Abstract

Evolutionary solutions to the physiological challenges of life in highly variable habitats can span the continuum from evolution of a cosmopolitan plastic phenotype to the evolution of locally adapted phenotypes. Killifish (*Fundulus* sp.) have evolved both highly plastic and locally adapted phenotypes within different selective contexts, providing a comparative system in which to explore the genomic underpinnings of physiological plasticity and adaptive variation. Importantly, extensive variation exists among populations and species for tolerance to a variety of stressors, and we exploit this variation in comparative studies to yield insights into the genomic basis of evolved phenotypic variation. Notably, species of *Fundulus* occupy the continuum of osmotic habitats from freshwater to marine and populations within *Fundulus heteroclitus* span far greater variation in pollution tolerance than across all species of fish. Here, we explore how transcriptome regulation underpins extreme physiological plasticity on osmotic shock and how genomic and transcriptomic variation is associated with locally evolved pollution tolerance. We also show that convergent evolution of locally adapted pollution tolerance involves complex patterns of gene expression and genome sequence variation, which is confounded with body-weight dependence for some genes. Similarly, exploiting the natural phenotypic variation associated with other established and emerging model organisms is likely to greatly accelerate the pace of discovery of the genomic basis of phenotypic variation.

Key words: AFLPs, comparative transcriptomics, *Fundulus heteroclitus*, gene expression, phenotypic plasticity, microarrays, natural populations

*Fundulus heteroclitus* is a well-established model species in physiology, ecology, toxicology, and evolutionary biology and is an emerging model for research in environmental genomics. *Fundulus* species are characterized by their extreme euryhalinity, eurythermia, tolerance to hypoxia, and evolved tolerance to pollution. Much is known about their ecology and evolutionary history (Whitehead 2010), as well as their physiology, developmental biology, and molecular biology (Burnett et al. 2007). Populations occupy diverse ecological niches across a wide geographical distribution and along strong environmental clines in temperature, salinity, and pollution stress, thereby serving as models for studies in acclimation and adaptation. This wide range of beneficial characteristics positions *F. heteroclitus* to serve as an important model for studying the role of gene expression variation in physiological acclimation and evolutionary adaptation in diverse environments.

The estuaries in which *F. heteroclitus* have evolved and thrive are subject to multiple environmental extremes. The evolution of physiological resilience and alternately the evolution of locally adapted phenotypes are different evolutionary solutions that may have enabled persistence in these dynamic environments. Whether or not physiological resilience or local adaptation evolves depends partly on the spatial and temporal scale at which environmental extremes are experienced in the lifetimes of individuals. Interestingly, *F. heteroclitus* exhibit plastic phenotypes to compensate for some environmental stressors that are experienced sporadically but locally adapted phenotypes to compensate for other environmental stressors that are experienced continuously.
Here, we focus on this species’ contrasting evolutionary solutions to 2 different types of stressors: natural osmotic stress and anthropogenic pollution.

Within estuaries, *F. heteroclitus* populations occupy habitats from the freshwater (FW) to seawater extremes of the salinity continuum and demonstrate one of the widest ranges of osmotic compensatory abilities among fishes (Griffith 1974; Whitehead 2010). This extraordinary plasticity presumably evolved to enable exploitation of a wide niche breadth within dynamic coastal habitats. *Fundulus heteroclitus* populations also live and thrive in highly human-altered habitats, including US Environmental Protection Agency (EPA) designated Superfund toxic waste sites, which are contaminated with industrial pollution such as dioxin-like compounds and polycyclic aromatic hydrocarbons (PAHs). Relative to other species, *F. heteroclitus* are sensitive to dioxin-like compounds; however, some local populations have derived dramatic tolerance to these pollutants, allowing them to survive when exposed to concentrations of chemicals orders of magnitude beyond those that are lethal to populations resident in clean habitats (Van Veld and Nacci 2008; Nacci et al. 2010). In contrast to osmotic tolerance, this pollution tolerance is not a plastic trait but rather appears derived and fixed in different populations and is heritable.

*Fundulus* populations exhibit both physiological and adaptive responses to cope with the variable environments they inhabit. They have been used to explore the role of gene expression variation in physiological acclimation and evolutionary adaptation in diverse environments (Oleksiak et al. 2002; Oleksiak et al. 2005; Whitehead and Crawford 2005, 2006a, 2006b; Fisher and Oleksiak 2007; Oleksiak 2008; Bozinovic and Oleksiak 2010). We present new data on physiological acclimation to osmotic stress and further analysis of published data on adaptation to pollution to highlight the utility of *F. heteroclitus* for work on ecological genomics and genetics.

**Materials and Methods**

**Acclimation**

*Fundulus heteroclitus* from coastal New Hampshire were acclimated to 32 ppt artificial seawater for at least 3 months before experiments. Hypo-osmotic challenge involved transfer of acclimated fish to FW (reverse osmosis water reconstituted with ions to achieve final concentrations of NaCl to 400 μM, CaCl₂ to 350 μM, KCl to 50 μM, and MgCl₂ to 50 μM, and a pH of 7.1). This is a severe osmotic challenge but is ecologically relevant as salinities can fluctuate widely and quickly in Atlantic coast estuaries (e.g., Sanders et al. 1965). Six fish were sampled posttransfer at 6, 24, 72, 168, and 336 h, including a 32 ppt pretransfer control (considered 0 h).

Sampled fish were sacrificed by cervical dislocation, blood isolated from the caudal vein in heparinized capillary tubes and immediately centrifuged to isolate plasma, which was measured for osmolality by freeze-point depression (μOsmette, Precision Systems Natick, MA), Na⁺ by flame atomic absorption spectroscopy (Varian AA240FS, Melbourne, Australia), and Cl⁻ using a modification of the mercuric thiocyanate method (Zall et al. 1956). Gills were excised and preserved in RNAlater (Ambion Inc.). RNA was extracted from gill using Trizol reagent and antisense RNA (aRNA) prepared using the MessageAmp II aRNA amplification kit (Ambion), coupled with Alexa fluor dyes (Alexa flour 555 and 647; Molecular Probes, Inc.), competitively hybridized to custom oligonucleotide microarrays, enclosed in a SureHyb microarray hybridization chamber (Agilent Technologies), and incubated for 18 h at 60 °C. Microarray probes were designed using OligoWiz software (Wernersson et al. 2007) from *F. heteroclitus* expressed sequence tags (Paschall et al. 2004), with preference for probe position toward the 3’ end of target sequences. Custom arrays (Agilent 8X15K element design; design ID 021434) included probes for 6800 elements, each printed in duplicate, plus control elements. Five samples (biological replicates) per time point were competitively hybridized in a balanced loop design, where paired samples were balanced across treatments, including a dye swap.

Raw data were log₂ transformed, lowest normalized in JMP-Gemomics (SAS, Inc.), then normalized using mixed linear models. Of the 6800 unique probes on the array, 624 were excluded because of spot quality or hybridization intensity problems, leaving 6176 probes included in the final analysis. Median intensities for duplicate spots within an array/channel were averaged, and differential expression for each gene across time points was detecting using linear mixed models with false discovery rate (FDR) estimated using QVALUE (Storey and Tibshirani 2003). Genes showing a main effect of time (*P* < 0.01; *q* < 0.06) were then examined in more detail. First, principal components analysis was applied (JMP-Genomics; SAS, Inc.) to summarize the trajectory of gene expression change through time, and mean expression levels for each gene at each time point were regressed against each of the first 3 principal components to identify the genes most significantly associated with the major trajectories of transcriptome response through time. Gene-annotation enrichment analysis was performed using the Functional Annotation Clustering tool within the DAVID Bioinformatics Database (Huang et al. 2009; http://david.abcc.ncifcrf.gov/home.jsp).

**Pollution and Body Weight**

Genes previously identified as significantly differentially expressed in polluted versus flanking reference sites by analysis of covariance with log₁₀ body weight as the covariate (Oleksiak 2008) were clustered used Cluster 3.0 for Mac OS X (de Hoon et al. 2004) and Java TreeView version 1.0.8 (Saldanha 2004). Volcano plots (Figure 4) were plotted in MatLab version 7.2.

**AFLP Analyses**

Amplified fragment length polymorphism (AFLP) analyses were performed on each of the 3 polluted populations and
2 flanking reference populations (9 populations total, 288 individuals) as described (Williams and Oleksiak 2008). \( F_{ST} \) and allele frequency values were calculated for each AFLP locus for each polluted site compared with its respective pooled reference sites. Based on an Island model and an infinite alleles mutational model (Beaumont and Nichols 1996), we used Winkles software to model the 99th quantile of neutral \( F_{ST} \) values along all allele values. Five simulations were run on each pairwise comparison of a polluted site and 2 pooled reference sites to generate an expected null distribution of 25,000 values. Each simulation started with 500 simulation biallelic loci in each of the 2 populations with uniform random distribution and was allowed to drift for 10N generations. The 99th percentile of \( F_{ST} \) values within each of the 40 binned mean allele frequency values (each bin representing a set of 0.025 frequency values from 0 to 1) was calculated after removing monomorphic loci because \( F_{ST} \) is strongly dependent on allele frequencies (Beaumont and Nichols 1996).

Results and Discussion

Time Course Analysis of the Compensatory Response to Extreme Hypo-Osmotic Stress

Osmotic tolerance varies extensively among populations and species of Fundulus. For example, northern coastal populations of *F. heteroclitus* are more tolerant of hypo-osmotic stress than southern coastal populations (Scott et al. 2004), and populations of *F. heteroclitus* and *F. majalis* distributed along habitat salinity clines also exhibit within-species variations in osmotic tolerance (Whitehead A, Galvez F, Zhang S, unpublished data). In addition to population variation, species within the genus occupy habitats that span the continuum of salinities from FW, to brackish, to marine (Whitehead 2010). Importantly, different taxa occupy salinity habitats that are temporally and spatially stable (FW, marine) but also highly variable (estuaries). Differences in variability among these habitats have presumably driven evolved differences in physiological plasticity among species. Species that occupy highly dynamic estuaries have evolved extraordinary physiological abilities to compensate for extremes in osmotic shock, whereas species that occupy more stable FW or marine habitats exhibit limited physiological plasticity (Griffith 1974). Here, we present physiological and transcriptomic responses that underpin the compensatory acclimation response to a large change in environmental salinity in likely the most plastic *Fundulus* taxon: a northern coastal population of *F. heteroclitus*.

Physiological Plasticity

*Fundulus heteroclitus* from coastal New Hampshire proved highly resilient to extreme hypo-osmotic shock. Abrupt transfer from 32 ppt to FW initially resulted in loss of osmotic equilibrium in blood plasma, but equilibrium was quickly restored by 72 h posttransfer (Figure 1). Plasma osmolality dropped from 383 mOsM (pretransfer control) to 344 mOsM by 6 h, reached a minimum of 326 mOsM by 24 h (15% reduction from seawater controls) but recovered back to control levels by 72 h. Restoration of plasma osmolality after only 3 days post-FW transfer is likely attributed to the coordinated actions of the kidney, which would function to produce a hypo-osmotic urine, the reduction of water intake at the intestine, and most importantly, the transformation of the gill from a salt secreting to a salt absorbing epithelium. Plasma sodium
levels followed a similar pattern, dropping by approximately 15% from seawater controls by 24 h. Remarkably, *F. heteroclitus* retained plasma chloride equilibrium for at least 14 days after abrupt transfer from 32 ppt to FW. Scott et al. (2004) also found that northern *F. heteroclitus* populations were able to regulate Cl\(^-\) balance more tightly than Na\(^+\) levels after hypo-osmotic shock. This effect was attributed to the preferential regulation of paracellular Cl\(^-\) loss across epithelial tight junctions, helping to counteract the absence of unidirectional Cl\(^-\) uptake in the fish gill of *F. heteroclitus* (Patrick et al. 1997; Scott et al. 2004).

**Transcriptome Plasticity**

Four-hundred and ninety-eight genes (8.1%, \(P < 0.01\), FDR \(= 0.06\)) showed a significant change in expression during the time course of hypo-osmotic shock and acclimation. The primary temporal pattern of transcriptome response to extreme hypo-osmotic shock was characterized by a dramatic rapid response, followed by more subtle changes at later time points (Figure 2). The first principal component (PC) accounted for 89.1% of transcriptome variation among time points, whereas the second and third PCs explained 5.9% and 3.4%, respectively. PC1 describes the dominant pattern throughout the time course and clearly indicates the most dramatic transcriptome changes during the first 6 h posttransfer and smaller changes through to 7 days (168 h) posttransfer. Similarly, the most dramatic transcriptome changes were within the first 6 h for genes comprising PC2 and PC3. Together, the first 3 PCs account for 98.4% of the gene expression variation throughout the timecourse, and though PCs are orthogonal and independent, a common theme for each PC is large changes during the first few hours of osmotic challenge.

Two phases are apparent in the regulatory response of euryhaline fishes following osmotic shock. Phase one typically involves early rapid sensing of an extracellular hypotonicity, which triggers a stretch-induced alteration in transcription factor and signaling cascade activity. These events activate the second phase, which is associated with regulation of cellular effectors, functioning to return the cell to a state of osmotic homeostasis. This entails stabilizing the intracellular environment by protecting DNA and proteins during the initial hypo-osmotic conditions, then extensive remodeling of epithelial cells and tissues to restore cell volume and develop an FW phenotype (Fiol and Kultz 2007; Evans and Somero 2008).

**Phase One of Compensatory Response: Sensing and Early Effector Signaling**

Genes associated with the phase one sensing and signaling are expected to be upregulated strongly, early, but transiently. Indeed, the cluster of genes indicating such a temporal pattern (cluster data not shown) is enriched for gene ontology categories including nucleosome, intracellular signaling cascade, signal transduction, protein kinase activity, and negative regulation of cell differentiation (\(P < 0.05\)). Within this group, transcription factors including the recently discovered ostf1 (Fiol and Kultz 2005) and 14.3.3.a protein (Kultz et al. 2001) are both quickly but transiently upregulated reaching their maxima by 6 h (Figure 3A).

Phosphorylation cascades are major signal transduction pathways that are central to a cell’s ability to mount compensatory responses to external stress, including changes in osmolality (Kultz and Burg 1998), for example, by regulating apoptosis, cell cycle arrest, and cellular proliferation. Expression of mitogen activated protein kinase (MAPK) p38\(\gamma\) (SAPK-3) is highly correlated with PC1 (\(r = 0.95, P = 0.02\)), is induced early (Figure 3A), and leads to a dramatic, yet transient, increase in phosphorylated p38 MAPK in the opercular epithelium of *F. heteroclitus* (Hoffmann et al. 2007). Regulation of the MAPK p38 pathway is associated with mediation of DNA repair, cell cycle regulation, apoptosis, and negative regulation of cell proliferation by inhibiting the ERK1 pathway, and regulation of the p38\(\gamma\) isoform. Cyclin D1, which is necessary for cell cycle progression, is downregulated (Conrad et al. 1999). In addition, GADD45 is induced by the SAPK pathway (Kultz et al. 1998), is quickly, yet transiently upregulated, peaking at 6 h (Figure 3A), is highly correlated with PC3 (\(r = 0.93, P = 0.02\)), and is important in mediating apoptosis, DNA repair, and cell cycle arrest (Lieberman and Hoffman 2008).

Though the role of thyroid hormone signaling in osmoregulation is controversial (McCormick 2001), type I
Figure 3. Timecourse of log2 gene expression change on hypo-osmotic challenge in Fundulus heteroclitus gill, partitioned by functional category. 14.3.3.a, 14-3-3.a protein; APOC1, apolipoprotein C-I; APOM, apolipoprotein M; AQP-3, aquaporin-3; Arpc1a, actin-related protein 2/3 complex subunit 1A; ATP6V1E1, V-type proton ATPase subunit E 1; BCDO2, beta-carotene dioxygenase 2; CALM, calmodulin; CFTR, cystic fibrosis transmembrane conductance regulator; CLD3, claudin-3; CLD4, claudin-4; CMC1, calcium-binding mitochondrial carrier protein Aralar1; CP24A, 1,25-dihydroxyvitamin D(3) 24-hydroxylase; CX32, gap junction connexin-32.2 protein; CYLC1, cylicin-1; Dio1; DSC1, desmocollin-1; ECH1, delta(3,5)-delta(2,4)-dienoyl-CoA isomerase; ECHB, acetyl-CoA acyltransferase; ERG28, probable ergosterol biosynthetic protein 28; F16PA, fructose-1,6-bisphosphatase class 1; G6PI, glucose-6-phosphate isomerase; GADD45, growth arrest and DNA damage-inducible protein GADD45 beta; GSN, gelsolin; H1, histone H1; H2AX, histone H2A.x; H2B1, histone H2B.1; H4, histone H4; HMDH, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IMPA1, inositol monophosphatase; INO1, inositol-3-phosphate synthase; K6PF, 6-phosphofructokinase; KCRB, creatine kinase B-type; KCRM, creatine kinase M-type; KRT18, keratin, type I cytoskeletal 18; Marcks, myristoylated alanine-rich C-kinase substrate; MYL6, myosin light polypeptide 6; MYL7, myosin regulatory light chain 2, atrial isoform; NEFL, neurofilament light polypeptide; NKA, sodium/potassium-transporting ATPase subunit alpha-1; NKCC2, sodium/calcium exchanger 2; ODP2, pyruvate dehydrogenase complex E2 subunit; ODPB, pyruvate dehydrogenase E1 component subunit beta; Ostf1, Oreochromis mossambicus osmotic stress transcription factor 1; OTOP1, ototetrin-1; PGM2, phosphoglucomutase-2; PLCD1, phospholipase C-delta-1; PLEC1, plectin-1; PYGM, glycogen phosphorylase; REDD-1, DNA damage-inducible transcript 4 protein; RHOA, transforming protein RhoA; S10AD, S100 calcium-binding protein A13; SAPK-3, MAP kinase p38; SCMC2, small calcium-binding mitochondrial carrier protein 2; SIAS, sialic acid synthase; TBCA, tubulin-specific chaperone A; TnnT3, troponin T, fast skeletal muscle; TPI1, triosephosphate isomerase; TUB1, tubulin alpha chain.
iodothyronine deiodinase (Dio1) is upregulated over 2-fold by 24 h and over 3-fold by 72 h in F. heteroclitus gills after FW transfer (Figure 3A). This protein catalyzes the first step in the mechanism of thyroid hormone action and the promoter for the type II paralog expressed in F. heteroclitus liver encodes a response element binding site (Lopez-Bojorquez et al. 2007). The rapid induction of Dio1 that we observe supports the possibility that some compensatory transcriptional responses to hypo-osmotic stress may be regulated through thyroid hormones, well-known transcriptional mediators (Evans 1988), and may specifically regulate expression of the Na\(^+\),K\(^+\) ATPase transporter (Subash Peter et al. 2000), which is important for long-term osmotic acclimation.

Calcium signaling is likely an important signal transduction mechanism on osmotic stress in fish gills (Fiol et al. 2006; Fiol and Kultz 2007, but see (Marshall et al. 2000). Otopetrin 1 regulates intracellular calcium concentrations (Hughes et al. 2007) and is dramatically upregulated early in a pattern tightly correlated with PC2 (r = −0.94, P = 0.02) (Figure 3B), though the specific role of this gene in osmoregulation is unknown. Furthermore, genes of many calcium-interacting proteins are upregulated on hypo-osmotic stress (Figure 3B).

**Phase 2 of Compensatory Response: Cell Stabilization and Remodeling**

On acute hypo-osmotic shock, gill epithelia face a crisis of cell swelling that must be relieved quickly to prevent massive tissue injury. Rapid regulatory volume decrease is associated with basolateral transport of osmolytes out of the cell, which establishes the ionic gradient for water to then follow. This is largely achieved by nonregulatory mechanisms that are initiated within minutes, but if osmotic stress is sufficiently severe and of sufficient duration, gene regulatory mechanisms may contribute to the compensatory response. Indeed, one of the most dramatically regulated early-response genes is aquaporin-3, which is upregulated 16-fold by 6 h (Figure 3C), and is highly correlated with PC1 (r = 0.98, P < 0.01). Aquaporin-3 is permissive to water and perhaps small osmolytes (e.g., glycerol and urea) in F. heteroclitus embryos (Tingaud-Sequeira et al. 2009) and localizes to the basolateral chloride cell membrane in tilapia (Watanabe et al. 2005), implying that membrane insertion of aquaporin channels may be important for regulatory volume decrease (Cutler et al. 2007).

Gill epithelial restructuring is a well-known core component of the long-term osmotic acclimation strategy in euryhaline fishes. On hypo-osmotic challenge, our experimental fish transitioned from a seawater to an FW gill morphology by 3 days (data not shown). Interestingly, fish from populations less tolerant of FW exposure were unable to completely acquire an FW morphology within 14 days (data not shown). In our study, many genes associated with cell cycle regulation, cell growth, and cell morphology were upregulated early and transiently. As mentioned above, the p38 MAP kinase signaling pathway that regulates apoptosis and cell cycle is induced (Figure 3A). Also, DNA histones H1, H2B.2, and H14, were transiently upregulated up to 4.7-fold by 6 h, reaching their maxima of up to 6.5-fold induction by 24 h before declining (Figure 3D). Histones play a central role in chromatin condensation and regulation of transcription, DNA repair, and DNA replication. Interestingly, northern F. heteroclitus exposed to FW show a proliferation of cuboidal mitochondrion-rich cells in the gills, which are characterized by their unique peripheral chromatin distribution (Laurent et al. 2006). REDD-1 is a repressor of mTOR-mediated cell growth (Brugarolas et al. 2004), is quickly upregulated nearly 6-fold, and is highly correlated with PC3 (r = −0.95, P = 0.01), indicating a transient repression of cell growth (Figure 3D). Gill cell morphology changes dramatically during this tissue restructuring, and indeed, we detect many cytoskeleton-related genes transiently or permanently upregulated including actin-related proteins, myosin, tubulin, tropomin T, keratin, and plectin-1 (Figure 3E). Genes involved in polyamine synthesis are also strongly upregulated at early timepoints (P < 0.01; data not shown). Though polyamines serve diverse cellular functions (Childs et al. 2003), their role in hypo-osmoregulation in fish gill has not been described.

**Phase 2 of Compensatory Response: Osmotic Balance and Energetics**

In F. heteroclitus, the transition from seawater to FW requires transformation of active and passive mechanisms to maintain plasma sodium and chloride homeostasis (Evans et al. 2005). The seawater morphology supports paracellular loss of sodium from the blood driven by an electrochemical gradient established by active chloride extrusion. In acclimating to hypo-osmotic conditions, the epithelial morphology must change such that sodium is actively taken up from the environment, but because F. heteroclitus does not appear to actively take up chloride, regulation of chloride flux is thought to be primarily achieved by regulating paracellular loss (Laurent et al. 2006). The morphological transition that supports this physiological transition takes several days and represents a long-term strategy to deal with this altered environment. Accordingly, we observe a mix of early-timepoint changes in gene expression associated with shutting down the components of ion transport in seawater and later-timepoint regulation of genes that support FW-appropriate mechanisms. For example, the cystic fibrosis transmembrane regulator (CFTR) and the Na\(^+\),K\(^+\),2Cl\(^–\) cotransporter (NKCC), which together are responsible for moving chloride from plasma to the environment in hyper-osmotic conditions, are both permanently (at least more than 14 days hypo-osmotic exposure) downregulated by 6 and 24 h, respectively (Figure 3F), and CFTR expression is correlated with PC1 (r = 0.89, P = 0.04). Paracellular tight junctions are a major site of ion flux across gill epithelia and are likely particularly important for regulating chloride loss in FW because F. heteroclitus appear to lack active uptake mechanisms for this ion (Patrick et al. 1997; Scott et al. 2004; Laurent et al. 2006). Indeed, cell adhesion and tight junction proteins are quickly upregulated in response to
hypo-osmotic shock, including transcripts for proteins connexin-32.2, desmocollin-1, claudin-3, and claudin-4 (correlated with PC1; $r = 0.92$, $P = 0.03$) (Figure 3G). Synthesis of organic osmolytes such as myo-inositol is important for maintaining osmotic balance in hyper-osmotic conditions (Hochachka and Somero 2002), and we detect gradual downregulation of genes in the myo-inositol synthesis pathway (myo-inositol-1-phosphate synthase and inositol monophosphatase), appropriate for the transition to hypo-osmotic conditions (Figure 3F). In contrast to these rapid early-response mechanisms, those that reflect the altered function of the remodeled gill epithelium do not appear until later in the timecourse. For example, Na$^+$,K$^+$-ATPase (subunit $\alpha$-1) and V-type proton ATPase (subunit E1) are not upregulated until 72 and 168 h posttransfer, respectively (Figure 3F), likely reflecting the time required for the proliferation of FW-type mitochondrion-rich cells in the gill and their associated transporters.

Osmoregulation is a costly process in terms of energetics, requiring an estimated 6–10% of the total energy budget in $F$. heteroclitus (Kidder et al. 2006). The physiological transition during osmotic acclimation is likely to require significant energy, and this expectation is supported by consistent upregulation of genes involved in glycolysis, lipid metabolism, and creatine kinase proteins (Figure 3H–I).

**Genomic Variation among Locally Adapted Populations**

Although $F$. heteroclitus is able to physiologically acclimate to salinity challenges in a short time period, its tolerance to anthropogenic pollutants has evolved over time within locally altered habitats. Populations of $F$. heteroclitus inhabiting clean estuaries are relatively sensitive to dioxin-like compounds compared with most species tested and exhibit a narrow tolerance to these dioxin-like compounds (Van Veld and Nacci 2008). This sensitivity is in stark contrast to resistant populations of $F$. heteroclitus, which are more than 2 orders of magnitude more tolerant to environmental pollution stress than other fish species tested (Van Veld and Nacci 2008) and more than 3 orders of magnitude more tolerant than sensitive populations of the same species. That is, the variation in pollution tolerance among populations of $Fundulus$ far exceeds variation in tolerance among all species of fish tested.

Some of the more striking examples of locally adapted populations are populations inhabiting Superfund sites: sites identified by the EPA that contain high levels of a variety of lipophilic, persistent and toxic contaminants worthy of remediation using Federal funds (part of the federal government’s program to clean up the nation’s uncontrolled hazardous waste sites). Three notoriously polluted sites $F$. heteroclitus inhabit include New Bedford Harbor (Massachusetts), Newark Bay (New Jersey), and Elizabeth River (Norfolk, VA). New Bedford Harbor is polluted with extremely high levels of polychlorinated biphenyls (PCBs) (Pruell et al. 1990), as well as polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, PAHs, and several trace metals (Pruell et al. 1990; Bergen et al. 1998). Newark Bay is most notorious for containing 2,3,7,8-tetrachlorodibenzo-p-dioxin, as well as other dioxins (Prince and Cooper 1995; Weis 2002) and also is contaminated with heavy metals, pesticides, PCBs, and PAHs (Iannuzzi et al. 2005). The Elizabeth River is predominantly contaminated with creosote, comprised a complex mixture of PAHs (Bieri et al. 1986; Huggett et al. 1992; Padma et al. 1998).

$Fundulus heteroclitus$ from these chronically polluted areas are resistant to the pollutants in their environment as compared with nearby fish from relatively clean environments (Vogelbein et al. 1990; Black et al. 1998; Elskus et al. 1999; Nacci et al. 1999, 2002; Meyer and Di Giulio 2002, 2003; Ownby et al. 2002). Resistance in first and second generation embryos from New Bedford Harbor and Elizabeth River and first generation embryos from depurated Newark Bay fish suggests that differential survival is due to genetic adaptation rather than physiological induction. Investigating $F$. heteroclitus from these 3 sites and comparing them with $F$. heteroclitus from surrounding reference (clean) sites provides the opportunity to study similarities and differences in adaptation to differing chemical pollutants and resistance to general stress conditions among populations.

Two complementary genomic approaches have been taken to explore resistance in the New Bedford Harbor, Newark Bay, and Elizabeth River $F$. heteroclitus populations: microarrays to yield insights into the mechanistic basis of adaptive variation and AFLP analyses to yield insights into genomic regions that are evolving differently in pollution-tolerant populations. Both approaches used the same experimental design: contrasting individuals from each polluted site with individuals from 2 flanking reference sites, one north and one south of the polluted site. This triad design is experimentally robust (Oleksiak 2010) because it controls for both genetic drift and clinal effects (the well defined north–south divergence). It controls for genetic drift because without selection, one expects geography and genetic distance to be correlated. Thus, geographically more distant reference populations will be genetically less similar to each other than to the closer polluted population. Each triad comparison also controls for clinal effects by comparing the polluted population with both a northern and a southern population, relative to the polluted population. Thus, effects of pollution are no longer confounded with either genetic drift or the north–south cline. Hence, divergence in a polluted population compared with both paired reference populations suggests that pollution might be causative.

**Gene Expression Analyses among Polluted Populations**

Gene expression analyses using targeted metabolic arrays define sets of genes that are significantly differentially expressed in liver tissues in the 3 polluted populations as compared with clean reference populations (Figure 4). The New Bedford Harbor comparison identifies 16% of genes that are significantly different ($P < 0.01$) from both of the
clean reference sites, the Newark Bay comparison identifies 32% of genes, and the Elizabeth River comparison identifies 8% of total genes (Oleksiak 2008). Among the 3 polluted sites, these gene sets have little overlap: 3 genes are in common among all 3 sites, 8 genes are shared between Elizabeth River and New Bedford Harbor, 10 between New Bedford Harbor and Newark Bay, and 7 between Newark Bay and Elizabeth River (Oleksiak 2008). Yet, there are differences even among the overlapping genes. For instance, the 3 genes in common among all 3 sites are all more highly expressed in the Elizabeth River population and less highly expressed in the New Bedford Harbor and Newark Bay populations. Thus, even though the genes in common are all significantly differentially expressed in polluted versus reference populations, they do not show a similar response among the 3 polluted populations. This lack of overlap in either specific genes or direction of response could be due to differences in the complexity of the chemical pollutants or due to different independent evolutionary solutions or both.

A potentially confounding factor in comparisons among populations is the potential for physiological induction. Fish from all 9 populations were common gardened in the laboratory for 4 months to minimize physiologically induced differences due to, for example, temperature, salinity, and diet. Yet this common garden design cannot account for irreversible developmental effects and early environmental effects (Clutton-Brock et al. 1984). Furthermore, particular to these populations, this common garden design cannot fully control for differential body burdens of pollutants. A striking observation is the number of genes for which body weight significantly covaries with gene expression in these populations (Figure 4). Previous studies in *F. heteroclitus* from nonpolluted sites have shown no relationship between body weight and metabolic gene expression even among fish ranging in weight from 4.8 to 31.9 g (Oleksiak et al. 2005). The fish from the polluted triads have a similar range in size from 1.5 to 16.0 g. In these fish, likely body weight per se does not affect gene expression. More probably, the toxic load associated with body weight affects gene expression. This supposition is substantiated by the observation that body weight does not similarly affect the fish from the 3 different polluted populations: the fewest effects of body weight occur in the smallest fish, the Elizabeth River fish, where body weight is a significant covariate for only 14 genes (6%) compared with 28 (9%) in the New Bedford Harbor comparison and 89 (34%) in the Newark Bay comparison. The Elizabeth River fish have an average weight of 3.5 g compared with 7.1 and 12.5 g for New Bedford Harbor and Newark Bay fish, respectively. Although differential effects of body weight may also reflect site-specific differences in pollutants and resulting differences in solubility, bioavailability, susceptibility to degradation, and capacity for metabolism by *Fundulus*, the different numbers of genes affected by body weight coupled with the differently sized fish suggest that the 4-month

**Figure 4.** Gene expression differences between polluted and reference populations. Significances of differences as $-\log_{10}(P\text{ values})$ are plotted against $\log_{2}$ differences in expression for the New Bedford Harbor, Newark Bay, and Elizabeth River triads. Gray points are genes for which body weight is a significant covariate.
common garden period may have been insufficient to depurate many of these fish.

These patterns of gene expression suggest that significant divergence among populations is due to both physiological effects of long-term latency of pollutants and genetic divergence. One can infer that physiologically induced changes in gene expression are not solely responsible for the divergence among populations considering that when genes affected by body weight are removed, individuals in the Newark Bay comparison cluster into 2 groups: one the Newark Bay individuals and the other both the southern and northern reference site individuals (Figure 5). Thus, differentially expressed genes that are not affected by body weight show patterns of expression in which the polluted population is different from both reference populations even though the reference populations are more geographically distant from each other. These differences are potentially evolved.

**Genome Scans among Polluted Population**

Gene expression differences among polluted and paired reference populations may be due to both evolved and physiologically induced differences though these are not mutually exclusive. In contrast, population genomic approaches specifically target changes in the genome sequence. Population genomics can be broadly defined as the simultaneous study of numerous loci across a genome to better understand the effects of evolutionary processes that influence variation across genomes and populations, and one can use population genomic approaches to separate locus-specific effects such as selection from genome-wide effects such as genetic drift or bottlenecks (Luikart et al. 2003). A powerful population genomic approach for species without a sequenced genome is AFLPs analyses because one does not need prior sequence information for this technique (Vos et al. 1995). AFLP analyses target genetic differences among populations by definition (i.e., barring technical artifacts, different AFLP banding patterns are due to different genotypes). Thus, AFLP analyses, coupled with analyses to identify loci with nonneutral patterns, can be used to more directly identify evolved differences among populations.

Identification of evolved differences among populations due to chronic pollutant exposure was the goal of AFLP analyses using the same triad comparison described above. AFLP analyses were performed on each of the 3 polluted populations and 2 flanking reference populations (9 populations total, 288 individuals), and a modeling approach was used to identify loci with nonneutral patterns in polluted populations compared with the flanking reference populations (Williams and Oleksiak 2008, Figure 6).

Similar to the sets of different genes with expression differences in the 3 polluted populations, AFLP analyses also show largely unique genetic differences in the 3 polluted population comparisons. Among the 24 loci with nonneutral patterns in each polluted site compared with both reference sites (Figure 6) but not in comparisons between the reference site populations, only 2 loci are shared between the New Bedford Harbor and Elizabeth River triads and only 2 are shared between the Newark Bay and Elizabeth River triads. None is shared among all 3 triads. Notably, unlike the gene expression studies in these populations, the lack of a clear signal of convergence cannot be attributed to physiological effects due to the use of genomic DNA. Outlier loci that are shared between triads may be responsible for general evolved stress responses. Loci significant within a triad may be involved in a specific adaptive response to particular mixture of toxicants.

The strength of these analyses is that they account for demographic effects because the polluted and reference populations are interspersed along the east coast of the United States. This is especially important in the Newark Bay comparison because Newark Bay occurs on the historical break at the Hudson River (Powers et al. 1991). Based on single nucleotide polymorphism analyses, the Newark Bay population is more similar to the Northern haplotype, whereas its southern reference site groups with the Southern haplotype (Williams et al. 2010). This is unfortunate because signals due to pollutants can be overwhelmed by the strong North–South clinal signal. Yet even with the strong clinal signal, in both gene expression analyses and AFLP analyses, we distinguish patterns that appear to be due to pollution.

**Concluding Remarks**

Multiple *Fundulus* populations exhibit different gene by environment interactions, some physiological and some adaptive. Thus, comparative analyses among these natural *Fundulus* populations provide a powerful approach to understand biological variation. We found multiple transcriptional mechanisms that underlie both physiological and adaptive resilience found in *F. heteroclitus*. Although the immediate crisis of cell swelling is initially mitigated by mechanisms other than transcription in fish subjected to osmotic shock, by 6 h some of the most dramatically regulated genes are associated with regulatory volume decrease and cell stabilization. Increase in calcium signaling is implicated early in the time course, as is regulation of transcription factors and signaling pathways associated with osmoregulation. As the fish struggle to regulate cell volume and initiate epithelial restructuring, expression patterns indicate repression of cell growth, regulation of cell cycle, modification of tight junctions and the cytoskeleton. Seawater-appropriate ion transport proteins and organic osmolyte synthesis pathways are quickly downregulated, whereas FW-appropriate ion transport gene regulation requires more time as this is likely dependent on successful completion of tissue remodeling. The successful completion of tissue remodeling and acclimation to the new osmotic environment requires upregulation of glycolysis and mobilization of energy reserves stored as lipid and phosphocreatine.

Comparative studies among multiple populations and species with different physiological tolerances can be used...
Figure 5. Hierarchical clustering of genes significantly differently expressed in Newark Bay individuals versus reference individuals. (A) All significantly differently expressed genes. Genes with black squares are not affected by body weight and are clustered separately in B. NB-# are Newark Bay individuals and Ref1-# and Ref2-# represent individuals from the 2 reference populations. (B) Hierarchical clustering of genes significantly differently expressed in Newark Bay individuals for which body weight is not a significant covariate.
to target the genetic bases of these gene expression changes. Such comparative studies with multiple polluted populations of *F. heteroclitus* identify an unexpected complexity in gene expression, even among specifically targeted metabolic genes. This complexity is confounded by an apparent physiological response for genes that covary with body weight. A similar complexity among polluted populations is found for substitutions in genomic DNA where few outlier AFLPs are shared across polluted populations and none are shared between all 3 polluted populations. Thus, potentially adaptive mechanisms among *F. heteroclitus* from different polluted populations are not well conserved at either the level of gene expression or the level of DNA polymorphisms. One simple explanation for this complexity is the heterogeneity of the stress (different polluted environments) coupled with the underlying genetic variation of the populations. Similar ongoing comparative experiments within *Fundulus* seek to test whether evolved mechanisms of osmotic tolerance exhibit comparable complexity among populations and species.

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