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Genetic basis for rapidly evolved tolerance in the wild: adaptation to toxic pollutants by an estuarine fish species

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Abstract

Atlantic killifish (*Fundulus heteroclitus*) residing in some urban and industrialized estuaries of the US eastern seaboard demonstrate recently evolved and extreme tolerance to toxic aryl hydrocarbon pollutants, characterized as dioxin-like compounds (DLCs). Here, we provide an unusually comprehensive accounting (69%) through quantitative trait locus (QTL) analysis of the genetic basis for DLC tolerance in killifish inhabiting an urban estuary contaminated with PCB congeners, the most toxic of which are DLCs. Consistent with mechanistic knowledge of DLC toxicity in fish and other vertebrates, the aryl hydrocarbon receptor (*ahr2*) region accounts for 17% of trait variation; however, QTL on independent linkage groups and their interactions have even greater explanatory power (44%). QTL interpreted within the context of recently available *Fundulus* genomic resources and shared synteny among fish species suggest adaptation via interacting components of a complex stress response network. Some QTL were also enriched in other killifish populations characterized as DLC-tolerant and residing in distant urban estuaries contaminated with unique mixtures of pollutants. Together, our results suggest that DLC tolerance in killifish represents an emerging example of parallel contemporary evolution that has been driven by intense human-mediated selection on natural populations.

Keywords: adaptation, contemporary evolution, dioxin, evolved tolerance, quantitative trait locus

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Footnote on nomenclature and definitions: We use the zebrafish genome conventions when referring to genes (*ahr2*) and proteins (Ahr2); however, we use a more generic form (AHR) to define a pathway or gene without species specificity. We also use the term 'shared synteny' or 'conserved synteny' to describe genes on the same chromosome in one species and their orthologs residing on the same chromosome in another species.

Introduction

The Atlantic killifish (*Fundulus heteroclitus*) has been described as a model species for studying physiological, ecological and evolutionary responses to environmental stressors (reviewed in Burnett *et al.* 2007). Populations comprised of tens of thousands of effective breeders reside in estuaries along the US eastern seaboard (Duvernell *et al.* 2008 and references therein) that vary extremely in a myriad of biotic and abiotic factors (Nordlie 2006; Nacci *et al.* 2010). They also exhibit a very narrow home range: migration distances, estimated through mark–recapture studies and confirmed by

population genetics, are estimated at less than 1 km (Lotrich 1975; Duvernell *et al.* 2008). Together, large population sizes and limited migration suggest minimal effects of genetic drift and gene flow and great potential for adaptive evolution (e.g. see review Hemmer-Hansen *et al.* 2014). In fact, killifish provide a classic example of evolution at large spatial (1000s of km) and temporal (10 000s of years) scales: clinal variation coincident with the wide temperature gradient across its species range has been characterized at the genetic, molecular and physiological levels (e.g. review Mitton 1997; but see Strand *et al.* 2012). However, in addition to the historical contingencies that have influenced large-scale patterns of genetic variation among killifish populations (Adams *et al.* 2006; Duvernell *et al.* 2008), contemporary processes are certainly important drivers of killifish evolutionary dynamics at finer geographic scales. Specifically at smaller spatial (10s of km) and temporal (10s of years) scale, killifish populations have adapted to shoreline habitats ranging from undeveloped to urban and industrialized conditions listed as among the most contaminated in the United States (e.g. review Nacci *et al.* 2010). It is the genetic basis for this contemporary microgeographic divergence and adaptation (Richardson *et al.* 2014) that we explore in this study.

In fact, this killifish example provides further evidence dismissing the notion that rapid evolution is rare (e.g. Hendry 2013). Well-described examples of contemporary evolution of fishes include earlier maturation and a smaller adult body size in intensely harvested Atlantic cod, and the evolution of the nonmigratory rainbow trout from an isolated population (since 1910) of migratory steelhead trout (review Schoener 2011). In a review of 28 recent studies, contemporary evolution of three-spine stickleback in all but three populations occurred in response to human disturbance; examples include the evolution of body armour in response to predation and the colonization of freshwater habitats by oceanic populations (review Bell & Aguirre 2013). Yet historically, contemporary evolution has been typecast as 'product(s) of unnaturally powerful selection', where industrial melanism and antibiotic and pesticide resistance represent some of these agent-specific adaptations (review Bell & Aguirre 2013).

Like these earlier examples of adaptation to potent human-mediated stressors, some Atlantic killifish populations have rapidly evolved tolerance to extremely toxic and widely distributed industrial pollutants characterized as 'dioxin-like compounds' (DLCs) (Nacci *et al.* 1999; Van Veld and Nacci 2008; Nacci *et al.* 2010; Whitehead *et al.* 2012). 'Dioxin' (TCDD, 2,3,7,8 tetrachlorodibenzo-p-dioxin) is the most potent member of this eponymous class that also includes some polychlorinated biphenyl (PCB) congeners, and is highly toxic to all vertebrates, especially fishes during early development (see reviews including King-Heiden *et al.* 2012). The defining characteristic of this group is the ability to bind the aryl hydrocarbon receptor (AHR), which mediates all or most DLC toxicity (Denison *et al.* 2011). Although the exact mechanism of DLC toxicity or tolerance is not known, DLC–AHR binding initiates a series of events reflecting complex interactions between the AHR signal transduction pathway and other regulatory pathways (Denison *et al.* 2011). As might be expected from an initiating event, variation in strength of ligand binding to Ahr1 largely explains interspecific differences in DLC sensitivity among avian species (Farmahin *et al.* 2013). However, vertebrate species vary in the number and functionality of Ahr isoforms and paralogs (Hahn 2002; Hahn *et al.* 2006; Goodale *et al.* 2012), and, for example, Ahr2 plays an important role in the DLC sensitivity of many fishes (e.g. Prasch *et al.* 2003). In fact, poor Ahr2-DLC binding characterizes a DLC-tolerant population of tomcod residing in the urban Hudson River system (NY, USA) (Wirgin *et al.* 2011). And, in contrast to the species-level differences in DLC sensitivity that have been observed in avian species, variation in the DLC sensitivity among NY tomcod populations has evolved recently in apparent response to intense urban pollution (Wirgin *et al.* 2011).

Like the Hudson River tomcod, this study's focal killifish population also resides in an extremely contaminated urban estuary. Upper New Bedford Harbor (NB), MA, USA, has been designated by the US EPA as a Superfund site for PCB sediment contamination more than 10 000 times higher than concentrations (Pruell *et al.* 1990) associated with adverse ecological effects (Long *et al.* 1995). Consistent with adaptation to this highly contaminated environment, NB killifish are tolerant of >2500 fold higher concentrations of a model DLC than killifish from a nearby uncontaminated site, Block Island (BI), RI, USA (Nacci *et al.* 2010). Yet unlike Hudson River tomcod, efforts to date have not confirmed Ahr binding as a specific target in NB killifish tolerance (Hahn *et al.* 2004, 2005). However, NB killifish results may be confounded by multiple Ahr paralogs and isoforms in this species (Hahn *et al.* 2006). Furthermore, genetic and genomic efforts targeting AHR pathway components in one or more tolerant killifish populations have not fully explained tolerance (Van Veld and Nacci 2008; Oleksiak *et al.* 2011; Whitehead *et al.* 2012; Proestou *et al.* 2014). Thus, these wild killifish populations provide a unique model to investigate realized genetic mechanisms for contemporary evolution to intense human-mediated stress and, more specifically, to infer the basis of variation in DLC sensitivity across fish species.

To investigate the genetic basis of DLC tolerance in NB killifish, we used a quantitative trait locus (QTL) mapping approach: 'a classical top-down genetic approach that starts with the phenotype and aims to map genetic variants linked to phenotypic variation' (Schielzeth & Husby 2014). Here, we linked variation in a dense panel of genetic markers to well-characterized phenotypic evidence of tolerance to DLC exposures in embryos from the segregating populations (F2) (Whitehead *et al.* 2010) and interpreted QTL using newly published gene models (National Science Foundation, DEB-1120512) and genetic map for *F. heteroclitus* (Waits *et al.* 2016) (see details below and in SI). There are relatively few QTL analyses of ecologically valuable traits in marine fishes, with exception of several studies to identify the basis for repeated evolution concurrent with transition to freshwater habitats among three-spine stickleback populations (review Nielsen *et al.* 2009). And, just as sticklebacks have provided a unique opportunity to characterize genetic mechanisms underlying parallel ecological adaptation in nature (e.g. Nielsen *et al.* 2009; Jones *et al.* 2012), we have taken advantage of large, persistent populations of the Atlantic killifish residing in estuaries along the US east coast that display inherited and adaptive tolerance to DLCs that has evolved concurrently with urban industrialization (Nacci *et al.* 2010). To date, four geographically disparate killifish populations resident to US urban estuaries have been characterized that display relatively similar magnitudes and phenotypes of tolerance to a model DLC (Nacci *et al.* 2010; Whitehead *et al.* 2012). Thus, we follow up this detailed analysis of QTL for DLC-tolerant NB killifish by genotyping for major QTL in fish from these other three independently evolving DLC-tolerant killifish populations. This example provides us with opportunity to identify regions of the genome involved in this unique adaptation across multiple populations and interpret these findings within a context of evolutionary adaptation in natural populations over observable time.

Materials and methods

The QTL approach employed here was used to infer the genetic basis of DLC tolerance in NB killifish, and is described in greater detail in Supporting Information. Briefly, source populations of wild killifish were collected from NB and BI and maintained under uncontaminated laboratory conditions (e.g. Nacci *et al.* 2010): individual female fish from NB and male fish from BI were used as the parental (P) generation in the classic F2 intercrosses. Three crosses (A–C) of mature sibling: sibling F1 progeny from independent parental lines produced large families of F2 recombinant embryos; we selected the largest families, which resulted from female NB × male BI crosses. F2 embryos from each of three NB × BI crosses were exposed to a discriminating concentration of a prototypical DLC, 3,3′,4,4′,5-pentachlorobiphenyl, PCB 126, and then observed for teratogenic effects, which were summed as phenotypic rating, PR (Fig. 1). A total of 95 embryos representing the tails of the PR distributions, that is embryos classed as most and least sensitive to DLC effects, were selected from each cross for genotyping. Genotyping was conducted using microsatellite and single nucleotide polymorphism (SNP) markers, identified as polymorphic in the parental (P) generation in each cross, selected from the dense panel of genetic markers used to create the first genetic map for this species (Waits *et al.* 2016). Statistical analysis identified highly significant phenotype-to-genotype associations, characterized as logarithm of odds of linkage (LOD) scores, which measure the strength of evidence for the presence of a QTL (Van Ooijen 1999). Analyses were conducted on a marker-by-marker basis (one-dimensional QTL scan) and using pairwise combinations of markers (two-dimensional QTL scan) to identify main and interacting QTL. QTL were annotated using recently developed killifish genomic resources, including gene models for *F. heteroclitus* (https://my.mdibl.org/display/FGP/Home; NSF DEB-1120512) and the first killifish genetic map (Waits *et al.* 2016). In addition, orthology and conserved syntenic relationships between fish species provided further support for the identification of putative tolerance genes.

To test for enrichment of QTL in geographically disparate populations, about 40 killifish fish per site were sampled for genotyping from three non-NB DLC-tolerant killifish populations (Whitehead *et al.* 2012; Proestou *et al.* 2014) (Bridgeport, CT (BP); Newark, NJ (NW); Elizabeth River, VA (ER)) and their respective local DLC-sensitive populations (Flax Pond, NY (FP); Sandy Hook, NJ (SH); Kings Creek, VA (KC)), as described (Proestou *et al.* 2014). Details on the site locations and contamination levels and the sensitivities of these killifish to a model DLC are published elsewhere (reviewed in Proestou *et al.* 2014). Details of genotyping and population genetic analyses are provided in Supplemental Materials.

Results and discussion

Each of the genetic crosses revealed multiple QTL associated with DLC tolerance (Table 1). A one-dimensional QTL scan identified seven main QTL intervals on three linkage groups (LG1, LG2 and LG8), with significant associations between phenotypes and markers producing LOD scores ranging up to 12.51 (Table 1). Furthermore, two-dimensional QTL scans identified interactions between QTL that contributed significantly

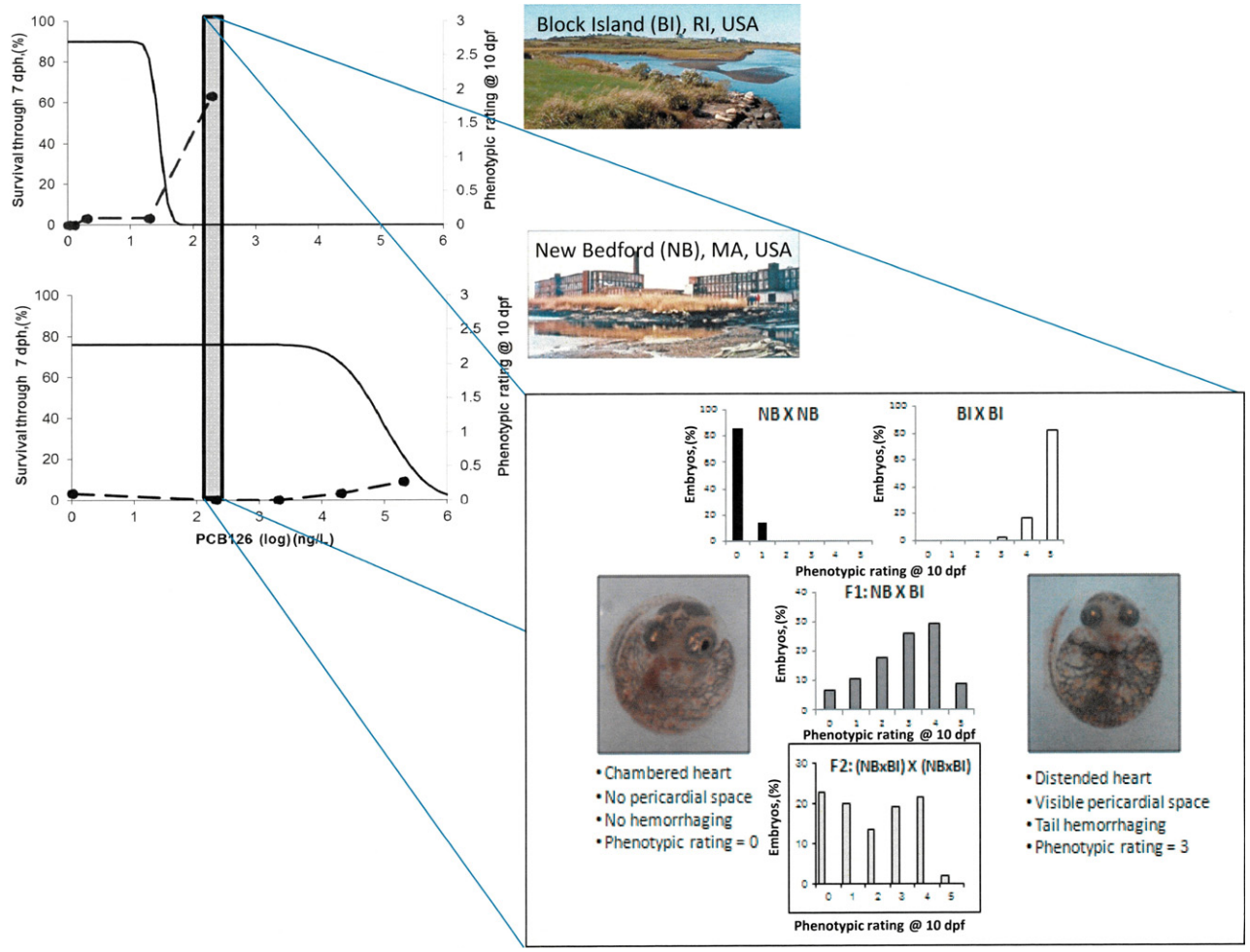

Fig. 1 Responses to a prototypical dioxin-like-chemical (DLC, 3,3′,4,4′,5-pentachlorobiphenyl, PCB 126) diverge dramatically in DLC-sensitive (Block Island, BI) and DLC-tolerant (New Bedford, NB) killifish populations. Upper left: Exposure concentrations of PCB 126 (ng/L) resulting in decreased larval survival (to 7 days posthatching, dph) (solid lines) and increased developmental abnormalities (phenotypic rating observed at 10 days postfertilization, dpf) (dashed lines) vary by orders of magnitude in BI (top panel) vs. NB (bottom panel) killifish embryos. Lower right: A single discriminating exposure concentration of PCB 126 (200 ng/L) produced 10 dpf embryos that range from normal (0–1 phenotypic rating) to highly aberrant (4–5 phenotypic rating), including dioxin-typical lesions, among the F2 embryos genotyped in this study.

to the explanatory power of these analyses (Table 1). In the full QTL models, which allow for main, additive and epistatic interactions, the total amount of variance in DLC tolerance that is explained by all QTL in this study is 68.56% (LOD = 23.87, $P = 8.33 \times 10^{-15}$), 38.89% (LOD = 10.16, $P = 4.5 \times 10^{-6}$) and 54.71% (LOD = 16.17, $P = 4.83 \times 10^{-8}$) for crosses A, B and C, respectively. This comprehensive accounting suggests that killifish DLC tolerance is largely explained by the QTL identified here, and provides a quantitative assessment of their relative contribution to this adaptive phenotype.

To support testable hypotheses for the genetic basis of DLC sensitivity and tolerance, we evaluated the identity of genes represented by the QTL markers, which themselves may only serve as locational proxies for the underlying causative genes (Table 2). This may be especially important to bear in mind with respect to microsatellite markers, which were selected without knowledge of their gene identifications; however, some markers (single nucleotide polymorphisms (SNPs) were selected because of their known or suspected association with the AHR pathway. Therefore, the potential roles in DLC tolerance for genes identified as QTL markers were considered in context of a conceptual model of DLC toxicity (Fig. 2). In addition, we inferred the value of genes within or nearby to QTL intervals through shared synteny with genomes of other fish species because a fully assembled killifish genome was not available. Thus, we focused on the identity and potential function of genes nearby to QTL in the killifish

Table 1 Quantitative trait loci (QTL) associated with sensitivity to a dioxin-like chemical (DLC) in three killifish crosses (A, B and C) as characterized by summarized developmental abnormalities (phenotypic rating) in response to DLC exposure, where boxed shaded area indicates QTL interval and bold signifies most highly supported QTL marker within interval; logarithm of odds (LOD), *P*-value and % variance (var) explained for single (one-dimensional analysis) marker or paired markers (two-dimensional analysis)

				Cross A			Cross B			Cross C		
LG	cM	Locus	QTL interval	LOD	P-Value	% Var	LOD	P-Value	% Var	LOD	P-Value	% Var
1	0	AHR2_1929		0.81	1.64E-01	3.86	0.51	3.23E-01	2.43	3.66	2.87E-04	16.41
1	**0**	**AHR2_792**	**1**	0.81	1.63E-01	3.87	–	–	–	**3.75**	**2.36E-04**	**16.77**
1	81.6	Fhe_1114		—	—	—	—	—	—	1.86	1.57E-02	8.73
1	81.6	FhCA-22		—	—	—	—	—	—	0.34	4.67E-01	1.66
1	**86.9**	**F105**	**2**	—	—	—	—	—	—	**3.58**	**3.40E-04**	**16.10**
2	**11**	**Fhe_2046**	**3**	**12.51**	**7.63E-13**	**45.50**	1.68	2.37E-02	7.81	—	—	—
2	39.6	Fhe_68		—	—	—	4.28	7.69E-05	18.61	—	—	—
2	**41.3**	**Fhe_2337**	**4**	—	—	—	**4.35**	**6.08E-05**	**19.03**	0.21	6.28E-01	1.02
2	66.2	CYP3a_1166		6.30	8.00E-07	26.30	2.18	7.76E-03	10.02	1.42	4.24E-02	6.71
2	68.4	Fhe_504					2.27	6.28E-03	10.44	—	—	—
2	**74.8**	**Fhe_2092**	**5**	6.58	4.46E-07	27.23	—	—	—	**2.73**	**2.27E-03**	**12.52**
2	74.8	xTC18885_458		6.55	4.49E-07	27.22	—	—	—	—	—	—
2	74.8	xTC23138		6.55	4.49E-07	27.22	—	—	—	—	—	—
2	87.1	Fhe_2254		2.40	4.77E-03	10.97	—	—	—	2.01	1.14E-02	9.37
8	0	Fhe_2196		1.01	1.04E-01	4.79	—	—	—	—	—	—
8	6	xTC19113_397			—	—	—	—	—	1.14	7.93E-02	5.42
8	**10.7**	**Hepcidin2_399**	**6**	**2.20**	**7.36E-03**	**10.12**	—	—	—	—	—	—
8	**11.2**	**xTC20491_128**	**6**		—	—	—	—	—	**1.65**	**2.55E-02**	**7.75**
8	11.3	Fhe_124		2.04	1.05E-02	9.42	—	—	—	—	—	—
8	12.2	Fhe_1035			—	—	0.89	1.37E-01	4.23	1.65	2.55E-02	7.75
8	27.8	Fhe_2101		—	—	—	1.14	7.89E-02	5.37	—	—	—
8	32.1	xTC18973_332		0.86	1.46E-01	4.10	—	—	—	—	—	—
8	35	Fhe_2003		0.21	6.30E-01	0.99	—	—	—	—	—	—
8	38.1	Fhe_2235		—	—	—	1.73	2.13E-02	8.03	0.80	1.70E-01	3.82
8	43.3	xTC17350_917		—	—	—	1.80	1.79E-02	8.37	—	—	—
8	44.3	x510_115		0.26	5.48E-01	1.30	1.73	2.09E-02	8.06	—	—	—
8	51	Fhe_2148		—	—	—	2.12	8.78E-03	9.78	0.34	4.66E-01	1.66
8	**55.8**	**Fhe_645**	**7**	—	—	—	**2.24**	**6.81E-03**	**10.28**	—	—	—
8	63.4	Fhe_2026		0.24	5.80E-01	1.16	2.10	9.15E-03	9.70	0.52	3.12E-01	2.53
Epistatic interactions												
2	29.4	Fhe_462	IA	4.57	1.28E-03	7.79	—	—	—	—	—	—
21	103.3	Fhe_1205	IB									
19	48.2	Fhe_2267	IIA	—	—	—	4.62	3.80E-03	15.32	—	—	—
11	46.3	Fhe_2179	IIB									
21	185.3	Fhe_2277	IIIA	—	—	—	—	—	—	4.72	1.23E-03	11.77
15	25.3	Fhe_2083	IIIB									
Additive interactions												
2	29.4	Fhe_462	IVA	3.75	7.33E-03	5.23	—	—	—	—	—	—
22	83.8	HSP90_775	IVB									

genome and two other fish species with relatively well-developed genomic resources, Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). This perspective supports our understanding of killifish DLC tolerance as well as potentially broader generalizations across fish species.

Because of the central role of the AHR receptor in the signal transduction pathway (Fig. 2), we were not surprised that AHR2_792 (the aryl hydrocarbon receptor 2; Table 1) on LG1 was identified as a QTL(1) associated with DLC tolerance, explaining 16.77% PR variance for cross C (Table 1). The emergence of an *ahr* gene as a significant QTL reflects its known requirement for DLC toxicity in fish (Prasch *et al.* 2003; Billiard *et al.* 2006; Incardona *et al.* 2006; Clark *et al.* 2010). However, in context with vertebrate-wide mechanisms of DLC toxicity, the relatively low explanatory power of the *ahr* with respect to DLC tolerance is unexpected, although perhaps not atypical for other fish species. Specifically, a parallel QTL study in zebrafish demonstrated that only

Table 2 Quantitative trait loci (QTL) markers identified by killifish map position (Waits *et al.* 2016) and gene model in *Fundulus heteroclitus* genome (https://my.mdibl.org/display/FGP/Home; NSF DEB-1120512); gene ontology (GO) terms inferred from *Danio rerio or Homo sapiens** homolog

QTL	Locus	LG	cM	Killifish gene model	GO term: biological process
1	AHR2_792	1	0	AHR2a	Cellular response to organic cyclic compound, cellular response to xenobiotic stimulus, intracellular receptor signalling pathway
2	F105	1	86.9	Rho GTPase-activating protein 20	Signal transduction
3	Fhe_2046	2	11	Nuclear factor 1 X-type	DNA replication, regulation of transcription (DNA-templated), skeletal muscle fibre development, skeletal muscle tissue development
I A, IVA	Fhe_462	2	29.4	MAX dimerization protein 4	Negative regulation of cell proliferation, negative regulation of transcription from RNA polymerase II promoter, transcription (DNA-templated)*
4	Fhe_2337	2	41.3	Solute carrier family 44	Choline transport, glycerophospholipid and phosphatidylcholine biosynthetic process, phospholipid and small-molecule metabolic process*
5	Fhe_2092	2	74.8	Soc-2 suppressor of clear	Positive regulation of Ras protein signal transduction
6	hepcidin2_399	8	10.7	Uncharacterized	Cellular iron ion homoeostasis, defence response to Gram-negative and Gram-positive bacterium, response to bacterium
	xTC20491_128	8	11.2	Glyceraldehyde-3-phosphate dehydrogenase	Apoptotic process, glucose metabolic process, glycolytic process, microtubule cytoskeleton organization, oxidation–reduction process
7	Fhe_645	8	55.8	Dynactin 3	G2/M transition of mitotic cell cycle, antigen processing and presentation of exogenous peptide antigen via MHC class II, cytokinesis*
IB	Fhe_1205	21	103.3	HNF1 homeobox B	Anterior/posterior pattern specification, digestive tract morphogenesis, embryonic digestive tract development, endoderm formation
IIA	Fhe_2267	19	48.2	TOX high-mobility group box family member 2	Chromatin remodelling, regulation of transcription (DNA-templated), transcription (DNA-templated)*
IIB	Fhe_2179	11	46.3	GTPase-activating Rap/Ran-GAP domain-like protein 3	Positive regulation of GTPase activity, small GTPase regulator activity*
IIIA	Fhe_2277	21	185.3	n/a	
IIIB	Fhe_2083	15	25.3	Vertebrate B-cell CLL/ lymphoma 11A	B-cell differentiation, T-cell differentiation, negative regulation of axon extension, regulation of collateral sprouting, regulation of dendrite development*
IVB	HSP90_775	22	83.8	Heat-shock protein HSP90-beta-3, putative	Blood vessel development, muscle organ development, protein folding, response to stress

14% of the total variation in dioxin embryotoxicity was explained by variation in *ahr2*, while other QTL had greater explanatory power (Waits & Nebert 2011). In teleosts, pathway roles may be complicated by the diversity of *ahr* genes that resulted from a tandem gene duplication event and established two distinct *ahr* lineages, *ahr1* and *ahr2* (Karchner *et al.* 1999; Hahn *et al.* 2006; Hansson & Hahn 2008). The *ahr2* gene is believed to be responsible for the toxicological functions of *ahr* in (at least some) fish species (Prasch *et al.* 2003; but also see Bak *et al.* 2013); knockdown of *ahr2*, but not *ahr1*, provided partial protection against the teratogenic effects of PAHs and PCBs in killifish embryos (Clark *et al.* 2010). In killifish, initiating events may be further complicated by the recent discovery of multiple *ahr1* and *ahr2* homologues. These homologues include those originally isolated killifish *ahr* genes, *ahr1a* and *ahr2a*, and novel *ahr* genes, *ahr1b* and *ahr2b*. These latter homologues are presumed to have arisen from a subsequent whole-genome duplication (Hahn *et al.* 2006; Reitzel *et al.* 2014); their roles in DLC sensitivity or tolerance are not known. Recent field studies also demonstrate *ahr* loci as under selection in wild killifish. Specifically, the *ahr2* variants (now known to be *ahr2a*) of NB killifish included a SNP within the PAS domain and several near the C-terminal transactivation domain, which emerged as potentially under selection and with distinct allelic patterns in NB fish as compared to the reference sites (Proestou *et al.* 201; Reitzel *et al.* 2014). Interestingly, the *ahr2* variant in DLC-tolerant tomcod, which was characterized by a 2-amino acid deletion just downstream from the PAS domain, was proposed to

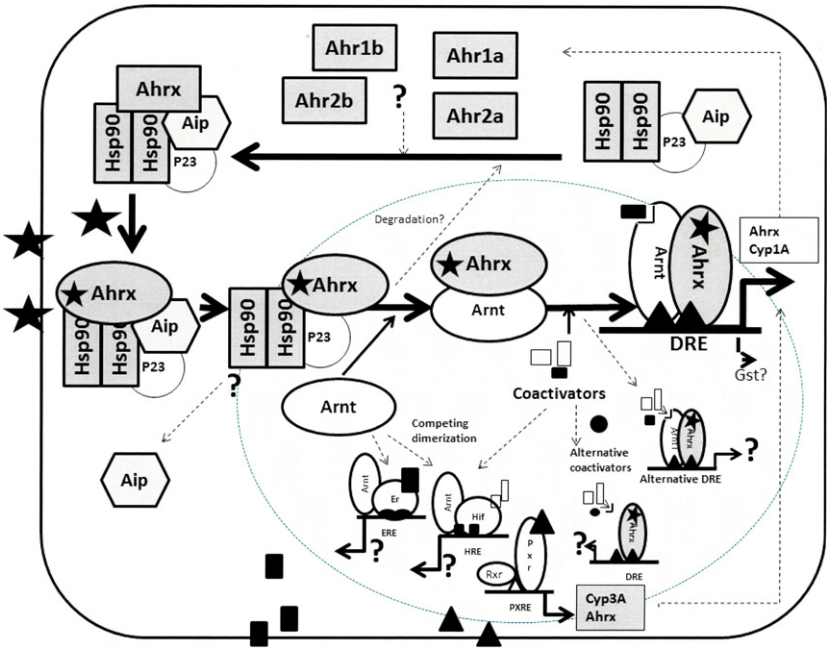

Fig. 2 Conceptual model representing some responses to dioxin-like compounds (DLC, stars) via the aryl hydrocarbon receptor (AHR) signal transduction pathway, and potential alterations (dashed) in DLC-tolerant killifish, such as in expression of Ahr(s), cytochrome p450 1a (Cyp1a) and glutathione S-transferase (Gst). Loci associated with (medium grey) or near loci associated with (light grey) DLC tolerance identified in this and companion study (Proestou *et al.* 2014) include Ahr (aryl hydrocarbon receptors, QTL1), Hsp90 (heat-shock protein 90, QTL IVb), aryl hydrocarbon interacting protein (Aip, presumed to be near QTL IA/IVA), cytochrome p450 3A (Cyp3A, QTL5). Pathways known to interact with the AHR pathway include those with the estrogen receptor (ER, and its ligands (squares)) and hypoxia response (Hif) pathways that share the AHR nuclear translocator (Arnt) cofactor with the AHR pathway, the pregnane X receptor (pxr, and its non-dioxin-like compound ligands (triangles)) which may be involved in regulating the expression of the Ahr as well as Cyp3A. Transcriptional regulation occurs via response elements (REs) for dioxin (DRE), estrogen (ERE), hypoxia (HRE) and pregnane (PxRE), as in Denison *et al.* (2011).

alter the ligand-binding affinity or stability of the Ahr2 protein and was associated with DLC tolerance in this fish species (Wirgin *et al.* 2011).

The specific roles of *ahr* homologues in DLC tolerance may not be resolvable by QTL studies because of the tandem occurrence of the *ahr* genes in killifish and other fish species (Karchner *et al.* 2005; Hahn *et al.* 2006) (Fig. 3), and may be best addressed through targeted mutagenesis and *in vitro* bioassay approaches. Orthologous relationships between killifish genes and those of other species may also be informative; however, the genetic basis for DLC tolerance has only been investigated in a limited number of fish species for which genomic resources are sparse, for example tomcod (Wirgin *et al.* 2011) and seabream (Bak *et al.* 2013). While the zebrafish genome is fairly well annotated and zebrafish DLC tolerance has been studied most extensively (Waits & Nebert 2011), determining *ahr* orthologs between killifish and zebrafish is complicated due to the extensive sequence divergence of the zebrafish *ahr1a* gene relative to other fish species, and the subsequent loss of an *ahr2* gene following whole-genome duplication (Hahn *et al.* 2006). Comparative genomic analyses performed by us and others (M. Hahn, personal communication) confirm that the single *ahr2* gene in zebrafish is orthologous to *ahr2b* not *ahr2a* in killifish (LG2; Fig. 4); however, our identification of an *ahr2* homologue as a significant QTL marker is consistent with the importance of *ahr2* in fish responses to DLCs. Recent population genetics studies (Proestou *et al.* 2014; Reitzel *et al.* 2014) also support the role of killifish *ahr2a* in DLC tolerance.

Unlike QTL1, a direct role in DLC sensitivity has not been proposed for QTL2 (Table 2; Rho GTPase-activating protein 20; *arhgap20*). Shared synteny was used to identify nearby genes of potential interest. For example, regions of zebrafish Chr 9 and medaka Chr 21 contain orthologs of QTL2 (Fig. 3), which may function in signal transduction (Table 2). Both regions also include pregnane X receptor (*pxr*; *nr1i2*) (Fig. 3; Table S1, Supporting information), another nuclear receptor known to interact with the *ahr* in some species. Recent evidence suggests a role for *pxr* in altered responsiveness to the non-DLC congeners that predominate PCB mixtures from contaminated sites and their resident killifish (Gräns *et al.* 2015); however, it is not known whether alterations in responses to this class of compounds

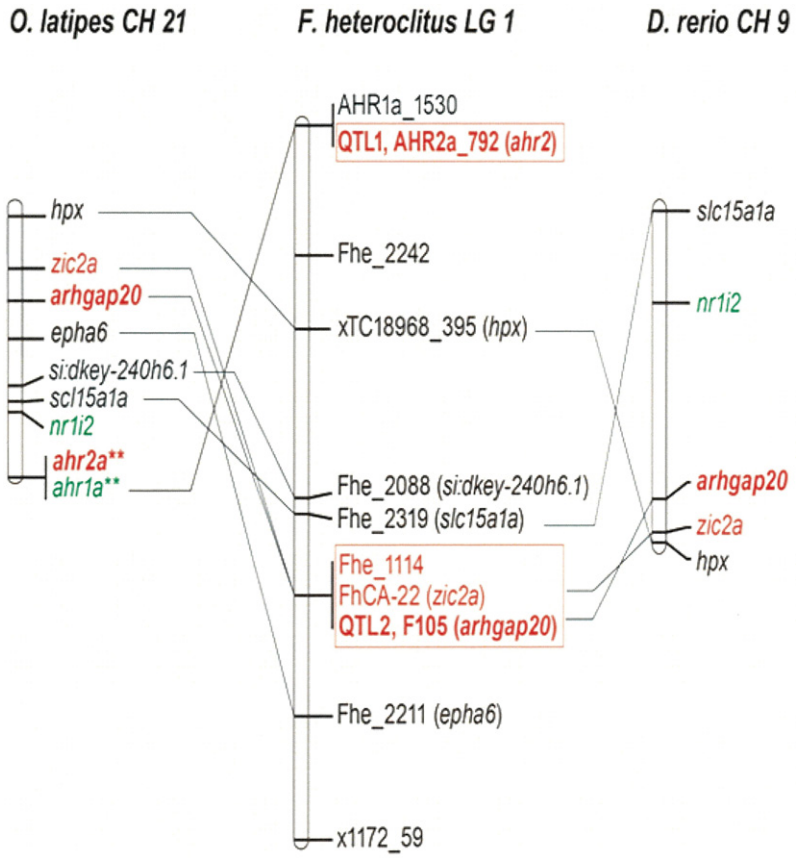

Fig. 3 Shared synteny between regions of killifish (*Fundulus heteroclitus*) linkage group (LG) 1, and medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) chromosomes. Shown are killifish quantitative trait locus (QTL) intervals (boxed red) and strongest marker within the interval (red bold), killifish mapped genetic markers (black) and genes of interest (green), including QTL identified for zebrafish DLC tolerance (ZF*) (Waits & Nebert 2011). Genes are identified using zebrafish gene symbols shown in Table S1 (Supporting information).

mechanisms is related to alterations to the AHR pathway (Fig. 2).

While LG1 includes areas of expected interest, LG2 accounts for a larger proportion of the variance in DLC tolerance, with significant QTL in all three crosses (Table 1). For example, within LG2, QTL3 (Fhe_2046) alone explained 45.50% of 68.60% total variance for DLC tolerance in cross A, QTL4 (Fhe_2337) alone explained 19.03% of 38.89% total variance for DLC tolerance in cross B, and QTL5 (Fhe_2092) alone explained 12.52% of 54.71% total variance in cross C (Table 1). Significant additive and epistatic interactions with LG2 at marker Fhe_462 contribute additional explanatory power to this region. In sum, LG2 is a 'hot' genomic region for killifish DLC tolerance that resolves into multiple, strongly supported QTL, suggesting that several genes in these regions may play important functional roles in DLC tolerance.

The strongest QTL revealed in LG2 and within this entire study, QTL3 (Fhe_2046, nuclear factor 1 X-type, *nfixa*), is known to play roles in replication and transcription (Table 2), and has been implicated more specifically in neurological development (Heng *et al.* 2014). Yet its statistical significance may be related partly to the nearby occurrence in both medaka and zebrafish of *aip*, the gene coding for aryl hydrocarbon receptor interacting protein (also known as HBV X-associated protein 2 (XAP2), and AH receptor-activated 9 (ARA9)) (Cai *et al.* 2011; Trivellin & Korbonits 2011) (Fig. 2). Generally (e.g. Trivellin & Korbonits 2011), Aip has been described as a cochaperone, presumably maintaining properly folded Ahr in the cytosol and improving the stability, subcellular localization and ligand-binding ability of the receptor in vivo (Fig. 2). More specifically, unliganded cytosolic Ahr is described as locked in an inactive state as a multiprotein complex with Aip, cochaperone protein p23 and heat-shock protein 90 (Hsp90) dimers. Within this complex, Hsp90 shapes the Ahr's ligand-binding domain into a state competent for ligand binding, and it also negatively regulates Ahr until ligand binding occurs (Nukaya *et al.* 2010; Denison *et al.* 2011). Upon ligand binding, Ahr undergoes a conformational change that exposes a nuclear localization sequence, resulting in translocation of the complex into the nucleus, where it forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator 1 (Arnt, also known as HIF1b), which leads to the activation of AHR-responsive genes; Aip's translocation into the nucleus with the AHR complex seems to be species-dependent (Trivellin & Korbonits 2011).

The specific role of Aip in DLC toxicity is not well known and may vary by species. Upon exposure to DLCs, Aip is thought to contribute to an increase in the

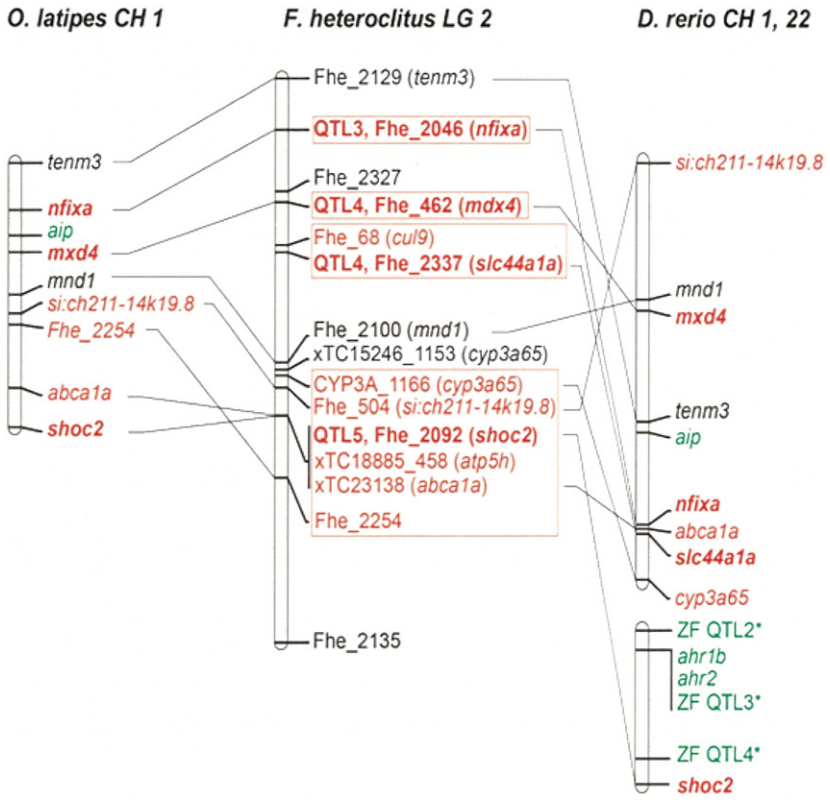

Fig. 4 Shared synteny between regions of killifish (*Fundulus heteroclitus*) linkage group (LG) 2, and medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) chromosomes. See Fig. 3 for further information.

concentration of nuclear transcriptionally effective Ahr complexes by a variety of mechanisms such as interacting directly with Hsp90 or influencing Ahr–Arnt complexing (Denison *et al.* 2011) (Fig. 2). Consistent with such a normal function, alterations in Aip have been hypothesized to affect DLC tolerance by mechanisms such as keeping Ahr 'locked' into an inactive cytosolic state even in the presence of ligand, or inhibiting the dynamics of Ahr-partner complexing and/or nuclear translocation (Trivellin & Korbonits 2011). In addition, it has been shown in mammals that Aip's role or its importance differs among AHR-mediated DLC effects; that is, toxic effects require Aip, while cyp1A induction does not (Nukaya *et al.* 2010). While the correlation between reduced toxicity and poor cyp1A induction in response to a model DLC has been observed consistently in tolerant killifish (Nacci *et al.* 2010; Whitehead *et al.* 2012), this relationship is uncoupled upon exposure to some other AHR agonists (Bello *et al.* 2001), a phenomenon that might be exploited to further tease out the role of Aip in DLC killifish tolerance. Thus, the central (although unclear) role of Aip in the AHR pathway (Fig. 2) and its occurrence in chromosomal regions showing conserved synteny to killifish's LG2 QTL (Fig. 4) make *aip* a strong candidate gene for killifish DLC tolerance. Furthermore, Aip's proposed role(s) in other pathways (Cai *et al.* 2011) also support hypotheses relating mechanisms of AHR pathway crosstalk and potential costs of DLC tolerance.

Between QTL3 and QTL4 within LG2, Fhe_462, *mxd4* (*max*, myc-associated factor X, dimerization protein 4) (Table 2), is particularly noteworthy because of epistatic and additive interactions with other markers, some of which are important components of the AHR pathway (see Discussion below). However, Mxd4, itself, has been proposed to interact with the AHR pathway; Mxd4 is upregulated by DLCs in fish (Carney *et al.* 2006). Interestingly, Ahr/Arnt and Max/interacting proteins are all basic Helix Loop Helix transcription factors that act by binding E-boxes (McFerrin & Atchley 2011), lending further speculation concerning a potential role for this gene in DLC tolerance. But the detection of *mxd4* as a QTL may also be related to its potential proximity to strong candidate genes of interest, that is *aip* as inferred from comparative genomic analyses with both zebrafish (Chr 1) and medaka (Chr 1) (Fig. 4).

Another strong QTL interval in LG2, QTL5, encompasses genes known to be regulated via AHR, that is cytochrome P450 3A (*cyp3A65*) (Table 2), which, like *cyp1*, is a member of the cytochrome P450 super family known for their primary role in the catabolism of xenobiotics (Fig. 2). The potential importance of *cyp3A65* is reinforced by evidence from field surveys indicating significant differentiation at this locus between NB and

BI killifish (Proestou *et al.* 2014). Furthermore, *cyp3A* is upregulated by exposure to DLC and non-DLC pollutants via the pregnane X receptor (Pxr) which dimerizes with retinoid X receptor (Rxr), and is purported to interact with the AHR pathway (Gräns *et al.* 2015) (Fig. 4). Alternatively, alterations in Pxr/Cyp3a65 in NB killifish (Wassur 2012; Gräns *et al.* 2015) have been interpreted as an AHR-independent mechanism of tolerance to contaminants (but also see Chang *et al.* 2013). While speculative, a well-supported candidate for QTL5 (ATP synthase subunit D, *atp5h*), or more generally the ATP synthase complex, has been proposed as interacting with the Ahr via Aip and/or Hsp90, providing a mechanistic basis for DLC-based wasting syndrome (Tappenden *et al.* 2011). Another strong candidate within the QTL5 interval, Fhe_2092, Soc-2 suppressor of clear, *shoc2* (Table 2) functions in *ras* signal transduction. Interestingly, the region where zebrafish *shoc2* resides has also been linked to DLC tolerance in zebrafish (zebrafish QTL4), and resides on a segment of Chr 22 that includes two *ahr*s as well as zebrafish QTL2 and QTL3 (Waits & Nebert 2011) (Fig. 4).

Two large QTL intervals (6 and 7) resolve in LG8, each with lower strengths of association (LOD) than those on LG2 or LG1 (Table 1), and each demonstrating conserved synteny with Chr 16 in both medaka and zebrafish (Fig. 5). Both *hepcidin2_399* and xTC20491_128 (glyceraldehyde-3-phosphate dehydrogenase, *gapdh*) are equally well-supported candidates for QTL6 (Table 1). Hepcidin has also been noted for significantly differentiating NB and BI killifish in a recent population genetic study (Proestou *et al.* 2014). The strongest marker within QTL7 interval is Fhe_645, *dynactin 3*, which is involved in cell cycling (Tables 1 and 2). Although it is unclear what effect (if any) this locus has on DLC sensitivity, shared syntenic relationships between killifish LG8 and medaka Chr 16 (Fig. 5) suggest it is likely that the retinoic X receptor (*rxr*; Fig. 2) gene that potentially interacts with the AHR pathway, is located near this QTL region.

In addition to the identification of main QTL through one-dimensional analysis, two-dimensional analysis tested interactions between paired markers for their association with DLC tolerance. Of particular interest are interactions with Fhe_462 (QTL IA; IV A, *mdx4*), which include epistasis with QTL IB, Fhe_1205 (HNF1 homeobox B, *hnf1ba*) and additivity with QTL IVB, HSP90_775 (heat-shock protein 90 β, *hsp90ab1*) (Tables 1 and 2). These additive and epistatic interactions are shown on a heat map of LOD scores (Fig. 6, upper left), and as they relate to phenotypic outcomes (Fig. 6, lower). For example, individuals heterozygous (AB) at Fhe_462 when genotype AA at Fhe_1205 locus are relatively DLC-sensitive (high average PR), but when genotype BB at Fhe_1205 are relatively DLC-tolerant (low average PR), where the A allele is derived from the DLC-sensitive (BI) and the B allele is derived from the DLC-tolerant (NB) parental population (Fig. 6, lower left). Tabulating the phenotypic outcome of all three genotypes shows, for example, that when Fhe_462 reflects the tolerant parental genotype (BB), then almost all embryos are tolerant (36/43); however, some

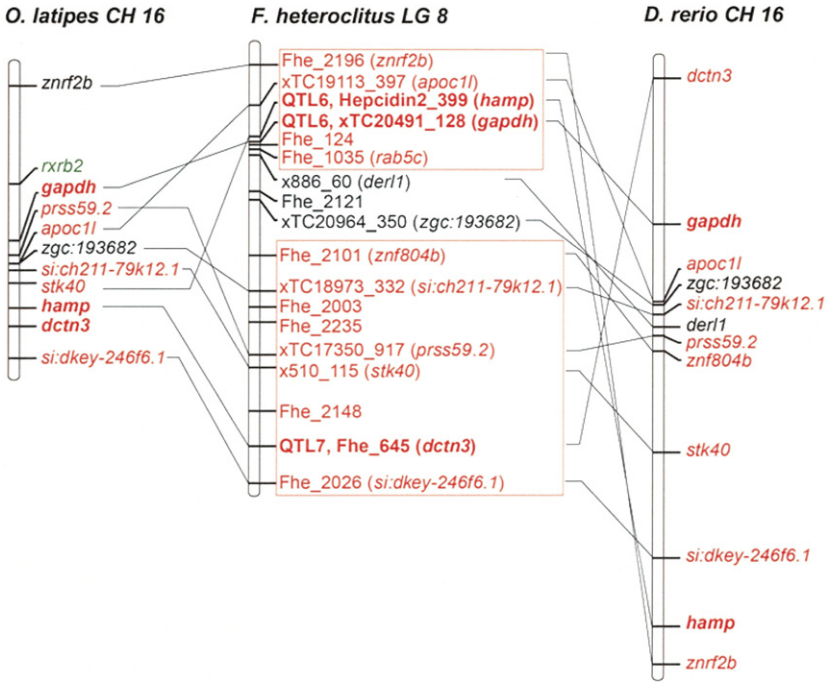

Fig. 5 Shared synteny between regions of killifish (*Fundulus heteroclitus*) linkage group (LG) 8, and medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) chromosomes. See Fig. 3 for further information.

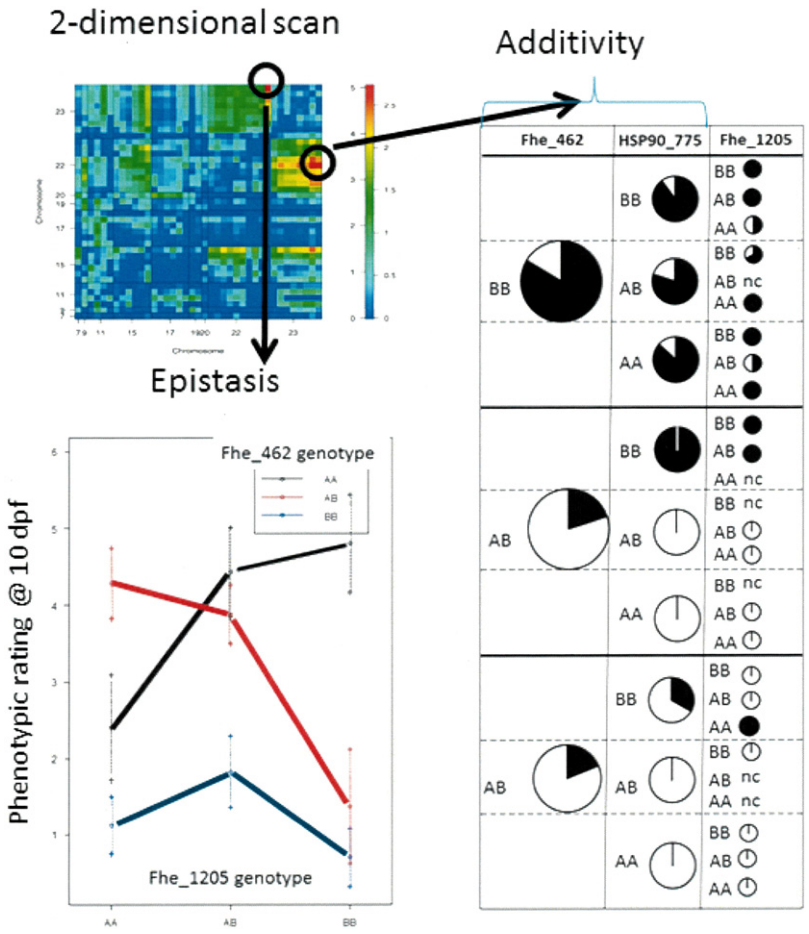

Fig. 6 Interactions between quantitative trait loci (QTL) associated with tolerance to dioxin-like compounds measured as developmental abnormalities, phenotypic rating (PR), at 10 days postfertilization (dpf). Upper left: A two-dimensional scan for additive (below diagonal) and epistatic (above diagonal) interactions resulting in logarithm of odds (LOD) scores characterizing strength of association between paired markers and trait. Lower left: Average (SD range dashed) PR associated with combinations of genotypes, where BB reflects the tolerant (NB) genotype and AA represents the sensitive (BI) genotype in cross A. Lower right: Proportion tolerant individuals for genetic markers acting additively (Fhe_462 x HSP90_775) or epistatically (Fhe_462 x Fhe_1205). NC, none counted.

embryos are also tolerant when they are heterozygous at Fhe_462 (6/30) but possess the tolerant genotype at the other two interactive loci (Fig. 6, lower right). Overall, this accounting suggests the importance to DLC tolerance of NB genotypes in at least three critical genes; however, multiple combinations of genotypes at these loci, potentially including those not found in the wild NB population, can produce the DLC-tolerant phenotype. More broadly, these results suggest that mechanisms of DLC tolerance may be flexible, and different alterations among interacting components could affect the phenotypic outcome similarly. Complementary *in vitro* studies might reveal the mechanism(s) by which combinatorial effects of pathway component variants, Ahr, Aip and Hsp90, affect signal transduction events such as agonist binding, complex translocation and response element binding.

It is particularly intriguing to speculate that if the Fhe_462 QTL actually reflects the nearby gene *aip*, then significant interaction with QTL IVb (*hsp90ab1*) would be consistent with a functionally interactive relationship between these two AHR pathway genes (Fig. 2). Specifically, genetic alterations in *ahr*, *aip* and *hsp90ab1* might affect the dynamic process of ligand binding, nuclear translocation and gene regulation contributing to variations in efficiencies of signal transduction, and ultimately DLC sensitivity. Although not as obviously connected to the AHR toxicity pathway, the epistatic relationship between Fhe_462 and QTL Ib, a homeobox gene, important in early development, may also contribute functionally to the development of DLC-mediated embryotoxicity. More generally, and perhaps most importantly for genes with widespread influence on fundamental (developmental) processes, it may be worth speculating that their association with adaptive responses in contemporary populations may reflect gene changes that have compensated for indirect deleterious effects of primary DLC-specific responses (see later discussion).

Analysis of shared synteny was also applied to implicate other nearby genes contributing to these combinatorial effects. In the case of *hsp90ab1*, a large number of genes known to cross-talk with the AHR pathway including estrogen receptors alpha and beta and retinoic acid x receptor alpha (Fig. 2) are clustered in the general vicinity of *hsp90ab1* on zebrafish Chr 20 and medaka Chr 24, regions with evidence of conserved synteny with killifish LG22 (Fig. S3, Supporting

information). Genetic variation in this region might be consistent with other studies that have described differences between NB and reference killifish in form (splice variants) and function (estrogen responsiveness) of estrogen receptors (Cotter *et al.* 2015). The importance of marker Fhe_1205 (*hnf1ba*, LG21) might also be inferred by this gene's position relative to AHR -related genes (hypoxia-inducible factor alpha (*hif1a1*) and zebrafish QTL1) in medaka Chr 13 and zebrafish Chr 15 which share synteny with killifish LG21 (Fig. 2; Table S1, Supporting information). It is important to note that zebrafish QTL1 explained most of the DLC tolerance in zebrafish (Waits & Nebert 2011). Other significant epistatic interactions (Table 1) are less easily interpretable relative to DLC tolerance, with the exception of the location on LG15 of QTL IIIB (Fhe_2083) which is nearby hypoxia-inducible factor 2 alpha (*epas1b*) in regions of shared synteny between medaka Ch 15 and zebrafish Ch 13 (Table S1, Supporting information), and known to cross-talk with the AHR pathway in yet undefined ways (Fig. 2).

Taken together, these results suggest that several individual genes and several interacting genes that include the *ahr*, *hsp90ab1* and other AHR pathway-related genes, potentially including *aip*, largely explain DLC tolerance in NB killifish. This contrasts with current knowledge of variation in DLC sensitivity among bird species, which can be largely explained by genetic variation in the *ahr* alone, but does not contradict results for tomcod, where the absolute importance of *ahr* variation was identified but its importance relative to other (yet unknown) genetic alterations was not. Striking orthology between several critical QTL for DLC tolerance in killifish (this study) and zebrafish (Waits & Nebert 2011) suggest that this complex trait may be similarly controlled across fish species.

To further assess the relevance of QTL identified in this study for intraspecific DLC sensitivity, killifish collected from NB, BI, and three additional independently evolving DLC-tolerant populations and their geographically proximate reference (DLC-sensitive) killifish populations were genotyped using a limited set of markers. These additional DLC-sensitive–tolerant killifish population pairs ranging from N to S along the US eastern Atlantic Coast are Flax Pond, NY (FLX)–Bridgeport Harbor, CT (BP); Sandy Hook Bay, NJ (SH)–Newark Bay, NJ (NWK); and Kings Creek, VA (KC)–Elizabeth River, VA (ER), whose relevant site and killifish population characteristics are reviewed elsewhere (Nacci *et al.* 2010). The eight selected markers represent LG1 and LG2 QTL (except QTL3) and included four SNP markers genotyped in these same killifish (Proestou *et al.* 2014) (Table S2, Supporting information).

Supporting the concept of parallel evolution for this apparently converging trait, prior studies have defined these four killifish populations screened in this study as 'DLC-tolerant' based on their insensitivity to a model DLC, even at very high exposure concentrations, as measured by adverse biological effects and CYP1A induction. The commonality of this latter phenomenon, a hallmark of ahr pathway activation (Nacci *et al.* 2010; Whitehead *et al.* 2012), suggests broad mechanistic similarities in tolerance among these geographically disparate populations. Yet residence sites vary greatly in pollutant profiles, suggesting that each has adapted to a specific suite and temporal pattern of exposure to toxic chemicals acting as selective agents; thus, a single adaptation that is beneficial in such diverse conditions is not easily envisioned. In fact, DLC-tolerant killifish populations differ in their tolerance to certain chemicals and other stressors (Fisher & Oleksiak 2007; Clark *et al.* 2013). Thus, it is unsurprising that modest to great population differentiation ($P < 0.02$ for all comparisons) was revealed by pairwise F_{ST} analysis when all markers were included. Consistent with neutral expectations, significant isolation by distance (IBD) was detected across populations when all eight markers were included in the analysis ($r = 0.536$, $P = 0.005$) and the partial correlation between genetic and geographic distance remained significant ($r = 0.3984$, $P = 0.0409$) after controlling for DLC sensitivity, suggesting that genetic differences among populations are largely driven by geographic distance. However, the analysis also showed a high degree of genetic differentiation between each tolerant–reference pair despite geographic proximity (e.g. 0.34985 for BI–NB, 0.20264 for FLX–BP) which indicates that genetic differences may be associated with differences in DLC sensitivity.

Grouping populations by DLC sensitivity, the majority of the observed molecular variation across all eight loci was attributed to differences within populations (73.45%); only 6.31% ($P = 0.088$) of the variation was explained by differences between the sensitive and tolerant population groups. However considered singly, each locus differentiated sensitive from tolerant population groups, with the highest ranking marker located at QTL4, Fhe_2337, where F_{ST} values ranged from 0.302 (KC–ER comparison) to 0.708 (BI–NB). In addition, QTL1 (ahr2_1929) differentiated sensitive and tolerant in three of four population comparisons (SH–NWK excluded), with F_{ST}s ranging from 0.183 (FLX–BP comparison) to 0.254 (BI–NB comparison) (Table S3, Supporting information). The ability to detect such a strong signal from multiple QTL in four outbred populations is remarkable and lends further support to their association with DLC tolerance in killifish.

When considering only the highest ranked marker QTL4 (Fhe_2337), a significant amount of the molecular variation (26.92%) was explained by the difference between sensitive and tolerant groups. Variation in this marker across all populations (Mantel's test) did not support isolation by distance, but indicated significant relationships between genetic distance and DLC sensitivity ($P = 0.007$), measured as published values for LC_{20} of the model DLC, PCB 126 (Nacci *et al.* 2010). However, two different alleles were associated with DLC sensitivity; rather, allele 315 in the three northern tolerant populations and 311 in the southern tolerant population exhibit frequencies ≥75% suggesting an association of these alleles with DLC tolerance (Fig. 7). Together with additional genotyping results for these same field populations (Proestou *et al.* 2014; Reitzel *et al.* 2014), tolerance appears to correlate with variation among a suite of loci including those known to be related to the AHR pathway (Fig. 2). Specifically, alterations were noted in *ahr2*, *cyp1A*, whose poor inducibility by DLC is a hallmark of tolerance in studied tolerant killifish populations, and *cyp3A*, whose activity may correlate with responses to non-DLC-like PCBs (Wassur 2012; Chang *et al.* 2013; Gräns *et al.* 2015), which predominate PCB-contaminated sites.

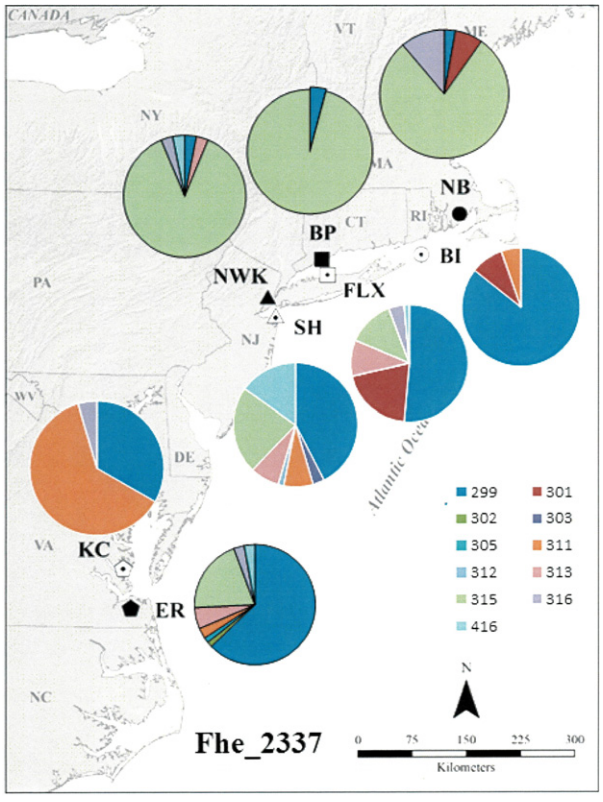

Fig. 7 Allelic variation for quantitative trait locus 4 (genetic marker Fhe_2337) in eight killifish populations known to vary adaptively in sensitivity to dioxin-like compounds, with locally paired tolerant populations (filled symbols; black-lined pies) resident to contaminated and sensitive populations (open symbols; white-lined pies) resident to relatively uncontaminated US Atlantic coastal estuaries. BI, Block Island, RI; NB, New Bedford Harbor, MA; FLX, Flax Pond, NY; BP, Bridgeport Harbor, CT; SH, Sandy Hook, NJ; NWK, Newark Bay, NJ; KC, Kings Creek, VA; ER, Elizabeth River, VA.

Prior studies documenting phenotypic similarities, or trait convergence, among these recently and independently evolving killifish populations suggested some parallelism in evolutionary patterns (e.g. Conte *et al.* 2012). Our study further suggests similarities in the genetic basis for DLC tolerance, which may be largely accounted for by a small number of loci. Given the disparate environments and presumed selective agents this similarity was unexpected among these urban killifish population. However, classic examples of rapid adaptation, such as pesticide resistance in insects and freshwater adaptation in sticklebacks, have often arisen through 'soft selective sweeps' of multiple adaptive alleles (Messer & Petrov 2013). Our results suggest that this killifish adaptation is a model example for such sweeps, that is where (i) traits are controlled by few genes of major (vs. many genes of minor) effect, (ii) altered genes affect more specialized vs. generalized pathways (Stern & Orgogozo 2009) and/or (iii) adaptation has 'a large mutational target, for example, when every loss-of-function mutation in a gene is adaptive' (Messer & Petrov 2013). In fact, large geographically structured killifish populations with high standing genetic variation are also ideally suited for soft sweeps resulting in parallel adaptation that emerges independently in distant locations (Messer & Petrov 2013). Such a description is consistent with a species range spanning the US East Coast, an 'abrupt selection mosaic created by human land use and pollution' (Richardson *et al.* 2014), and a mean individual home range of less than 1 km (Lotrich 1975; Duvernell *et al.* 2008). In addition to the parallel evolution in DLC tolerance among the four *Fundulus heteroclitus* populations explored here, phenotypically similar tolerance has been documented in recently a sister species, *Fundulus grandis*, residing in the highly contaminated Houston Ship Channel (Oziolor *et al.* 2014). Thus, the *Fundulus* genus provides new opportunity to investigate actualized mechanisms of contemporary evolution.

Evidence presented here suggests that DLC tolerance in *F. heteroclitus* may be largely explained by a limited number of genes representing proteins that interact to influence the dynamics of the AHR signal transduction pathway. However, how gene alterations translate into functional differences remains to be determined. More specifically regarding DLC tolerance, empirical

determinations of functional characteristics of the diverse *ahr1* and *ahr2* allelic variants are important to discern their role(s) in killifish DLC adaptation. Clearly, additional research will be needed to determine the possible role(s) of all four *ahr* genes and their potential partners, especially *hsp90* and *aip*, in evolved resistance to PCBs and related chemicals.

We have shown that several interacting genes known to be involved in stress responses contribute to DLC adaptation in killifish; some of what remains to be determined are the temporal sequence and functional implications of each alteration. For example, multiple changes might contribute to the magnitude of DLC tolerance. Specifically, the intraspecific variation in tolerance demonstrated by killifish, which is extreme in comparison to intraspecific tolerance in other fish species, such as tomcod and zebrafish, could be dependent upon the incremental effects of gene alterations that became fixed in tolerant populations in temporal sequence as pollution increased. Sequential changes might also reflect primary adaptation followed by secondary compensatory responses. Alternatively, co-adaptation may have occurred to the multiple stressors that commonly co-occur in these highly polluted environments, such as non-DLC PCBs and hypoxia. These questions of evolutionary interest can be tested directly, for example using core sampling that permits historical reconstruction of pollution and genetic alterations. Complementary research to characterize how gene alterations affect the magnitude of tolerance can also take advantage of killifish populations resident to less extreme pollution and demonstrating less extreme tolerance (Van Veld and Nacci 2008; Nacci *et al.* 2010; Clark *et al.* 2013; Oziolor *et al.* 2014). In summary, genomic knowledge/tools are contributing to the advancement in understanding the full genetic basis for rapidly evolved tolerance to DLCs among and within fish species, which ultimately, will contribute to our understanding of rapid evolution to environmental changes.

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Data accessibility

All genetic markers are described fully elsewhere (Proestou *et al.* 2014; Waits *et al.* 2016). *Fundulus heteroclitus* gene models and draft assembly can be accessed via www.fundulus.org.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Killifish (*Fundulus heteroclitus*) genetic marker locations (Quantitative Trait Loci identified in this study shown in bold) in centiMorgans (cM) on linkage groups (LGs)[13], and identities of gene models from the killifish genome version 1 (National Science Foundation, DEB-1120512), and symbol and location (Chr, chromosome) for zebrafish (ZF, *Danio rerio*) and Medaka (Med, *Oryzias latipes*) orthologs, displayed in figures.

Table S2 Allele frequencies at genotyped Quatitative Trait Locus markers for killifish (*Fundulus heteroclitus*) populations known to vary adaptively in sensitivity to dioxin-like compounds.

Table S3 Single locus F_{ST} values (se) demonstrating genetic differentiation at Quantitative Trait Loci 1 and 4 (shown in bold) across killifish population pairs known to vary adaptively in sensitivity to dioxin-like compounds.