Development of the morpholino gene knockdown technique in *Fundulus heteroclitus*: A tool for studying molecular mechanisms in an established environmental model

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ABSTRACT

A significant challenge in environmental toxicology is that many genetic and genomic tools available in laboratory models are not developed for commonly used environmental models. The Atlantic killifish (*Fundulus heteroclitus*) is one of the most studied teleost environmental models, yet few genetic or genomic tools have been developed for use in this species. The advancement of genetic and evolutionary toxicology will require that many of the tools developed in laboratory models be transferred into species more applicable to environmental toxicology. Antisense morpholino oligonucleotide (MO) gene knockdown technology has been widely utilized to study development in zebrafish and has been proven to be a powerful tool in toxicological investigations through direct manipulation of molecular pathways. To expand the utility of killifish as an environmental model, MO gene knockdown technology was adapted for use in *Fundulus*. Morpholino microinjection methods were altered to overcome the significant differences between these two species. Morpholino efficacy and functional duration were evaluated with molecular and phenotypic methods. A cytochrome P450-1A (CYP1A) MO was used to confirm effectiveness of the methodology. For CYP1A MO-injected embryos, a 70% reduction in CYP1A activity, a 86% reduction in total CYP1A protein, a significant increase in β-naphthoflavone-induced teratogenicity, and estimates of functional duration (50% reduction in activity 10 dpf, and 86% reduction in total protein 12 dpf) conclusively demonstrated that MO technologies can be used effectively in killifish and will likely be just as informative as they have been in zebrafish.

1. Introduction

The Atlantic killifish (*Fundulus heteroclitus*) is one of the most abundant estuarine fish along the Atlantic coast of the United States; as such it is ecologically very important (Bigelow and Schroeder, 1953; Meredith and Lotrich, 1979; Lee et al., 1980; Kneib, 1986; Burnett et al., 2007). Although killifish are widespread, they have small home ranges, which make them ideal for studying the effects of local stressors (Lotrich, 1975). They are tolerant of significant changes in many environmental conditions, including salinity (Griffith, 1974; Wood and Marshall, 1994; Nordlie, 2006), pH (Gonzalez et al., 1989), temperature (Umminger, 1971; Dunson et al., 1993; Smith and Able, 1994; Nordlie, 2006), and oxygen (Wannamaker and Rice, 2000; Smith and Able, 2003; Stierhoff et al., 2003; Nordlie, 2006). Specific killifish populations have adapted to a variety of anthropogenic stressors, including polycyclic aromatic hydrocarbons (PAH)(Meyer et al., 2002; Ownby et al., 2002; Meyer and Di Giulio, 2003), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Prince and Cooper, 1995a,b), polychlorinated biphenyls (PCB)(Nacci et al., 1999; Bello et al., 2001), and mercury (Weis et al., 1981). For these reasons, killifish have become one of the premier teleost environmental models, and certainly one of the most well-studied estuarine environmental models (Burnett et al., 2007). While we know a great deal about killifish physiology, a lack of genomics data and the resulting paucity of genetic tools available hinder our ability to investigate mechanisms of adaptation and chemical response. To adapt existing genetic tools for use in this valuable environmental model, we developed and evaluated antisense morpholino oligo (MO) gene knockdown methods for use in killifish. Morpholino oligos are chemically modified oligonucleotides (generally 25 bases) that bind to specifically targeted mRNA (Nasevicius and Ekker, 2000). Morpholinos reduce

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gene expression via steric blocking of translation or pre-mRNA splicing.

Of the killifish populations known to have adapted to environmental contaminants, several have adapted specifically to chemicals that act via AhR-dependent mechanisms, and alterations to the AhR pathway seem to be a critical component of the adaptation (Prince and Cooper, 1995a,b; Nacci et al., 1999; Powell et al., 2000; Bello et al., 2001; Ownby et al., 2002; Meyer and Di Giulio, 2003; Bacanskas et al., 2004; Hahn et al., 2004). Specifically, all of these populations exhibit a marked reduction in CYP1A inducibility in response to a variety of AhR agonists (Prince and Cooper, 1995a; Elskus et al., 1999; Nacci et al., 1999; Bello et al., 2001; Arzuaga and Elskus, 2002; Meyer et al., 2002, 2003). To more fully understand alterations to the AhR pathway, CYP1A was chosen as the target for morpholino gene knockdown in killifish from a reference site.

CYP1A is an excellent target gene for assessing the utility of MO technology in killifish for several reasons. First, CYP1A is known to be a critical determinant of embryotoxicity caused by PAH mixtures (Wassenberg and Di Giulio, 2004; Billiard et al., 2006). Second, we can easily test for protein activity via the *in ovo* EROD assay. This is particularly important given the inability to use mRNA to quantify the effectiveness of MOs that act by inhibiting translation. Third, knockdown of CYP1A via chemical inhibition in *Fundulus* (Wassenberg and Di Giulio, 2004) or with morpholinos in zebrafish (Billiard et al., 2006) has been shown to exacerbate the embryotoxicity of AhR agonists and PAH mixtures. Therefore, we could also use deformity screens to test whether the MO gene knockdown was successful. Finally, immunochemical techniques have been shown to be useful in assessing induced CYP1A protein levels in *Fundulus* embryos (Toomey et al., 2001).

Antisense morpholino oligo technology has primarily been used in zebrafish (*Danio rerio*) and clawed frogs (*Xenopus laevis* and *X. tropicalis*). While MO based experiments are most often conducted in zebrafish, there are several significant differences between zebrafish and killifish eggs. Killifish eggs are larger, have a more durable chorion, have higher internal pressure, have mucous-like material on the exterior surface of the chorion that can contain large amounts of debris, and develop more slowly (Armstrong and Child, 1965; Brummett and Dumont, 1981; Morin and Able, 1983; Marteinsdottir and Able, 1988; Taylor, 1999). Egg size does not pose a significant challenge for microinjection of morpholinos; however, the tougher chorion and high internal pressure are considerable challenges. The mucous-like membrane surrounding the chorion is a significant problem with regard to clogging injection needles.

Killifish have a long development time (∼14 days) compared to zebrafish (2–3 days). The slower developmental rate in killifish can be both an advantage and disadvantage. The several hours that it takes embryos to reach the 8-cell stage allows for the injection of large numbers of eggs, but the extended development period greatly increases the amount of time a morpholino must remain effective. Since zebrafish develop rapidly, the duration of morpholino effectiveness has not been a primary concern. However, reports of successful use of this technology in rainbow trout, *Oncorhynchus mykiss* (Boonanuntanasarn et al., 2002) and sea lamprey, *Petromyzon marinus* (McCauley and Bronner-Fraser, 2006), which have significantly longer development times (>2 weeks), suggest that MO usage in killifish would be feasible. Antisense morpholino oligo technology remains the only thoroughly tested method of *in vivo* gene expression knockdown in fish embryos. Thus, the development of MO technologies in *Fundulus* would provide a powerful tool for studying gene function in this established environmental model. Subsequent research will make use of this technology to further our understanding of the aryl hydrocarbon receptor (AhR) pathway and its role in killifish adaptation to PAH-contaminated environments.

2. Materials and methods

2.1. Fish care

Adult killifish were collected at a reference site on King's Creek, a tributary of the James River which feeds into the lower Chesapeake Bay in southeastern Virginia (37°17′52.4″N, 76°25′31.4″W). Fish were maintained at 23–25 °C in 25 ppt artificial seawater (ASW; Instant Ocean, Foster & Smith, Rhinelander, WI) and fed a mixture of TetraMin® Tropical Flakes (Tetra, Blacksburg, VA) and freshly hatched brine shrimp (*Artemia*, Brine Shrimp Direct, Ogden, UT). Eggs were manually collected from females and fertilized *in vitro* by milking sperm from males directly into a beaker containing the eggs in ASW. Eggs were set aside for 30 min for fertilization, and then washed in 0.3% hydrogen peroxide. Eggs are subsequently "rolled" on durable wet paper towels with wet fingers to remove mucous-like material and debris from the exterior of the chorion, as per standard medaka egg separation techniques (Marty et al., 1990). At this point the embryos are ready for injection. Adult care and reproductive techniques are non-invasive and have been reviewed and approved by the Duke University Institutional Animal Care & Use Committee (A250-04-09).

2.2. Morpholino and microinjection

Morpholino antisense oligos were designed and manufactured by Gene Tools (Philomath, OR). The CYP1A morpholino was designed to target *F. heteroclitus* cytochrome P4501A (Morrison et al., 1998) (GenBank, AF026800); the CYP1A MO sequence was 5′-ATGCCATGATGACAACTTTTCTCTG-3′. This morpholino overlaps the start codon (underlined) and acts as a translational blocker. Gene Tools' standard control morpholino (control MO, 5′-CCTCTTACCTCAGTTACAATTTATA-3′) was used as a morpholino injection control. All morpholinos used were tagged with fluorescein to monitor embryonic incorporation. Morpholinos were diluted to 250 μM injection stocks in RNase free water. Working injection stocks were maintained at 4 °C. Morpholino injection stocks were briefly vortexed and centrifuged for 5 min prior to use.

Microinjection system consisted of a Nikon SMZ-1500 zoom stereomicroscope (Nikon Instruments Inc., Lewisville, TX), MDI PM 1000 Cell Microinjector (MicroData Instrument Inc., S. Plainfield, NJ), course manipulator (Narishige International USA, Inc., New York, NY), and a three-axis joystick oil hydraulic micromanipulator (Narishige). A PC-10 Puller (Narishige) was used to pull filtered ethanol-rinsed 1.0 mm o.d., 0.5 mm i.d., 10 cm borosilicate glass tubing (Sutter Instrument, Novato, CA) into microinjection needles using a single pull with a heater output setting of 53. Embryos were placed in a glass Petri dish filled with ASW. For injections, embryos were immobilized by wedging them between a 44.7 μL calibrated "holding" pipette (VWR, West Chester, PA) and the bottom of a glass Petri dish. Eggs were oriented so that the blastodisc was aligned for injection directly into the cell. Morpholino injection volumes ranged from 2 to 5 nL. Injection pressure was varied to compensate for variable needle opening size, thus insuring an appropriate injection volume. Confirmation of proper injection and full incorporation was performed immediately following injections and at 24 hpf, respectively, using a Leica MZ FLIII fluorescence zoom stereomicroscope (Leica Microsystems Inc., Bannockburn, IL) and GFP/FITC filter. Only normally developing embryos that displayed strong uniform fluorescence were included in subsequent dosing experiments.

2.3. Chemicals and dosing

β-naphthoflavone (BNF), benzo[*a*]pyrene (BaP), ethoxyresorufin, and dimethyl sulfoxide (DMSO) were purchased from

Sigma–Aldrich (St. Louis, MO), and 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) was purchased from AccuStandard (New Haven, CT). BNF, BaP, PCB126, and ethoxyresorufin were all dissolved in DMSO. Embryo dosing was conducted in 25 ppt ASW, with individual embryos being exposed in 10 mL of solution in 20-mL glass scintillation vials (VWR). Ethoxyresorufin was added to all exposure vials for a final concentration of 21 μg/L for subsequent EROD assays. BNF exposures were performed at a final concentration of 10 μg/L. DMSO concentrations were held constant at <0.002% across all experimental groups. Individual embryos were placed in 10 mL of dosing solution from 24 hpf through deformity screening at 144 hpf. Morpholino duration experiments were conducted using a sliding window approach. In addition to the standard dosing described above, independent groups of embryos were dosed at 96, 144, 196, or 240 hpf, with CYP1A protein activity assessed 48 h after dosing. A later larval window was added in view of the fact that morpholino duration exceeded the normal 14-day incubation period. Hatchlings were dosed at 18 dpf and screened at 20 dpf. Although primary dosing was performed with the model AhR agonist BNF, we also performed dosing experiments with both BaP (100 μg/L) and PCB126 (1 μg/L).

2.4. EROD assay

CYP1A activity was measured via an *in ovo* EROD (7-ethoxyresorufin-*O*-deethylase) assay modified from Nacci et al. (1998, 2005). A detailed description of the modified method is provided in Wassenberg and Di Giulio (2004). This assay quantifies the fluorescence of resorufin, the product of CYP1A metabolism of ethoxyresorufin, which accumulates in the bi-lobed urinary bladder in *Fundulus* embryos. Upon hatching, resorufin no longer accumulates in the urinary bladder. Therefore, an *in vivo* EROD assay was used to measure CYP1A activity in *Fundulus* larvae. The *in vivo* and *in ovo* EROD assays differ only in that resorufin fluorescence is quantified in the gall bladder of larvae, rather than the urinary bladder of embryos. EROD assays were performed 48 h after dosing for all time points except the 24 hpf dosing, for which larvae were screened 72 h later. The *in vivo* EROD assay required anesthetization of larvae with MS-222 (Sigma–Aldrich) and immobilization in 3% methylcellulose for proper orientation during quantification (Billiard et al., 2006).

2.5. Deformity assessment

Embryos were dosed with BNF at 24 hpf as described previously. Heart deformities were scored blind at 144 hpf. Deformities were scored as 0, 1, or 2. Common heart abnormalities with BNF exposure were heart elongation and pericardial edema. This scale represented normal (0), mild deformities (1), and severe deformities (2). Representative images for each deformity class have been included in Fig. 4 inset. Our distinction between mild and severe deformities is important in that embryos with severe heart abnormalities do not hatch, whereas mild deformities prevent hatching in roughly one half of affected embryos (data not shown). However, we do not have data regarding the long-term survival of *Fundulus* with mild heart abnormalities. Embryos scored as a 1 on the deformity scale have a range of heart abnormalities from moderate orientation changes to slight elongation of the atrium.

2.6. Confirmation of CYP1A morpholino efficacy by in vitro protein synthesis

The TNT® T7 Quick Coupled Reticulocyte Lysate System (Promega, Madison, WI) was used to synthesize [^{35}S]methionine-labeled proteins as per manufacturer protocols. Briefly, 20 μL of T7 TNT Quick Master Mix was combined with 1 μL of [^{35}S]methionine

(>1000 Ci/mmol at 10 mCi/mL), 2 μL *Fh*CYP1A-pBluescript II KS(−) (0.5 μg/μL), and adjusted to a final volume of 25 μL with H$_2$O. To test the efficacy of the CYP1A MO, 0.5 μL of a 25 μM stock of the standard control morpholino (Control MO) or CYP1A MO was added to the reaction, for a final concentration of 500 nM. Mixtures were incubated at 30 °C for 30 min to allow for sufficient *in vitro* transcription of CYP1A. To facilitate translation of the membrane-associated CYP1A protein, 1 μL of canine pancreatic microsomal membranes (Promega) was added to each reaction and incubated at 30 °C for an additional 60 min. Fifteen microliters of the labeled protein were resolved by SDS-PAGE. Fluorography was used to amplify the signal and visualize proteins on film. Densitometric analysis was performed with the ImageJ software package (National Institutes of Health, Bethesda, MD).

2.7. Western blot analysis

Whole embryos were homogenized in 2× sample treatment buffer (15 μL of buffer per embryo) and proteins were further denatured by boiling samples for 3 min. Homogenates were centrifuged at 14,000 rpm for 5 min. Equivalent volumes of lysate were subjected to SDS-PAGE and blotted to nitrocellulose. Blots were probed with monoclonal antibody 1-12-3 (3 μg/mL) against *Stenotomus chrysops* (scup) CYP1A (Park et al., 1986; Kloepper-Sams et al., 1987). Blots were subsequently probed with goat anti-mouse IgG horseradish peroxidase (Bio-Rad, Hercules, CA) secondary antibody (1:1000 dilution). The blots were visualized on film by enhanced chemiluminescence (ECL Plus™, GE HealthCare, Piscataway, NJ). Densitometric analysis was performed with the ImageJ software package. The relative densitometric units were determined by normalizing the data from PCB-only and PCB plus CYP1A MO treatments to the values from DMSO treatments after all densitometric values were adjusted for local background.

2.8. Data analysis

All data were analyzed using SPSS ver. 15 (Chicago, IL). EROD data were not normally distributed according to the Kolmogorov–Smirnov test. As such, EROD data were rank transformed and a non-parametric ANOVA was performed to test for differences among treatments. A Bonferroni-corrected post hoc comparison was conducted to determine which pairwise comparisons were statistically significant. Deformity data were non-parametric in nature and as such were analyzed similarly to our rank transformed EROD data. Statistical significance was accepted at $p \leq 0.05$ for all tests. A curve estimation was also performed to determine which type of curve best fit our EROD knockdown duration data.

3. Results and discussion

3.1. Assessment of injection techniques and morpholino effectiveness

To examine the effectiveness of MOs in *Fundulus* embryos, we tried using standard zebrafish injection techniques, which involve morpholino injection into the yolk up to the 4-cell stage (Nasevicius and Ekker, 2000). Fluorescence screening of these embryos at 24 hpf revealed that the morpholino failed to fully incorporate into the embryo; the vast majority of fluorescence was observed distributed throughout the yolk. In fact, fluorescence remained in the yolk throughout the 14–16 day incubation period. Early hatchlings (18 dpf) still had observable fluorescence in what was left of their yolk, clearly showing that yolk-injected morpholino was not efficiently incorporated into the developing embryo. The results of EROD assays used to test for functional knockdown of CYP1A activity are shown in Fig. 1. Embryos injected into the yolk with
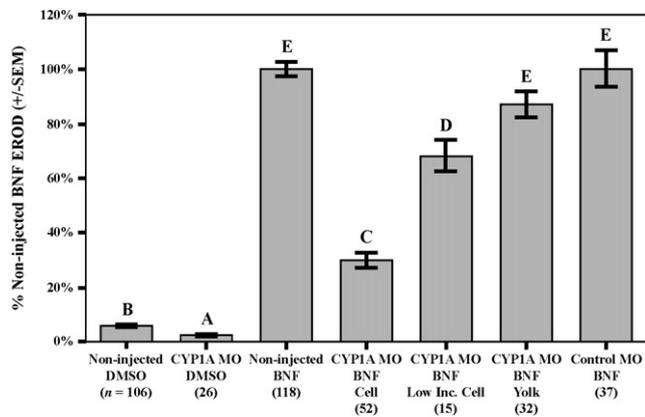
Fig. 1. CYP1A activity as measured via *in ovo* EROD assay for both non-injected and morpholino-injected *Fundulus* embryos. EROD data are presented as percent activity relative to non-injected BNF control. BNF exposures were conducted at a concentration of 10 μg/L, and DMSO was used as a vehicle control. EROD activity was measured in 4 dpf embryos. Letters above bars represent statistically significant groupings ($p \leq 0.05$; Bonferroni corrected non-parametric ANOVA). Cell and yolk denotations represent location of morpholino injections. Low inc. represents cell-injected embryos that had incomplete or low incorporation of the morpholino.

the CYP1A MO showed a slight, non-significant reduction in BNF-induced CYP1A activity relative to non-injected embryos.

In light of these results showing that the CYP1A MO was ineffective when injected into the yolk, we altered our protocols to inject MO directly into the blastomere, similar to the technique used to inject MO into *Xenopus* embryos (Heasman et al., 2000). Injections were performed on embryos up to the 4-cell stage, with 4-cell embryos being injected into two separate cells. When morpholino was injected directly into the cell, it fully incorporated into the developing embryo. Fluorescence screening at 24 hpf was used to confirm normal embryonic development and full morpholino incorporation. Individuals with evenly distributed but minimal fluorescence were evaluated separately as "low incorporation" embryos representing a reduced-dose morpholino group.

CYP1A MO function was tested in cell-injected embryos via an *in ovo* EROD assay. EROD assay results for high-incorporation embryos demonstrate that the CYP1A MO significantly reduced the induction of CYP1A activity in 4 dpf BNF-exposed embryos (Fig. 1). CYP1A activity in BNF-exposed morphants was reduced by 70%, relative to non-injected or control morpholino-injected embryos exposed to BNF. This level of knockdown is critical, given that previous work has shown that greater than 50% knockdown of CYP1A activity is associated with the synergistic teratogenicity of some PAH combinations (Wassenberg and Di Giulio, 2004). Induction of EROD activity was also significantly reduced in low incorporation embryos relative to controls; however, the knockdown was not as efficient as seen in the high-incorporation embryos (Fig. 1).

3.2. Confirmation of CYP1A morpholino efficacy by in vitro and in vivo analysis

Reduced EROD induction in CYP1A MO-treated embryos suggested that the MO was effective at reducing CYP1A translation in the embryos. To directly assess the effectiveness of the MO, we measured its effect on CYP1A protein synthesis *in vitro* and *in vivo*.

A TNT® assay system was used to confirm the MO efficacy at inhibiting the *in vitro* translation of CYP1A. Membrane associated proteins do not perform optimally in the *in vitro* TNT reactions (see manufacturer protocols); therefore, microsomal membranes were added to the reactions after the initial transcription phase to facilitate translation. Although the protein yield was low compared to non-membrane associated proteins, detectable amounts of CYP1A
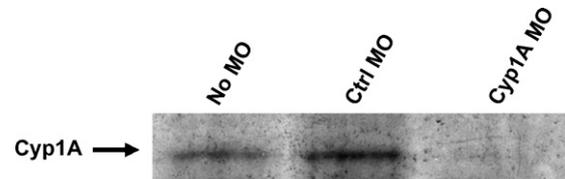

Fig. 2. Inhibition of *in vitro* translation of CYP1A by CYP1A MO. The ability of a CYP1A MO targeting the translation start site for CYP1A to inhibit translation was tested via an *in vitro* translation assay using the TNT® Coupled Reticulocyte System (Promega). The standard control MO (Ctrl MO) from Gene Tools was used to detect any non-specific interference in the transcriptional/translational process. A final MO concentration of 500 nM was used in each of the reactions. Densitometric analysis was used to calculate percent inhibition of the CYP1A MO compared to No MO (97.7%) or Ctrl MO (98.8%).

protein were observable by SDS-PAGE of [^{35}S]Met-labeled proteins (Fig. 2). Based on densitometric analysis, the CYP1A MO was able to inhibit translation by approximately 98% compared to the No MO and Ctrl MO control treatments.

Western blot analyses were performed to confirm the knock-down of endogenous CYP1A protein in *Fundulus* embryos at various developmental time points. CYP1A protein was not detectable in initial experiments with BNF treated embryos (4 and 6 dpf) in which lysate from 6 embryos was loaded in each lane. Additional gels with lysate from 12 embryos per lane allowed for the minimal detection of CYP1A in both DMSO controls and BNF treatments but were not sensitive enough to detect any changes in protein levels (data not shown). To optimize detection capabilities, *Fundulus* embryos were exposed to a stronger AHR agonist (PCB126). While a strong induction of CYP1A was observed in 8 dpf embryos exposed to PCB126, CYP1A protein was barely detectable in PCB126-exposed embryos injected with the CYP1A MO (Fig. 3A). To further assess the degree of inhibition over time, Western blot analyses for CYP1A were performed on PCB126-treated embryos at 6, 8, and 12 dpf. Significant decreases in induced CYP1A expression (79–86%) were observed in embryos injected with the CYP1A MO at these time points (Fig. 3B).
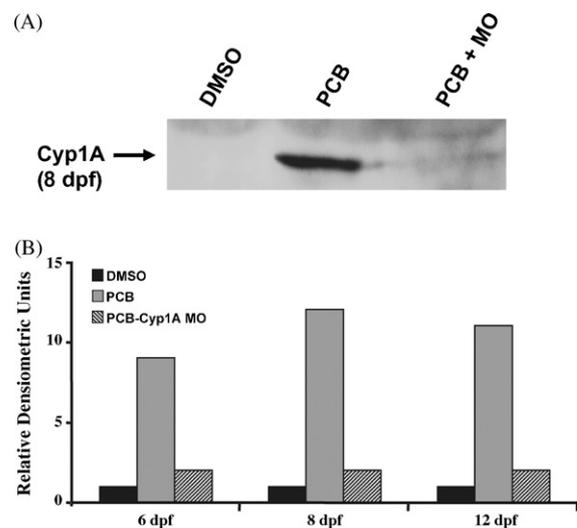

Fig. 3. Inhibition of *in vivo* CYP1A translation by CYP1A MO. The ability of a CYP1A start site MO to inhibit translation *in vivo* was assessed by Western blot analysis using lysates from pooled embryos. (A) Representative CYP1A Western blot for 8-day post-fertilized embryos exposed to PCB126 (1 μg/L). Lysate from 4 embryos was loaded in each lane. Equal protein loading was confirmed by Ponceau staining of nitrocellulose membrane and Coomassie blue staining of SDS-PAGE gels after transfer. (B) Densitometric analysis of CYP1A Western blots for three different time points during *Fundulus* embryo development. Percent reduction of CYP1A induction at each time point: 6 dpf, 79%; 8 dpf, 82%; 12 dpf, 86%. Lysate from 6 embryos was loaded in each lane. For each time point, data from PCB-only and PCB + CYP1A-MO-treated embryos were normalized to the value for the DMSO control.
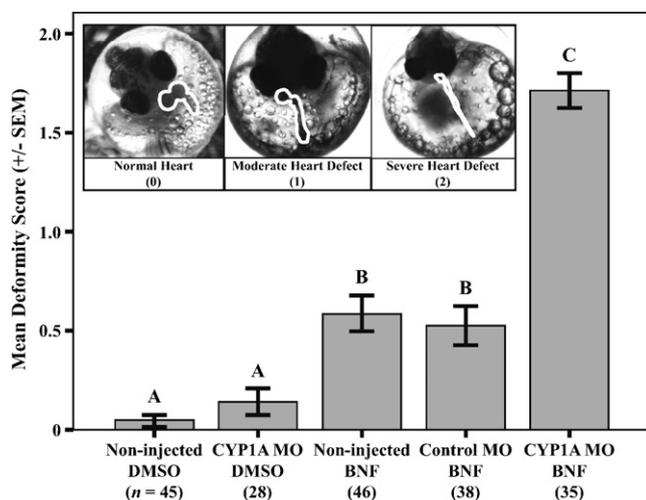
Fig. 4. Mean deformity scores for both non-injected and morpholino-injected *Fundulus* embryos. Increasing deformity score represents increasing severity of deformities. BNF exposures were conducted at a concentration of 10 μg/L, and DMSO was used as a vehicle control. Letters above bars represent statistically significant groupings ($p \leq 0.05$; Bonferroni corrected non-parametric ANOVA). Inset contains representative images for each deformity class. Hearts have been traced in white for visualization purposes.

3.3. Phenotypic evaluation of morpholino function

The functionality of the CYP1A MO was also tested in terms of its ability to enhance BNF-induced embryotoxicity. BNF has been shown to cause cardiovascular deformities in developing fish embryos at concentrations as low as 10 μg/L (Billiard et al., 2006). It has also been demonstrated that reducing CYP1A protein activity, via chemical inhibition or morpholino knockdown, during BNF exposures results in increased severity of cardiovascular developmental abnormalities in fish embryos (Wassenberg and Di Giulio, 2004; Billiard et al., 2006). We confirmed that 10 μg/L BNF is sufficient to induce cardiovascular abnormalities of mild to intermediate severity in *Fundulus* embryos (Fig. 4). More importantly, the morpholino-injected embryos revealed a dramatic increase in the number and severity of cardiovascular abnormalities in BNF-dosed, but not control, groups. The control morpholino had no observable effect on the number or severity of deformities in *Fundulus* embryos exposed to BNF.

3.4. Morpholino functional duration

Functional duration was tested by dosing with BNF and conducting EROD assays on embryos at increasing intervals following injection of the CYP1A MO. *In ovo* assays were performed at 2-day intervals from 4 to 12 dpf, and *in vivo* assays were performed on newly hatched *Fundulus* at 20 dpf.

Injection of CYP1A-MO at the 1–4-cell stage caused a persistent reduction in EROD induction by BNF, with 70% decrease at 4-dpf and a slow recovery of inducibility (Fig. 5). In newly hatched *Fundulus* larvae (20 dpf), BNF-induction of EROD activity was still reduced as compared to control (non-injected) larvae exposed to BNF. A curve estimation was performed to determine which model best fit the data (Fig. 5). A logarithmic model provided the best estimate of mean EROD activity as a function of time. The logarithmic model (Fig. 5) yielded a 10 dpf estimate for the point at which CYP1A activity in MO-injected embryos, as measured via EROD assay, was back to 50% of its normal response, with complete recovery predicted around day 58.

The longevity of morpholino-induced knockdown is a critical concern of scientists using morpholino techniques to study devel-
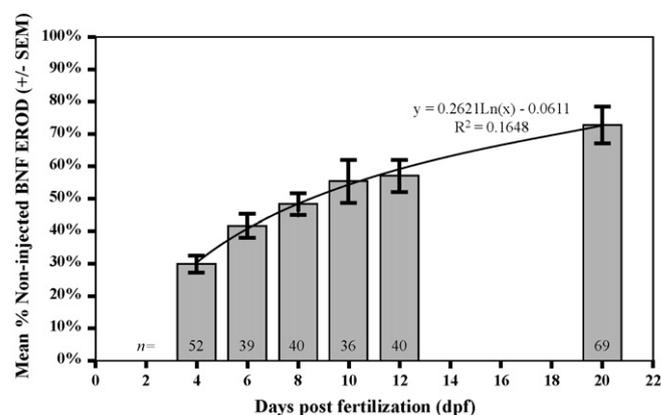

Fig. 5. Mean CYP1A activity in CYP1A MO-injected embryos in response to BNF exposure (10 μg/L), relative to non-injected BNF-exposed embryos. The data demonstrate the functional duration of the morpholino in *Fundulus* embryos. DMSO was used a vehicle control. Error bars represent standard error of the mean (S.E.M.). Sample sizes are included at the base of each bar. The logarithmic curve that best explains the data is also provided.

opment or processes that only occur after a certain developmental stage. Since development occurs much more slowly in *Fundulus* than in zebrafish, there was some concern that the duration of morpholino effects in *Fundulus* might be insufficient for practical use of this technique. However, these data clearly demonstrate that significant morpholino-induced knockdown lasts beyond hatching (~14 dpf), and thus persists throughout the development of all major organ systems. In combination, the EROD (Fig. 5) and Western blot (Fig. 3) data provide a convincing argument for the long duration of morpholino-induced knockdown in *Fundulus*. Thus, it appears that morpholinos will be just as useful in *Fundulus* as they are in zebrafish.

4. Conclusions

We have conclusively shown, via molecular and phenotypic methods, that MO technologies can be adapted to the teleost estuarine environmental model, *F. heteroclitus*. The development and evaluation of MO technologies in *Fundulus* has revealed several critical changes that must be made when working with this species, relative to zebrafish methods. The hardness of the chorion and high internal pressure make the use of an agarose ramp for injection ineffective. A holding pipette seems to be necessary to adequately immobilize *Fundulus* eggs for proper microinjection, because intracellular injections are necessary for proper incorporation of morpholino and thus proper alignment of the blastomere is critical. This has the consequence of slowing down the injection process. However, time is not a critical concern with *Fundulus* injections, given the slower rate of division. The hardness of the chorion is also important in this regard, as non-perpendicular injections often result in broken needles. We have also shown that the duration of MO-mediated gene knockdown in *Fundulus* is comparable to that achieved in zebrafish, in terms of developmental stages during which MO knockdown persists. Gene knockdown continues well into the larval stage, as seen in zebrafish. One additional point to note with regard to morpholino duration is that the gene we selected for evaluation of MO technologies in *Fundulus* (CYP1A) is highly inducible (Handley-Goldstone et al., 2005; Timme-Laragy et al., 2007). As such, it provides a very rigorous test of the duration of morpholino action.

The development of MO technologies in *Fundulus* will allow researchers to more fully investigate a number of interesting aspects of killifish, including their adaptation(s) to contaminated environments. The methodology developed here also paves the way

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