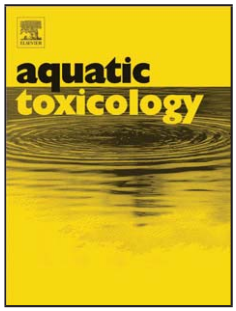

Title: Evaluation of water quality threats to the endangered Okaloosa darter (*Etheostoma okaloosae*) in East Turkey Creek on Eglin Air Force Base

Authors: R.E. Weil, D.J. Spade, I. Knoebl, J.M. Hemming, M.L. Tongue, N.J. Szabo, K.J. Kroll, W.B. Tate, N.D. Denslow

PII: S0166-445X(12)00020-3
DOI: doi:10.1016/j.aquatox.2012.01.007
Reference: AQTOX 3207

To appear in: *Aquatic Toxicology*

Received date: 17-6-2011
Revised date: 7-1-2012
Accepted date: 10-1-2012

Please cite this article as: Weil, R.E., Spade, D.J., Knoebl, I., Hemming, J.M., Tongue, M.L., Szabo, N.J., Kroll, K.J., Tate, W.B., Denslow, N.D., Evaluation of water quality threats to the endangered Okaloosa darter ({\it{Etheostoma okaloosae}}) in East Turkey Creek on Eglin Air Force Base, {\it{Aquatic Toxicology}} (2010), doi:10.1016/j.aquatox.2012.01.007

Evaluation of water quality threats to the endangered Okaloosa darter (*Etheostoma okaloosae*) in East Turkey Creek on Eglin Air Force Base

R. E. Weil[1#], D. J. Spade[1#], I. Knoebl[1], J. M. Hemming[2], M. L. Tongue[3], N. J. Szabo[4], K. J. Kroll[1], W. B. Tate[3], N. D. Denslow[1*]

[1]Department of Physiological Sciences and Center for Environmental and Human Toxicology, University of Florida, Gainesville, FL, USA.

[2]Ecological Services and Fisheries Resources, US Fish and Wildlife Service, Panama City, FL, USA.

[3]Fisheries Resources Office, US Fish and Wildlife Service, Eglin Air Force Base, Niceville, FL USA.

[4]Analytical Toxicology Core Laboratory, University of Florida, Gainesville, FL, USA.

[#]These authors contributed equally to the project.

*Corresponding author

Department of Physiological Sciences and Center for Environmental and Human Toxicology

University of Florida

PO Box 110885

Gainesville, Florida 32611

Phone: 352-294-4246

Fax: 352-392-4707

Email: ndenslow@ufl.edu

Abstract

The threatened Okaloosa darter (*Etheostoma okaloosae)* is found almost exclusively on the Eglin Air Force Base in the Choctawhatchee Bay watershed of Florida. Portions of this limited habitat are threatened with soil erosion, altered hydrology, and impaired water quality. In the present study, general water quality parameters (i.e., dissolved oxygen, specific conductance, pH, temperature, relative turbidity, and primary productivity) were characterized in East Turkey Creek, which is a body of water potentially impacted by treated wastewater sprayfields, and Long Creek, an adjacent reference stream that does not border the sprayfields. Water quality was assessed during a 30-day exposure using passive samplers for both non-polar and polar effluent parameters. Because the Okaloosa darter was listed as endangered at the time of sampling we chose a closely related species from the same creeks, the sailfin shiner (*Pteronotropis hypseleotris)* in which to measure metal body burdens. Additionally, fathead minnows (*Pimephales promelas*) were used for microarray analysis on gonad and liver tissue after 48 h exposures to water collected from the two creeks and brought into the laboratory. Waters from all sites, including reference sites, affected the expression of genes related to various biological processes including transcription and translation, cell cycle control, metabolism, and signaling pathways, suggesting that the sum of anthropogenic compounds in the site waters may cause a generalized stress response in both liver and testis, an effect that could be related to the generally low populations of the Okaloosa darter. Furthermore, effects of site waters on fish gene expression may be related to the impact of human activities other than the wastewater

sprayfields, as nearby areas are closed to the public for military testing, training, and administrative activities and due to ordnance contamination.

Keywords: Okaloosa darter, *Etheostoma okaloosae*, species recovery, wastewater effluent, microarrays, passive samplers

1. Introduction

The Okaloosa darter *(Etheostoma okaloosae)* is a small fish with adults ranging in size from 27- 49 mm standard length (Mettee and Crittenden 1979; Ogilvie 1980). The U.S. Fish and Wildlife Service (U.S.-FWS) has included this darter on the List of Endangered and Threatened Wildlife and Plants because it inhabits only six stream systems located in Okaloosa and Walton Counties, Florida (U.S.-FWS 1973). The extremely limited range of the darter and the amount of its habitat degraded by road and dam construction, as well as siltation from land clearing, were primary factors in the initial listing (U.S.-FWS 1998a). Continued threats include water quality impacts, ongoing increases in urbanization, ground and surface water withdrawal, and vulnerability to hazardous material spills (U.S.-FWS 1998a).

The areas inhabited by the Okaloosa darter are typically the margins of flowing streams where detritus, root mats, and vegetation are present. Okaloosa darters are not found in areas where there is no current, nor are they collected in the open, sandy areas in the middle of stream channels. Densities average about one darter in every 2.7 meters of stream length (Burkhead et al. 1994). Although brown darters occupy similar stream margins, they are capable of living in areas of little to no flow (Burkhead et al. 1994). Okaloosa darters feed primarily on fly (*Diptera*), mayfly (*Ephemeroptera*),

and caddis fly (*Trichoptera*) larvae (Ogilvie 1980). The breeding season extends from late March through October, although it usually peaks in April. Video recordings have shown spawning pairs attaching one or two eggs to vegetation, and they also have been observed attaching eggs to woody debris and root mats (Collette and Yerger 1962; Burkhead et al. 1994). Ogilvie (Ogilvie 1980) found a mean of 76 ova and 29 mature ova in 201 female Okaloosa darters. These numbers may under-represent annual fecundity as the prolonged spawning season is an indication of fractional spawning (i.e., eggs develop and mature throughout the spawning season). Estimates of longevity range from two to three years (Mettee and Crittenden 1979; Burkhead et al. 1992).

Ongoing population monitoring of the darter's range has indicated that water systems such as East Turkey Creek have consistently reported decreasing numbers of darters moving downstream (U.S.-FWS 1998a). To assess potential causes of these findings, water quality parameters of the impaired systems in comparison with productive streams have been examined. The current assessment was designed to evaluate potential negative influences on water quality in East Turkey Creek from the City of Niceville's municipal wastewater effluent after application to a designated wastewater sprayfield leased from Eglin Air Force Base. There are suspected, but unconfirmed, hydrologic connections from the karst sprayfield area to the seepage fed East Turkey Creek.

Due to the presence of diverse human activity other than the sprayfields, we chose three reference sites including a site just upstream of the sprayfields on East Turkey Creek, and two sites on an adjacent reference creek, Long Creek. Preliminary

reports have indicated possible impacts of water quality (indicated by elevated conductivity) (Thom and Herod 2005) on darter abundance in East Turkey Creek (U.S.-FWS 1998a). Because the Okaloosa darter is protected, we focused chemical and genomic investigations on other fish species. For contaminant analysis we chose Sailfin shiners (*Pternotropis hypselopterus*), a species which is found in the same waters and shares a similar trophic level as the darter. For genomic analyses, we chose the fathead minnow (*Pimephales promelas,* FHM) as this species is commonly used for ecotoxicologic assessment and we already had a well-annotated microarray available for this species. During this evaluation (1) ambient water quality parameters were measured, (2) non-polar and polar organic pollutants in ambient waters were evaluated by means of passive sampling devices, (3) the metal burdens in a similar native fish species that inhabits a similar trophic level were determined and (4) microarray responses were examined from gonad and liver tissues of a model fish species after laboratory exposure to stream waters.

2. Materials and Methods

2.1. Site selection

Sites were selected based on Okaloosa darter population monitoring locations, proximity to the wastewater treatment sprayfields, and watershed size (Figure 1). Three sites on East Turkey Creek, a watershed of 12.3 km^2 drainage, were selected; site B at the headwaters and the top edge of the sprayfield influence; site E in the middle of the sprayfields and site A below the sprayfields. Two reference sites (D and G) were located on Long Creek, a watershed 8.8 km^2 in size. Site G was selected only for

collection of sailfin shiners for metal analyses, as the shiners were abundant at this site. No microarray experiments were conducted with waters from site G. It should be pointed out that Long Creek originates in a large area that is closed to the public due to military testing, training, and administrative activities, or to the presence of unexploded ordnance. Thus, there is likely some anthropogenic impact at sites D and G, as well.

2.2. Collection of Water Quality Data

YSI water quality data loggers (model 6600) were placed in both creeks. Within the creeks, locations were chosen based on water depth and at sites where the loggers were least likely to be compromised by woody debris, flocculent substrate or algae. Once a suitable site was identified, the multiprobes were activated and then placed in a locked PVC housing which was then secured to a tree. The PVC housing was constructed by drilling ½" holes into a 4" diameter pipe with one end of the pipe permanently closed and the other end closable by a screw top with a key lock. The housing was developed in order to prevent the loggers from becoming smothered by sand during unattended data collection and to provide a means for protection against theft.

During deployment from August 2007 to June 2008, the loggers were programmed to take readings every 40 minutes in order to capture fluctuations in water chemistry during rain events. In December of 2007 the sondes were removed from the creeks, factory-inspected, and calibrated to validate function and ensure accuracy. Loggers measured pH, dissolved oxygen (mg/L), specific conductance (mS/cm), chlorophyll (µg/L), water temperature (^{o}C), and turbidity (NTU). Multiprobe calibration was carried out frequently but more often when measurement drift was observed. Data

was uploaded weekly to a YSI 85 and then onto a desktop computer where it could be synthesized and analyzed using EcoWatch software, provided by the manufacturer, and by Microsoft Excel.

2.3. Passive Sampling for Organic Compounds

Semi-permeable membrane devices (SPMD) and polar organic chemical integrative samplers (POCIS) supplied by Environmental Sampling Technologies, Inc. (St. Joseph, MO), were used, respectively, for the collection of non-polar (organochlorine pesticides, polyaromatic hydrocarbons, polychlorinated biphenyls) and polar (estrogens) organic compounds from stream flow in East Turkey Creek and Long Creek. Triplicate sets of SPMD membranes, low density polyethylene tubing containing thin layers of triolein, and flat POCIS membrane disks were secured in flow-through stainless steel canisters and deployed in the creek waters at sites E, D, and G for 31 days and at site A for 29 days. On recovery, the membranes were sealed in airtight cans and shipped to the St. Joseph, MO facility for extraction along with SPMD and POCIS field blanks that had traveled to the sites during deployment and recovery as a means of accounting for background contamination.

Polar contaminants were extracted at the St. Joseph, MO facility from each SPMD membrane via dialysis with the dialysates from each set of triplicate membranes combined into one composite sample. After concentration by Kuderna-Danish, and isolation by gel-permeation chromatography (GPC), the final SPMD extract for each site had an approximate volume of 1.25 mL in hexane and was stored at -20º C in a sealed ampoule. A dialysis blank and the field blank were prepared alongside the site samples. Polar contaminants were extracted into methanol from each set of triplicate

159 POCIS membrane disks using chromatography. The resulting composite had an
160 approximate volume of 1 mL in methanol and was stored at -20º C in a sealed ampoule.
161 All extracts were transferred to the University of Florida Analytical Toxicology Core
162 Laboratory (ATCL) for contaminant analysis.

163 For improved sensitivity, SPMD extracts were concentrated under nitrogen to 0.3
164 mL before quantitative analysis for 30 organochlorine pesticides (OCP), 16 polyaromatic
165 hydrocarbons (PAH), and total polychlorinated biphenyls (PCB; sum of mono-
166 substituted to octa-substituted chlorinated biphenyls) using the GC-MS method
167 described in Gelsleichter et al. (Gelsleichter et al. 2006). Sample concentrations were
168 converted to estimated concentrations in site waters using the SPMD Water
169 Concentration Estimator v4.1 by Environmental Sampling Technologies based on
170 algorithms developed by USGS (Alvarez et al. 2009)

171 For improved sensitivity, POCIS extracts were dansylated and concentrated to a
172 final volume of 0.2 mL before quantitative analysis of estrogenic compounds using
173 positive mode APCI LC-MS/MS in a method modified from Nelson et al. (Nelson et al.
174 2004). Sample concentrations were converted to estimated concentrations in site
175 waters using the POCIS Turbulent Systems Estimator (Environmental Sampling
176 Technologies, personal communication).

177 *2.4. Metals assessment*

178 To assess the darter's potential exposure to USEPA priority pollutant metals,
179 Sailfin shiners were collected from study sites E, A, and from reference site G. This
180 species of shiner is common to the study areas, is about the same size as the darter,
181 and shares similar habitat. Seined shiners from each site were sorted into composite

182 samples (19.8 g to 32.7 g) containing 12 to 23 individual whole fish, placed in plastic
183 bags, and shipped on ice to Columbia Analytical Services (Kelso, WA) for
184 extraction/analysis by USEPA methods (Ag, As, Be, Cd, Cu, Ni, Pb, Sb, Tl, and Zn via
185 200.8 (Creed et al. 1994)/ 6020A (U.S.-EPA 2007); Cr via 6010B-LL; Hg via 1631(U.S.-
186 EPA 2002); and Se via 7742 (U.S.-EPA 1994). Each homogenized composite was
187 analyzed in triplicate. Method reporting limits (MRL) and method detection limits (MDL)
188 are listed in Suppl. Table 1.

189 *2.5.* In Vivo *Site Water Exposure and Tissue Collection*

190 Water samples were taken from four study sites, including three (B, E, and A) in
191 East Turkey Creek and one reference site (D) in Long Creek. The creek water was
192 collected via repeated filling of glass vessels and transfer to fiberglass lined drums.
193 With gloved hands, water was collected mid water column. Water being transferred to
194 the drums was filtered through glass fiber to remove substrates that may sorb chemicals
195 in solution. Water collected from each site was placed in a 30 gallon drum which was
196 previously lined with fiberglass to prevent metal intrusion. The drums were weathered
197 and washed thoroughly and no leaching of fiberglass was expected. All water samples
198 for exposures were collected in one day over the course of 30-60 minutes per site.
199 Water was immediately transported to the University of Florida for exposure
200 experiments (48 h with static renewal) using FHM to assess the effects of the site
201 waters in a model teleost fish. All animal care procedures were approved by the
202 University of Florida IACUC.

203 Aquaria were equilibrated with test waters for 24 h prior to the introduction of fish.
204 Reproductively mature male FHM, (Andersen Minnow Farm, AR.) exhibiting secondary

sex characteristics (fat pads, tubercles, markings) were separated from the population and acclimated in the treatment aquaria for 48 h. The control water used for this study (UF Control) was carbon-filtered, de-chlorinated Gainesville, FL, city water. The exposure system consisted of 40 L glass aquaria supplied by a magnetic drive pump and Chemfluor PTFE tubing (Cole-Parmer). Each treatment was conducted in triplicate and each aquarium contained four male FHM in 8 L of treatment water. Test waters from each location and a lab control for each treatment group (120 L) were pumped from fiberglass coated barrels into each test aquarium using EPA approved tubing during the exposure. Test water was changed a total of 5 times during the experiment. Test waters were renewed to 90% twice daily (12, 24, 36, and 48 h after the start of the experiment). The positions of the treatment tanks were randomized and test initiation times were staggered to ensure an exposure/sampling interval of 48 h. The fish were not fed the day prior to or during the experiment. Temperature was maintained at 25 ^{o}C with a photoperiod of 16 h light: 8 h dark.

Following exposures, fish were anesthetized using Tricaine MS-222 (3-aminobenzoic acid ethyl ester). Liver and gonad samples were collected, flash-frozen in liquid nitrogen, and stored at -80 ^{o}C until they were processed.

2.6. RNA Extraction

Total RNA was isolated from 30-50 mg each FHM testis and liver sample with the RNA STAT-60 reagent (Tel-test, Friendswood, TX), as previously described (Garcia-Reyero et al. 2006). Total RNA was treated with DNase I (Turbo DNA-free, Ambion). RNA quality was assessed by the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA).

The quantity was determined on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA was stored at -80 °C until further use.

2.7. Microarrays

The 8x15k oligonucleotide fathead minnow microarray (GEO Accession no. GPL9248) was designed in the Denslow laboratory, manufactured by Agilent (Palo Alto, CA), and was first used by Villeneuve et al.(Villeneuve et al. 2010). The array consists of 15,744 oligonucleotide probes corresponding to 15,208 FHM genes and 536 control sequences. The sequences of the genes were obtained by compilation and bioinformatics analysis of 250,000 ESTs publicly available in NCBI databases and 1000 genes obtained by suppressive subtractive hybridization (Diatchenko et al. 1996). Annotation of the genes was done by BLAST against both the NR and NT databases.

For the microarray experiment, four biological replicates consisting of RNA samples from four different individuals were analyzed for each of the treatments (University of Florida control and each of the sites). The cDNA synthesis, cRNA amplification, and labeling with cyanine 3 (Cy3) were performed using the Agilent Low RNA Input Fluorescent Linear Amplification Kit and (Agilent, Palo Alto, CA), following the manufacturer's protocol for One-Color Microarray-Based Gene Expression Analysis (Agilent publication no. G4140-90040). Cy3 specific activity was determined using the NanoDrop ND-1000 spectrophotometer, and was a minimum of 9 pmol Cy3/μL cRNA. After labeling, the samples were hybridized to the microarrays for 17 h using Agilent's Gene Expression Hybridization Kit, washed, and scanned using the Agilent G2505B microarray scanner and Agilent Feature Extraction software, again according to the Agilent protocol. Raw and normalized data have been deposited to the Gene

Expression Omnibus database (GEO Accession no. GSE23521), in accordance with MIAME (Minimum Information About a Microarray Experiment) standards (Brazma et al. 2001)

2.8. Statistical and pathway analysis of microarray data

Microarray data were imported into JMP Genomics software v3.1 (SAS, Cary, NC) for analysis. Any rows not containing at least two data points for all treatment groups were deleted and the data were subjected to Loess normalization, with a smoothing parameter of 0.2. ANOVA was performed using the Loess-normalized dataset, and genes that differed significantly among the groups were identified ($p<0.01$ or $p<0.05$ for further analyses, with no *post hoc* correction because we were interested in including as many genes as possible for pathway analyses. The program Cluster (Eisen et al. 1998) was used to perform hierarchical clustering analysis based on all significantly differentially regulated genes ($p < 0.01$); the result was visualized in the program Java TreeView (Saldanha 2004). For this analysis, Loess-normalized microarray data were mean-centered by gene and array and Pearson correlation was used as the distance metric, based on average linkage. Functional enrichment analysis based on Gene Ontology (GO) Biological Process terms was performed in JMP Genomics, using the Column Enrichment function. This analysis was based on Fisher's exact test and was used to identify over-represented terms in the list of differentially regulated genes ($p < 0.05$). All terms with a nominal p-value of $p < 0.05$ in the Fisher's exact test (no *post hoc* correction) were considered to be enriched.

3. Results

3.1. Water quality

Differences in water quality were found between East Turkey Creek (Site E) and Long Creek (Site D) (Suppl. Table 2). Specific conductance was markedly higher in East Turkey Creek (median over five times more) when compared to the reference, Long Creek. Large differences in pH (1.6 pH units) were also found, with East Turkey Creek (pH 5.0) and Long Creek (pH 6.6). A smaller difference in temperature (0.6 ºC) showed East Turkey Creek to be warmer overall. Only very small differences in dissolved oxygen concentration, turbidity, and chlorophyll were observed.

3.2. Passive samplers

Chemical analyses were conducted for a suite of 37 chlorinated compounds (pesticides, PCBs, and PAHs). Three organochlorine pesticides were detected in the site waters of this study (Suppl. Table 3). Hexachlorobenzene and p,p'-DDE were identified at all sites, including the reference sites, at estimated average concentrations of 9.1 pg/L and 4.75 pg/L, respectively. Although detectable, levels of cis-chlordane at sites A and G were below the limit of quantification (11.5 pg/L). Two PAHs were detected at multiple sites and at quantifiable levels. Acenaphthene was present at all test sites and one reference site (D). Acenaphthylene was also present at all sites in East Turkey Creek and one site in Long Creek. The following chlorinated compounds were not detected in any SPMD sample from reference or test site waters at levels above the limit of quantitation, 0.7 pg/L: aldrin, α-BHC, β-BHC, lindane, δ-BHC, p,p'-DDD, p,p'-DDT, o,p'-DDD, o,p'-DDE, o,p'-DDT, dieldrin, endosulfan, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor

296 epoxide, kepone, methoxychlor, mirex, trans-chlordane, oxychlordane, cis-nonachlor,
297 trans-nonachlor, toxaphene, anthracene, benzo[g,h,i]perylene, dibenz[a,h]anthracene,
298 fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, pyrene,
299 and PCBs.

300 Most POCIS samples did not show detectable levels of estrogens. One
301 reference site (D) was estimated to have estrone at levels of 254 pg/L and one test site
302 (E) was estimated to have 17α-ethynylestradiol at levels of 51 pg/L.

303 *3.3. Priority metals*

304 The findings of USEPA priority metals in sailfin shiner composites are
305 summarized in Suppl. Table 4. Fish residues from both test and reference sites had
306 total Hg body burdens (ranging from 0.412 to 0.581 mg/kg dry weight) that were
307 comparable to the concentrations found in the fillets of larger and longer-lived predatory
308 fish species (~0.42 and ~0.58 mg/kg dry weight, respectively, in small and large mouth
309 bass) (Webber and Haines 2003). The highest Hg concentrations were found in shiners
310 from reference site G. Fish residues from all sites also contained Zn (ranging from 372
311 to 431 mg/kg dry weight), with the highest levels again at reference site G. Lead and
312 selenium were identified in fish samples from all sites (ranging from 0.251 to 0.504
313 mg/kg for lead and 3.22 to 4.63 mg/kg for selenium), but these metals tended to be
314 higher in the East Turkey Creek sites than in the reference site with the exception of
315 lead at site A, which was lower than in the reference site. Site E fish composites
316 exceeded all others in cadmium concentration (0.38 mg/Kg dry weight) and in chromium
317 concentration (2.71 mg/kg fish residue), respectively.

318

319 *3.4. Microarray analysis of fathead minnow liver*

320 Microarray data for the livers of fish exposed to water from sites A, B, D and E
321 were analyzed by one-way ANOVA, with Site as the factor, and using UF as control.
322 Genes that were significantly differentially expressed in at least one comparison ($p <$
323 0.01) are shown in the two-way unsupervised hierarchical cluster (Figure 2 A). In this
324 representation, each column is a separate gene and each row is a separate biological
325 sample. Genes that are expressed at a level greater than the column average are
326 shown in red and those that are lower than the column average are blue. Notably,
327 according to the cluster analysis, all of the sites are different from one another, but more
328 similar to each other than any of them are to the UF control (i.e. UF clusters as the out
329 group).

330 To better compare the gene expression fingerprints from fish exposed to water
331 from the four sites, the expression data ($p < 0.05$) were arranged in correspondence to
332 their locations on East Turkey Creek, starting with site D (reference site) on Long Creek,
333 to site B (above the sprayfields), to E (within the sprayfields), and to A (below the
334 sprayfields). For each site, the expression data was in the same order and arranged
335 from the most up-regulated to the most down-regulated genes (Figure 3). Expression
336 values are listed as $\log_2$ of the fold-expression compared to the UF control. If a
337 particular gene was not significant for a site (based on the ANOVA), its expression
338 value was set to zero. Thus, the only expression values plotted are those that are
339 significant. Of the four sites, it is clear that fish exposed to water from Site D (the site
340 on Long Creek) had some differences compared to the UF controls (526 genes). Gene
341 expression results from Site B (566 altered genes) are most similar to those from Site D,

342 but do contain an additional set of genes that are not differentially expressed in Site D.
343 Sites E and A presented a total of 492 and 808 altered genes, respectively.
344 Gene Ontology (GO) Biological Process categories significantly enriched at
345 several sites (Table 1) were related to transcription (sites B and D), chromatin structure
346 (D), ribosome biogenesis (site B), collagen catabolism (B), or Rho protein (B), among
347 other terms. At the two sites closest to the sprayfields, we saw numerous changes: for
348 Site E, ribosome biogenesis and assembly, rRNA processing, and regulation of GTPase
349 activity, and for Site A, tRNA aminoacylation for protein translation, protein import into
350 the nucleus, translation initiation, chromatin remodeling, phospholipid transport,
351 regulation of small GTPase mediated signal transduction, and growth, among others.
352 Despite the similarity in Gene Ontology terms enriched in each site water
353 comparison, there are only six genes that are differentially regulated for all sites. These
354 include RUN and FYVE domain-containing 2, a Zn binding protein that is down-
355 regulated at all sites, zgc:85677 (hmga1 high mobility group AT-hook 1), a homolog of a
356 *Danio rerio* transcription-related gene that is slightly up-regulated at every site, FK506
357 binding protein 5, which is related to heat shock response and protein folding and is up-
358 regulated at every site, hypoxia-inducible factor 1a (HIF-1α), which is induced at every
359 site, and two hypothetical proteins.
360 *3.4.2. Microarray analysis in testis*
361 Gene expression data for the testis is plotted as a two-way hierarchical cluster
362 (Figure 2B), as with the liver data. In this organ, each of the individual samples
363 clustered by site, suggesting that there was a significant site effect on gene expression.
364 As was the case in the liver, all animals from each of the treatment groups cluster

365 together with their respective treatments and are separate from the UF controls, with
366 sites B, E and A grouping closer together than to site D.

367 Gene expression fingerprints for the four sites were constructed for the testis
368 using expression data ($p < 0.05$) which was arranged as described for Figure 3, from
369 the most up-regulated to the most down-regulated genes for each of the sites (Figure
370 4). The reference site D is plotted first followed by sites B, E and A, in relation to their
371 position on East Turkey Creek. Of the four sites, it is clear that fish exposed to water
372 from Site D (the reference site) had the fewest differences (378 altered genes) when
373 compared with the UF controls. Exposure of fish to waters from sites B, E and A
374 presented with 474, 671, and 510 significantly altered genes. The general pattern of
375 gene expression was similar to what was described above for the liver with most of the
376 changes occurring at Sites E and A, which are closest to the spray fields.

377 Enriched GO Biological Process terms (Table 2) for site D fish included changes
378 in embryonic heart tube development, angiogenesis, ion transport and cell matrix,
379 among others, and in Site B fish phospholipid metabolic process and lipid catabolic
380 process were enriched, among others. In fish exposed to waters from the sites closest
381 to the spray fields, enriched terms included regulation of transcription, response to
382 stress, DNA replication initiation and calcium ion transport (Site E); ribosome biogenesis
383 and assembly, intracellular protein transport, microtubule-based movement, receptor-
384 mediated endocytosis and rRNA processing (Site A).

385

386

387

4. Discussion

The assessment at Eglin Air Force Base was designed to evaluate the potential for sprayfield-related municipal effluent to alter water quality in East Turkey Creek. The impact of municipal wastewater effluents on water quality has been long recognized, coming to the forefront with the amendments to the Clean Water Act in 1977 (CWA 1972). Municipal effluents are very complex and thus present challenges to the ecosystems that inhabit effluent receiving systems. Acute and subchronic toxicity bioassays on fish and in-stream invertebrate monitoring have provided information about the extreme variability of effluents and their toxicities on organisms in streams receiving the effluents (Garric et al. 1996; Middaugh et al. 1997; Doherty et al. 1999; Kosmala et al. 1999; Alvarez et al. 2009). In the case of the Okaloosa darter, it appears that it is endangered because of changes in water quality. While the two streams in the present study appear similar in their characteristics and both appear to be suitable habitat for Okaloosa darters, the Okaloosa darters are less prevalent in East Turkey Creek compared to Long Creek (U.S.-FWS, 1998a).

4.1. Polar and nonpolar organic pollutants and priority pollutant metals

Metals, anthropogenic hormones, and various consumer products such as pesticides, plastics, detergents, antibacterial agents, and pharmaceuticals are now routinely found in wastewater effluents at effective concentrations (Fent et al. 2006). These effluents are the source of both natural and synthetic substances including, but certainly not limited to, polychlorinated biphenyls, phthalates, heavy metals, alkylphenols, PAHs, 17β-estradiol, 17α-ethinylestradiol, and bisphenol A (Pait and Nelson 2002; Aguayo et al. 2004; Nakada et al. 2004; Iwanowicz et al. 2009).

411 SPMD and POCIS were used to collect organic residues in the East Turkey
412 Creek and Long Creek streams. The analytical advantages of these devices over living
413 tissues is that the membranes do not metabolize the analytes as live tissue would and
414 that the analytes are easier to extract from triolein (the lipid present within the SPMD
415 membrane) and from the POCIS extraction discs. Additionally, because passive
416 samplers are exposed to site waters for a period of time, in this case one month, a
417 larger portion of the water is sampled and a more complete profile of polar and non-
418 polar contaminants is collected and at magnified concentrations compared to
419 contaminants collected using a traditional grab sample.
420 Although a number of chlorinated compounds were found to be present at the
421 sites, including several PAHs, the levels were consistently low, below USEPA ambient
422 water quality criteria for fresh water aquatic systems (U.S.-EPA 1986). As Figure 1
423 shows, the sprayfields start just down-stream from site B and water likely runs off
424 toward sites E and A directly. The contaminant analysis panel was short due to cost
425 and there may be additional chemicals that are different among the sites that were not
426 tested.
427 The appearance of estrone at site (D) cannot be easily explained as this site was
428 deemed to be not impacted from the sprayfields. 17α-ethynylestradiol (EE2) was found
429 at a single test site (E), which is surrounded by the sprayfields, and this most likely is
430 from the municipal waste introduced through the sprayfields. Site E not only showed
431 EE2 contamination, but also elevated concentrations of acenaphthene and
432 acenaphthylene.

The whole body burdens for priority pollutant metals were below concentrations at which overt piscine toxicity would be anticipated, but the presence and trends, especially of the nonessential elements, are suggestive of potential subchronic impact on the animals (U.S.-FWS 1998b). Hg levels in shiners from all sites were higher than the mean whole body concentrations found in large, longer-lived bottom-feeders such as carp (0.11 mg/kg wet weight; ~0.14 mg/kg dry weight) and channel catfish (0.09 mg/kg wet weight; ~ 0.11 mg/kg dry weight) and comparable to the mean fillet concentrations found in large, longer-lived predatory species such as smallmouth (0.34 mg/kg wet weight; ~0.42 mg/kg dry weight) and largemouth bass (0.46 mg/kg wet weight; ~0.58 mg/kg dry weight) (U.S.-EPA 2001). Metal profiles showed that the reference site G had higher levels of Hg (total), Ag, and Zn (Suppl. Table 4) than did the test sites. Site E has slightly higher levels of some metals including cadmium, copper, lead, nickel, and selenium, suggesting these are of anthropogenic sources.

4.2. Gene expression analysis

Overall, the microarray data suggest that the waters from all sites, and especially the down-stream East Turkey Creek sites (E and A), induced potentially adverse genomic responses in both FHM livers and testis. Identifying changes in gene expression in these two tissues gives insight into how the Okaloosa darters may be affected. We consider Sites D and B as pseudo-reference sites because they are above the influence of the sprayfield. However, these sites may be influenced by nearby areas that are closed to public access, due either to active military testing and training, the presence of unexploded ordnance, or use as military administration areas. If munitions-related testing or training occurs on these sites or if unexploded ordnance is

456 present, contaminated soil or surface waters could influence creek waters at the sites in
457 the present study. Sites E and A, which are, respectively, surrounded by the sprayfield
458 influence and immediately south of the sprayfields appear to have slightly more gene
459 expression changes when compared to the reference sites. At a crude level of analysis
460 ($P < 0.05$), an average of 546 liver genes and 426 testis genes are altered at the
461 reference sites compared to 650 and 590, respectively at the sprayfield sites.
462 Interestingly, there appears to be a dampening of the magnitude of the change (fold
463 change) in gene expression going down-stream for genes that are common to B, E and
464 A, suggesting that a stressor is diluted as the water proceeds down-stream (Figures 3
465 and 4). The additional genes that are found at sites E and A may be due to the
466 influence of the sprayfields, which provide run off waters that enter the stream
467 surrounding Site E and just up-stream from Site A.

468 Site D was considered a useful reference site for the influence of sprayfields at
469 Eglin Air force Base, but it should be noted that, as stated earlier, there are several
470 compromised sites that are up-stream from this location (Figure 1). Site B was a
471 second pseudo-reference site located just up-stream from the spray fields in East
472 Turkey Creek, but it, too, is surrounded by sites that are closed to the public.
473 Interestingly, changes in gene expression at sites D and B did not cluster together
474 suggesting that there are some significant differences in these two sites.

475 Functional enrichment analysis based on GO Biological Processes in the liver
476 indicated that at all sites, the predominant pathways affected were related to
477 metabolism, cell cycle control, transcription, translation, remodeling of various cellular
478 structures, and cell signaling. This implies that the difference between UF control water

and site water at all sites is sufficient to cause changes in transcription of genes related to the basic processes of gene and protein expression and signal transduction. This is consistent with a generalized stress response. Many of the changes in gene expression seen at Sites E and A, both in the liver and in the testis were related to protein synthesis at multiple levels (initiation of translation, aminoacylation of tRNAs, ribosome biogenesis, etc). This suggests an acute response to stress, potentially through the mTOR (metazoan target of rapamycin) pathway. The mTOR signaling pathway is involved in regulating the protein translational machinery (Proud 2002), and has been shown to be inactivated by low nutrients, low energy reserves, and hypoxia, among other stressors (Fig 5 and (Wullschleger et al. 2006). The functional enrichment data for Site A identified 12 significantly enriched terms within the list of differentially regulated genes. Among these, tRNA aminoacylation was the most significantly enriched ($p = 0.006$). There are 45 genes on the array under this GO category, of which three were down-regulated and five were significantly ($p < 0.05$) up-regulated. In addition, other genes that could be related to the mTOR pathway have general effects on transcription, heat shock proteins, and hypoxia, among others.

While identifying changes in the expression of individual genes is not the overall goal of our microarray study, the four annotated genes that change consistently in FHM exposed to water from all four sites are relatively telling, as they relate to metal transport (albeit Zn, not a toxic metal), transcription, hypoxia and heat shock/protein folding. These findings are consistent with our enrichment analysis, and these may be important transcripts in the overall response to water from these four sites. HIF-1a is an interesting gene in particular because it is induced in response to hypoxia, and can

down-regulate the mTOR pathway (Zacchia et al. 2011; Wouters and Koritzinsky 2008). We have no reason to believe that any of the animals in our study were exposed to ambient hypoxic conditions because water samples from the sites were brought into the laboratory and aerated during exposure for all treatments and controls. However, it is possible that some characteristic of the effluent could inhibit proper oxygenation of the tissues, causing HIF-1a induction at the hepatocellular level. In mammals, HIF-1a induction has also been observed with acute hepatic toxicity (Ramadori et al. 2010; Wu et al. 2010), and therefore it is possible that the FHM exposed to the site waters are displaying preliminary signs of cytotoxicity in the liver. While all of these gene changes suggest that the mTOR pathway is affected, this hypothesis needs to be further tested in future work.

The testis is the major site of steroidogenesis and gamete maturation leading to reproduction in fish and thus is another organ that is essential for toxicity testing. Analysis of changes in gene expression in the testis suggests that the biological processes most affected in fish exposed to Sites E and A are related to transcription, translation, and stress response, which are similar effects to those observed in the liver microarray experiment. Again, however, there is considerable difference among sites. Changes in signaling and metabolism transcripts at site B indicate a possible response to stressors in the testis. The effects on the cell cycle at site D may be of particular interest due to the nature of testis tissue, in which mitosis and meiosis are factors in spermatogenesis. However, there is no direct indication of a significant negative impact on spermatogenesis-related transcripts in the testis enrichment analysis, and in the

absence of histological data, it is not possible to know whether site waters could negatively impact spermatogenesis.

Genes associated with stress response at site E included erythropoietin (two probes, one up- and one down-regulated), proliferating cell nuclear antigen (up-regulated), and myoglobin (down-regulated). Interestingly, all three of these transcripts were up-regulated in a study of zebrafish *Danio rerio* muscle response to endurance training (van der Meulen et al. 2005). Therefore, aberrant expression of these transcripts may be evidence of tissue reorganization.

Overall, the enrichment analysis consistently indicates that effects on transcription, translation, signaling, and metabolism are present in both testis and liver, and this may indicate a generalized stress response. The testis effects are more diffuse than liver effects, but include indicators of stress response and modulation of cell cycle control. The liver data meanwhile provide several key clues that exposure to site waters may result in transcriptional response consistent with liver damage. Though our contaminant analysis did not identify any smoking guns, it is possible that the combined effects of contaminants found in these site waters could be a net hepatotoxicity. This indicates a stronger impact than the effect of site waters on the testis; we have no direct transcriptional evidence of effects on spermatogenesis. Therefore, liver toxicity appears to be the major concern for fish exposed to waters from the sites included in the present study.

5. Conclusions

546 The Okaloosa darter is threatened in its home streams which run adjacent to the
547 main populated area of Eglin Air Force Base. Water quality measurements in East
548 Turkey Creek suggest that anthropogenic influences have negatively impacted the
549 stream system. Treated sewage water is sprayed on the land that surrounds sites E
550 and A, creating the possibility that some residues from this water make their way into
551 East Turkey Creek. Gene expression changes from microarray analysis demonstrated
552 that major biological processes were affected in fish exposed to waters from the creek
553 influenced by the spray fields, suggesting a stress response in both liver and testis, and
554 possibly early liver damage. However, a limited analytical examination of possible
555 contaminants including analysis of metal burdens in fish and passive sampling for
556 nonpolar organic (SPMD) and polar organic (POCIS) analytes failed to identify the
557 causative factors. This failure demonstrates the importance of a multifaceted approach
558 when attempting to reveal and understand complex ecological issues, and it highlights
559 the importance of bioassays in the assessment of contaminants in aquatic
560 environments. Results of these analyses have provided insight into the water quality
561 degradation in East Turkey Creek. Based on these data, recommendations have been
562 made for additional studies to improve understanding of water quality issues and adapt
563 management strategies to promote recovery of the Okaloosa darter.
564

565 **Acknowledgements**

566 This work was supported by Research Work Order 247 on the FL Cooperative
567 Fish and Wildlife Research Unit (US Geological Survey, University of Florida, FL Fish
568 and Wildlife Conservation Commission, US Fish and Wildlife Service, and Wildlife

569 Management Institute cooperating). We would like to thank Li Liu for expert help with
570 the fathead minnow annotation and microarray design and Carolyn Diaz for analytical
571 analyses.

572

572 **References**

573 Aguayo, S.,Munoz, M. J.,de la Torre, A.,Roset, J.,de la Pena, E.Carballo, M., 2004.
574 Identification of organic compounds and ecotoxicological assessment of sewage
575 treatment plants (STP) effluents. Sci Total Environ 328, 69-81.
576 Alvarez, D. A.,Cranor, W. L.,Perkins, S. D.,Schroeder, V. L.,Iwanowicz, L. R.,Clark, R.
577 C.,Guy, C. P.,Pinkney, A. E.,Blazer, V. S.Mullican, J. E., 2009. Reproductive
578 health of bass in the Potomac, U.S.A., drainage: part 2. Seasonal occurrence of
579 persistent and emerging organic contaminants. Environ Toxicol Chem 28, 1084-
580 1095.
581 Brazma, A.,Hingamp, P.,Quackenbush, J.,Sherlock, G.,Spellman, P.,Stoeckert,
582 C.,Aach, J.,Ansorge, W.,Ball, C. A.,Causton, H. C.,Gaasterland, T.,Glenisson,
583 P.,Holstege, F. C.,Kim, I. F.,Markowitz, V.,Matese, J. C.,Parkinson, H.,Robinson,
584 A.,Sarkans, U.,Schulze-Kremer, S.,Stewart, J.,Taylor, R.,Vilo, J.Vingron, M.,
585 2001. Minimum information about a microarray experiment (MIAME)-toward
586 standards for microarray data. Nat Genet 29, 365-371.
587 Burkhead, N. M.,Jelks, H.,Jordan, F.,Weaver, D. C.,Williams, J., 1994. The comparative
588 ecology of Okaloosa and brown darters in Boggy and Rocky Bayou stream
589 systems, Choctawhatchee Bay, Florida. Final Report to Eglin Air Force Base.**:**
590 90.
591 Burkhead, N. M.,Williams, J. D.Yerger, R. W., 1992. Endangered Okaloosa darter
592 in: C. R. Gilbert (Eds.) Book Endangered Okaloosa darter University Press of
593 Florida Gainesville, FL, p.23-30.
594 Collette, B. B.Yerger, R. W., 1962. The American percid fishes of the subgenus *Villora.*
595 Tulane Studies in Zoology 9, 213-230.
596 Creed, J. T.,Brockhoff, C. A.Martin, T. D., 1994. Method 200.8. Determination of Trace
597 Elements in Waters and Wastes by Inductively Coupled Plasma-Mass
598 Spetrometry. Cincinati, Ohio, U.S. EPA.
599 CWA, 1972. Clean Water Act (CWA). P.L. 92500, 86 Stat. 816,
600 http://www.epa.gov/agriculture/lcwa.html
601 Diatchenko, L.,Lau, Y. F.,Campbell, A. P.,Chenchik, A.,Moqadam, F.,Huang,
602 B.,Lukyanov, S.,Lukyanov, K.,Gurskaya, N.,Sverdlov, E. D.Siebert, P. D., 1996.
603 Suppression subtractive hybridization: a method for generating differentially
604 regulated or tissue-specific cDNA probes and libraries. *Proceedings of the*
605 *National Academy of Sciences U S A* 93, 6025-6030.
606 Doherty, F. G.,Qureshi, A. A.Razza, J. B., 1999. Comparison of the Ceriodaphnia dubia
607 and Microtox((R)) inhibition tests for toxicity assessment of industrial and
608 municipal wastewaters. Environmental Toxicology 14, 375-382.
609 Eisen, M. B.,Spellman, P. T.,Brown, P. O.Botstein, D., 1998. Cluster analysis and
610 display of genome-wide expression patterns. Proc Natl Acad Sci U S A 95,
611 14863-14868.
612 Fent, K.,Weston, A. A.Caminada, D., 2006. Ecotoxicology of human pharmaceuticals.
613 Aquat Toxicol 76, 122-159.
614 Garcia-Reyero, N.,Barber, D. S.,Gross, T. S.,Johnson, K. G.,Sepulveda, M. S.,Szabo,
615 N. J.Denslow, N. D., 2006. Dietary exposure of largemouth bass to OCPs

changes expression of genes important for reproduction. Aquat Toxicol 78, 358-369.

Garric, J.,Vollat, B.,Nguyen, D. K.,Bray, M.,Migeon, B.Kosmala, A., 1996. Ecotoxicological and chemical characterization of municipal wastewater treatment plant effluents. Water Science and Technology 33, 83-91.

Gelsleichter, J.,Walsh, C. J.,Szabo, N. J.Rasmussen, L. E., 2006. Organochlorine concentrations, reproductive physiology, and immune function in unique populations of freshwater Atlantic stingrays (*Dasyatis sabina*) from Florida's St. Johns River. . Chemosphere 63, 1506-1522.

Iwanowicz, L. R.,Blazer, V. S.,Guy, C. P.,Pinkney, A. E.,Mullican, J. E.Alvarez, D. A., 2009. Reproductive health of bass in the Potomac, U.S.A., drainage: part 1. Exploring the effects of proximity to wastewater treatment plant discharge. Environ Toxicol Chem 28, 1072-1083.

Kosmala, A.,Charvet, S.,Roger, M. C.Faessel, B., 1999. Impact assessment of a wastewater treatment plant effluent using instream invertebrates and the Ceriodaphnia dubia chronic toxicity test. Water Research 33, 266-278.

Mettee, M. F.Crittenden, E., 1979. A study on the distribution of Etheostoma okaloosae (Fowler) and Etheostoma edwini (Hubbs and Cannon) in the Swift and Rocky Creeks, Okaloosa and Walton Counties, Florida, during 1975-1978. , US Fish and Wildlife.

Middaugh, D. P.,Beckham, N.,Fournie, J. W.Deardorff, T. L., 1997. Evaluation of bleached kraft mill process water using Microtox(R), Ceriodaphnia dubia, and Menidia beryllina toxicity tests. Arch Environ Contam Toxicol 32, 367-375.

Nakada, N.,Nyunoya, H.,Nakamura, M.,Hara, A.,Iguchi, T.Takada, H., 2004. Identification of estrogenic compounds in wastewater effluent. Environ Toxicol Chem 23, 2807-2815.

Nelson, R. E.,Grebe, S. K.,DJ, O. K.Singh, R. J., 2004. Liquid chromatography-tandem mass spectrometry assay for simultaneous measurement of estradiol and estrone in human plasma. Clin Chem 50, 373-384.

Ogilvie, V. E., 1980. Unpublished Florida Game and Freshwater Fish Commission Endangered Wildlife Project E-1. Annual Progress Report. Tallahassee, Florida US Fish and Wildlife.

Pait, A. S.Nelson, J. O., 2002. Endocrine disruption in fish: Assessment of recent research and results. NOAA Technical Memorandum NOS NCCOS CCMA 149. Silver Spring, MD, USA., NOAA, NOS, Center for Coastal Monitoring and Assessment.

Proud, C. G., 2002. Regulation of mammalian translation factors by nutrients. Eur J Biochem 269, 5338-5349.

Ramadori, P.,Sheikh, N.,Ahmad, G.,Dudas, J.Ramadori, G., 2010. Hepatic changes of erythropoietin gene expression in a rat model of acute-phase response. Liver Int 30, 55-64.

Saldanha, A. J., 2004. Java Treeview--extensible visualization of microarray data. Bioinformatics 20, 3246-3248.

Thom, T.Herod, J., 2005. Aquatic inventory of Eglin Air Force Base and recommendations for long-term aquatic ecosystem management. Report

prepared for Eglin Air Force Base, Natural Resources Management Branch, U.S. Fish and Wildlife Service.

U.S.-EPA, 1986. Quality Criteria for Water. Washington, DC., Office of Water Regulations and Standards (Gold book; EPA 440/5-86-001; www.epa.gov/ waterscience/ <http://www.epa.gov/waterscience/>criteria)

U.S.-EPA, 1994. Method 7742: Selenium (Atomic Absorption, Borohydride Reduction).

U.S.-EPA, 2001. Fact Sheet: Mercury Update: Impact on Fish Advisories. Washington, DC, Office of Water.

U.S.-EPA, 2002. Method 1631, Revision E: Mercury in Water by Oxidation, Purge and Trap, and Cold Vapor Atomic Fluorescence Spectrometry

U.S.-EPA, 2007. Method 6020A.

U.S.-FWS, 1973. Conservation of endangered species and other fish or wildlife. Federal Register 38, 106.

U.S.-FWS, 1998a. Okaloosa Darter (*Etheostoma okaloosae*) Recovery Plan (Revised). Atlanta, Georgia, U.S. Fish and Wildlife Service 42.

U.S.-FWS, 1998b. A contaminants survey of three lentic systems within the Cypress Creek Watershed, Texas 1993 - 1995. . Arlington Ecological Services Field Office, Arlington, Texas, U.S. Fish and Wildlife Service (Region 2)**:** 20-25.

van der Meulen, T.,Schipper, H.,van Leeuwen, J. L.Kranenbarg, S., 2005. Effects of decreased muscle activity on developing axial musculature in nicb107 mutant zebrafish (Danio rerio). J Exp Biol 208, 3675-3687.

Villeneuve, D. L.,Garcia-Reyero, N.,Martinovic, D.,Cavallin, J. E.,Mueller, N. D.,Wehmas, L. C.,Kahl, M. D.,Linnum, A. L.,Perkins, E. J.Ankley, G. T., 2010. Influence of ovarian stage on transcript profiles in fathead minnow (Pimephales promelas) ovary tissue. Aquat Toxicol 98, 354-366.

Webber, H. M.Haines, T. A., 2003. Mercury effects on predator avoidance behavior of a forage fish, golden shiner (Notemigonus crysoleucas). Environ Toxicol Chem 22, 1556-1561.

Wouters, B.G., Koritzinsky, M. 2008. Hypoxia signalling through mTOR and the unfolded protein response in cancer. Nat Rev Cancer. 8,:851-864.

Wu, Y. L.,Jiang, Y. Z.,Jin, X. J.,Lian, L. H.,Piao, J. Y.,Wan, Y.,Jin, H. R.,Joon Lee, J.Nan, J. X., 2010. Acanthoic acid, a diterpene in Acanthopanax koreanum, protects acetaminophen-induced hepatic toxicity in mice. Phytomedicine 17, 475-479.

Wullschleger, S.,Loewith, R.Hall, M. N., 2006. TOR signaling in growth and metabolism. Cell 124, 471-484.

Zacchia, M.,Vilasi, A.,Capasso, A.,Morelli, F.,De Vita, F.Capasso, G., 2011. Genomic and proteomic approaches to renal cell carcinoma. J Nephrol 24, 155-164.

Figure 1. Water collection sites on Eglin Air Force Base, FL. Stars indicate the location of the sites. Collection sites A, E, and B are on East Turkey Creek, whereas sites D and G are located on Long Creek. Large areas indicated in light green show the location of the spray fields flanking East Turkey Creek. The topography along East Turkey Creek is such that runoff from the sprayfields flows into the creek. Map has been modified from www.maps.google.com and the Eglin Air Force Base 2005-2006 Outdoor Recreation, Hunting, and Freshwater Fishing Map.

Figure 2. Bi-directional hierarchical cluster analysis of gene expression changes in (A) the liver and (B) the testis. Blue indicates down-regulation relative to UF control and red indicates up-regulation relative to UF control. Fathead minnows were exposed for 48 h to waters from Sites A, B, D and E as well as to control water (UF). Each row represents a different array, and each column is a transcript that is differentially regulated in at least one site versus the control.

Figure 3. Fingerprint diagrams of overall gene expression in fathead minnow liver. (A) Site D, (B) Site B, (C) Site E, and (D) Site A gene expression relative to the control, as determined by microarray. The genes were ordered according to their fold difference at site D (most up-regulated to most down-regulated) and then at each of the remaining sites in order ($p < 0.05$). If expression of a gene was not significantly altered at a particular site, its expression level was set to zero.

723 **Figure 4.** Fingerprint diagrams of overall gene expression in fathead minnow testis.
724 (A) Site D, (B) Site B, (C) Site E, and (D) Site A gene expression relative to control, as
725 described for Figure 3.
726
727 **Figure 5.** Diagram of the mTOR pathway showing how environmental factors may
728 generally influence transcriptional and translational processes in tissues. Adapted from
729 (Wullschleger et al. 2006).
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745

745 **Table 1. Biological processes GO categories in the liver that were significantly**
746 **altered (P<0.05) by water collected at sites D, B, E and A**

GO ID	Biological Process	Not DR	DR	p-value	Type
	Site D				
GO:0006355	Regulation of transcription, DNA-dependent	803	47	0.012	enriched
GO:0006325	Establishment and/or maintenance of chromatin architecture	5	2	0.028	enriched
GO:0030097	Hemopoiesis	15	3	0.031	enriched
GO:0006412	Translation	188	2	0.036	under-rep
GO:0008203	Cholesterol metabolic process	7	2	0.045	enriched
	Site B				
GO:0007411	Axon guidance	13	4	0.007	enriched
GO:0030574	Collagen catabolic process	25	5	0.012	enriched
GO:0006353	Transcription termination	192	18	0.013	enriched
GO:0006897	Endocytosis	32	5	0.029	enriched
GO:0007266	Rho protein signal transduction	5	2	0.040	enriched
GO:0050930	Induction of positive chemotaxis	5	2	0.040	enriched
	Site E				
GO:0042254	Ribosome biogenesis and assembly	11	3	0.012	enriched
GO:0006364	rRNA processing	18	3	0.036	enriched
GO:0016998	Cell wall catabolic process	7	2	0.038	enriched
GO:0043087	Regulation of GTPase activity	19	3	0.040	enriched
	Site A				
GO:0006418	tRNA aminoacylation for protein translation	37	8	0.006	enriched
GO:0000059	Protein import into nucleus, docking	11	4	0.012	enriched
GO:0006413	Translational initiation	19	5	0.015	enriched
GO:0006338	Chromatin remodeling	7	3	0.021	enriched
GO:0015914	Phospholipid transport	7	3	0.021	enriched
GO:0051056	Regulation of small GTPase mediated signal transduction	30	6	0.023	enriched
GO:0048484	Enteric nervous system development	8	3	0.028	enriched
GO:0040007	Growth	9	3	0.035	enriched
GO:0008285	Negative regulation of cell proliferation	26	5	0.042	enriched
GO:0006821	Chloride transport	18	4	0.045	enriched
GO:0045893	Positive regulation of transcription, DNA-dependent	18	4	0.045	enriched
GO:0006412	Translation	171	19	0.047	enriched

747
748

748 **Table 2.** Biological processes GO categories of testis that were significantly (P<0.05) by
749 water collected at sites D, B, E and A

GO ID	Biological Process	Not DR	DR	p-value	Type
	Site D				
GO:0035050	Embryonic heart tube development	12	4	0.001	Enriched
GO:0001525	Angiogenesis	9	3	0.003	Enriched
GO:0006811	Ion transport	163	10	0.014	Enriched
GO:0007160	Cell-matrix adhesion	22	3	0.026	Enriched
GO:0009117	Nucleotide metabolic process	9	2	0.031	Enriched
GO:0009617	Response to bacterium	10	2	0.037	Enriched
GO:0045786	Negative regulation of cell cycle	28	3	0.045	Enriched
GO:0007050	Cell cycle arrest	29	3	0.048	Enriched
	Site B				
GO:0006644	Phospholipid metabolic process	12	4	0.002	Enriched
GO:0016042	Lipid catabolic process	14	4	0.003	Enriched
GO:0006468	Protein amino acid phosphorylation	453	6	0.004	Under-rep
GO:0009968	Negative regulation of signal transduction	137	0	0.010	Under-rep
GO:0045786	Negative regulation of cell cycle	27	4	0.024	Enriched
GO:0035023	Regulation of Rho protein signal transduction	42	5	0.026	Enriched
GO:0006182	cGMP biosynthetic process	6	2	0.031	Enriched
GO:0001756	Somitogenesis	20	3	0.048	Enriched
	Site E				
GO:0045449	Regulation of transcription	244	22	0.007	Enriched
GO:0006950	Response to stress	16	4	0.011	Enriched
GO:0006270	DNA replication initiation	9	3	0.015	Enriched
GO:0006816	Calcium ion transport	144	1	0.015	Under-rep
GO:0009190	Cyclic nucleotide biosynthetic process	5	2	0.037	Enriched
GO:0000723	Telomere maintenance	6	2	0.048	Enriched
GO:0030318	Melanocyte differentiation	6	2	0.048	Enriched
	Site A				
GO:0042254	Ribosome biogenesis and assembly	11	3	0.015	Enriched
GO:0006886	Intracellular protein transport	126	11	0.020	Enriched
GO:0007018	Microtubule-based movement	42	5	0.032	Enriched
GO:0006898	Receptor-mediated endocytosis	6	2	0.035	Enriched
GO:0006364	rRNA processing	18	3	0.044	Enriched

750
751

Highlights

- The habitat of the Okaloosa darter appears to be degraded by the presence of waste water sprayfields adjacent to East Turkey Creek at Eglin Airforce Base
- Wastewater sprayfields can permeate the creek and alter gene expression in test fish as measured by microarray analysis.
- Exposure of fish to the creek waters for 48 h induces changes in pathways related to general stress.