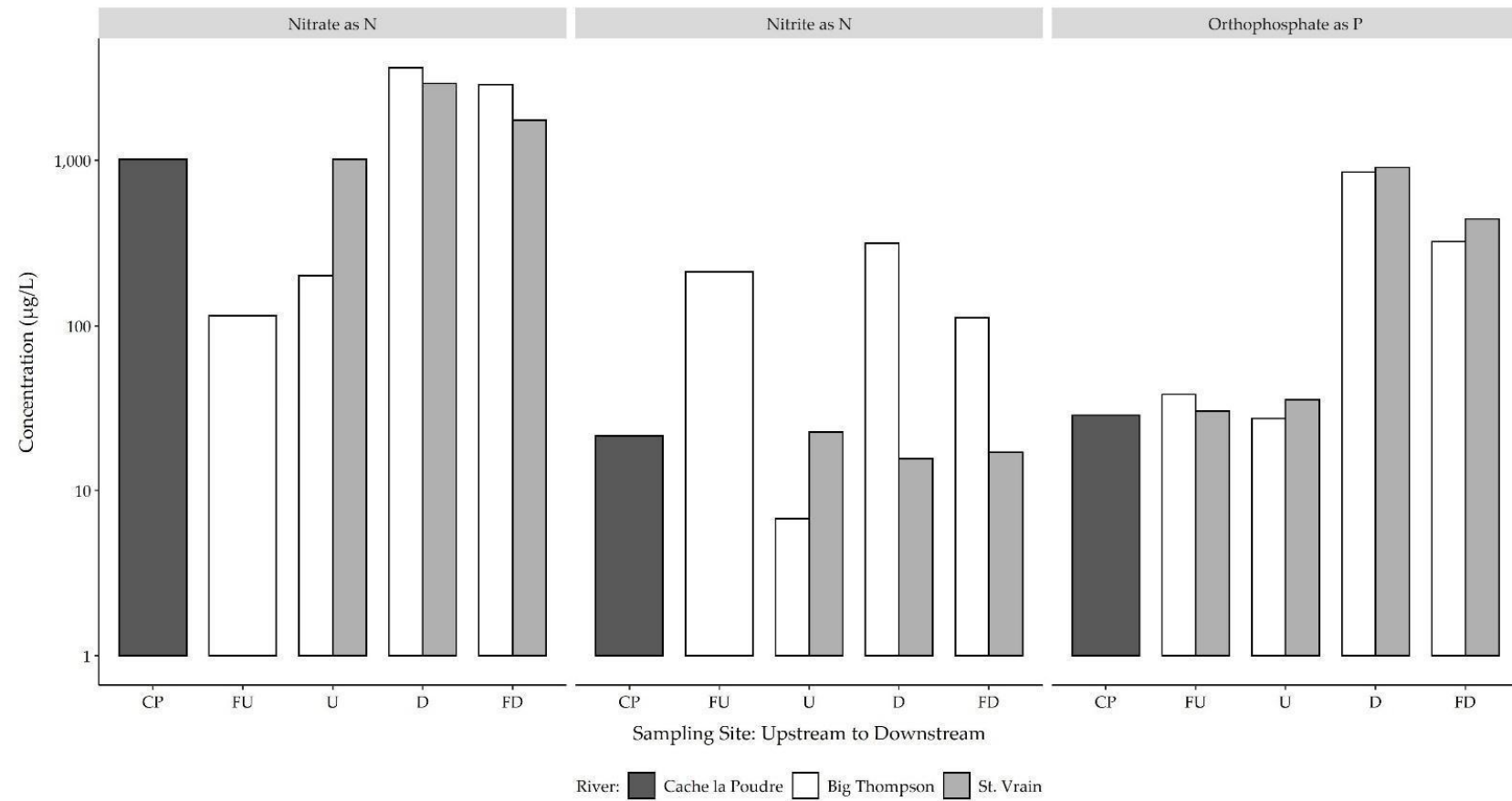


Figure S2. Nutrient concentrations at all study sites. If a site is missing on the figure, that nutrient was not detected at that site. The y-axis is transformed for graphical comparison.

Of the 96 PPCPs and 3 NNPs, only 3 PPCPs (caffeine, DEET, and gabapentin) and 1 NNP (orthophosphate) were detected at every site. Forty-one PPCPs were detected exclusively at all sites downstream of the WWTPs in BT and SV as well as CP, apart from 6 PPCPs not detected also in CP (3-Hydroxycarbofuran, Diclofenac, Monoethylglycinexylidide, Norquetiapine, Oxymorphone, Primidone; Table S9). There were 28 PPCPs detected in BT that were not detected in SV, 18 of which were only detected at site D–BT. Only 3 of those (Ritalinic acid, Phentermine, and Benzoylecgonine) were also in CP. The only chemical in SV that was not in the BT or CP was Carisoprodol and only detected at D–SV. There were no PPCPs detected in the CP that were not detected in BT or SV.

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