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The long amplicon quantitative PCR for DNA damage assay as a sensitive method of assessing DNA damage in the environmental model, Atlantic killifish (*Fundulus heteroclitus*)*

Dawoon Jung, **Youngeun Cho**, **Joel N. Meyer**, and **Richard T. Di Giulio****
Nicholas School of the Environment, Duke University, Durham, NC 27708, USA

Abstract

DNA damage is an important mechanism of toxicity for a variety of pollutants, and therefore, is often used as an indicator of pollutant effects in ecotoxicological studies. Here, we adapted a PCR-based assay for nuclear and mitochondrial DNA damage for use in an important environmental model, the Atlantic killifish (*Fundulus heteroclitus*). We refer to this assay as the large amplicon quantitative PCR (LA-QPCR) assay. To validate this method in killifish, DNA damage was measured in liver, brain, and muscle of fish dosed with 10 mg/kg benzo[*a*]pyrene. This exposure caused 0.4-0.8 lesions/10 kb. We also measured DNA damage in liver and muscle tissues from killifish inhabiting a Superfund site, confirming the utility of this method for biomonitoring. In both cases, damage levels were comparable in nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Since extensive nDNA sequence data are not readily available for many environmentally relevant species, but mitochondrial genomes are frequently fully sequenced, this assay can be adapted to examine mtDNA damage in virtually any species with little development. Therefore, we argue that this assay will be a valuable tool in assessing DNA damage in ecotoxicological studies.

Keywords

DNA damage; LA-QPCR assay; *Fundulus heteroclitus*; PAH; Benzo[*a*]pyrene; Elizabeth River

1. Introduction

DNA damage results from exposure to many contaminants, and is widely used as an indicator or biomarker of biological effects (van der Oost et al., 2003). In addition, DNA damage and repair is an important field of study within ecotoxicology (Theodorakis, 2001). The long amplicon quantitative PCR (LA-QPCR) assay, previously referred to as the QPCR assay, provides a sensitive way of assessing DNA damage and alterations to DNA that often lead to damage (Ayala-Torres et al., 2000). The assay measures the fraction of undamaged template DNA by comparing the amplification of very long PCR target (amplicon), under the assumption

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**Corresponding Author: Nicholas School of the Environment, Duke University, Box 90328, Durham, NC 27708, USA, Phone: (919) 613-8024, Fax: (919) 668-1799, Email: E-mail: richd@duke.edu.

that lesions and/or structural alterations in the target genomic DNA template will block or slow the progression of the DNA polymerase used in the reaction (Kalinowski et al., 1992). We can then mathematically transform the difference in amplification to represent lesion frequencies. With parallel amplification of shorter PCR target within the amplified region of the longer target, we can normalize for DNA copy number and compare DNA damage in different DNA sources. The LA-QPCR assay can detect DNA strand breaks, adducts, and many other types of structural modifications such as those caused by oxidative damage. Therefore, it is potentially of particular utility in biomonitoring contexts where the specific types of DNA damage may not be well-defined or predictable ahead of time. It has been used successfully in human cell lines (Yakes and Van Houten, 1997; Van Houten et al., 2000) and laboratory model organisms (Chan et al., 2006; Meyer et al., 2007). In these models, the assay has been shown to have a high sensitivity, with a limit of detection of approximately 1 lesion per 10^5 nucleotide (Santos et al., 2006). This assay is a relatively simple method of detecting damage in nuclear DNA (nDNA) and mitochondrial DNA (mtDNA), and if adapted, would be valuable for detecting DNA damage in wildlife populations. However, the use of this assay in an environmental model has not been published.

The Atlantic killifish (*Fundulus heteroclitus*) is an estuarine fish species distributed throughout the coastal marshes along the North American Atlantic Coast. This species has a limited home range (Lotrich, 1975), and is considered to be very adaptable to diverse local environments and various stressors that are present in their habitats (Burnett et al., 2007). Different populations show resistance to pollutants such as metals (Weis and Weis, 1989), dioxin-like compounds and PCBs (Prince and Cooper, 1995a, 1995b; Nacci et al., 1999), and PAHs (Meyer et al., 2002; Ownby et al., 2002; Meyer and Di Giulio, 2003). Furthermore, they are easy to collect and maintain in the laboratory (Burnett et al., 2007). Thus, killifish have been widely utilized in laboratory toxicology (Eisler, 1986; Wassenberg et al., 2002), genetic adaptation research (Nacci et al., 2002), ecological studies (Weis and Weis, 1989; Nacci et al., 1999), and as a model organism for estuarine system monitoring (Eisler, 1986).

Several assays have been developed for assessing contaminant exposures in killifish (Binder et al., 1985; Van Veld et al., 1992; Greytak et al., 2005). Most of these assays are specific to individual or groups of compounds, and do not address the downstream and potentially higher level biological effects of contaminants on this species. Considering the role of killifish as an important environmental model, an assay that can easily detect general DNA damage would be a valuable tool for assessing contaminant effects in this organism.

In this study, we explored the potential of the LA-QPCR assay to measure PAH-induced genetic damage in Atlantic killifish. First, we tested the utility of this method for use in laboratory exposures by quantifying DNA damage in killifish that had been injected with the well-established genotoxin benzo[*a*]pyrene (BaP). Second, in an environmental study, we used LA-QPCR to look for evidence of genotoxicity in Atlantic killifish inhabiting a highly contaminated Superfund site.

2. Materials and Method

2.1. Killifish care

Adult killifish (*Fundulus heteroclitus*, Fundulidae, Cyprinodontiformes) were captured using baited minnow traps from King's Creek, a tributary of York River in Gloucester County, VA. This is a relatively unpolluted site that we have used for several years as a reference site (Meyer et al., 2002; 2005). After capture, the fish were transported to the Duke University Ecotoxicology Laboratory. Fish were maintained in a recirculating system containing 23-25° C, 25 ppt artificial sea water (Instant Ocean®, Aquarium Systems, Forster & Smith, Rhinelander, WI, USA) with a 14:10 Light:Dark photoperiod. The fish were fed a mixed diet

of Tetramin® Tropical Fish Food (Tetra Systems, Blacksburg, VA, USA) and brine shrimp (*Artemia sp*, Brine Shrimp Direct, Ogden, UT, USA).

2.2. BaP treatment and DNA isolation

Male killifish were moved to individual aerated tanks with 3 L of artificial seawater 24h before treatment. Ten fish were injected intraperitoneally with BaP in corn oil. Fish were injected with 5 μL/g wet mass of 10 mg/kg BaP. Additional fish were injected with 5 μL/g wet mass of corn oil as a carrier control. The fish were fed everyday and sacrificed 72h post-treatment. Brain, liver, and muscle tissues were dissected out and flash frozen in 20% glycerol, and stored at -80°C. Tissues were later ground in liquid nitrogen, and total DNA was extracted with the Genomic-tip 20/G kit (Qiagen Inc., Valencia, CA, USA) according to the manufacture's protocol. In addition, fish from King's Creek and from the Atlantic Wood Superfund site at the Elizabeth River in Portsmouth, VA were collected and sacrificed within 24h of capture. Previous studies have shown that populations from these two sites are genetically suited to be used in comparison studies (Mulvey et al., 2002; 2003). Liver and muscle from the fish were dissected out. The tissues were stored at -80°C until total DNA was isolated as described above. Since this assay relies upon the amplification of long stretches of DNA, it is critical that the DNA template be extracted as carefully as possible. The extracted DNA should not be exposed to phenol, and should be of high molecular weight. Additional protocol details are available in Santos et al. (2006).

2.3. Ultraviolet radiation C (UVC) exposure

Total DNA was isolated from the liver and brain of adult male *Fundulus heteroclitus* using the isolation methods described above. Equal amounts of DNA (50 μL of 3 ng/μL) were exposed to 0, 5, 10, and 20 J/m^2 of ultraviolet radiation (254 nm; hereafter referred to as UVC) using either an ultraviolet lamp (UVLMS-38 EL Series 3UV Lamp, UVP, Upland, CA, USA) in conjunction with a UVX radiometer and UVX-25 sensor (UVP), or a CL-1000 Ultraviolet Crosslinker (UVP) with an emission peak at 254 nm. DNA was immediately frozen until further analysis.

2.4. Primer selection

Primers for large and small nuclear targets were designed for the cystic fibrosis transmembrane conductance regulator gene (CFTR, GenBank assession no. **AY028263**), and large and small mitochondrial targets were designed from a cDNA sequence for cytochrome c oxidase polypeptide VIa, mitochondria (**CN984995**). The CFTR gene was selected as it was the only published gene over 10kB long. Primers were designed using PRIMER3 (Rozen and Skaletsky, 2000). The primer sequences for the large mitochondrial target were obtained from Kim et al (2004). Primers and amplicaon sizes are described in Table 1. All primers were tested to confirm the amplification of a single band of the expected length.

2.6. LA-QPCR

LA-QPCR was performed according to a protocol modified from Santos *et al*. (2006). This assay has previously been referred to as the QPCR for DNA damage assay; we have chosen to refer to it as the LA-QPCR assay to avoid confusion with quantitative PCR (qPCR), the abbreviation frequently used to refer to real-time PCR-based measurement of mRNA levels.

Briefly, 10 ng DNA (5 μL of 2 ng/μL DNA) from each sample was amplified with *rTth* polymerase (Applied Biosystems) using the primers described above. Small nDNA and mtDNA targets were amplified for normalization/verification of DNA concentration and to account for mitochondrial copy number, respectively. We optimized the elongation temperature, $Mg(OAc)_2$ concentration, and cycle number for each PCR target. The PCR

conditions for each set of primers are as follows. For all targets, final concentrations of 1x buffer (provided in the *rTth* polymerase kit), 100μg/mL of BSA, 200 μM of each dNTP, and 0.4 mM of each primer were added in the PCR mix. Water volume was adjusted to make the volume 50 μL for each reaction. For both short targets, 1.2 mM of $Mg(OAc)_2$ was used in the PCR mix. The cycling conditions were 75°C for 2 min; 94°C for 1 min; 94°C for 15 s, 62°C for 45 s, and 72°C for 30s (repeated 24 cycles); and 72°C for 5 min. For the long nuclear target, 1.1 mM of $Mg(OAc)_2$ was used in the PCR mix. The cycling conditions were 75°C for 2 min; 94°C for 1 min; 94°C for 15 s and 68°C for 12 min (repeated 24 cycles); and 72°C for 10 min. For the long mitochondrial target, 1.2 mM of $Mg(OAc)_2$ was used in the PCR mix. The cycling conditions were 75°C for 2 min; 94°C for 1 min; 94°C for 15 s and 65°C for 12 min (repeated 16 cycles); and 72°C for 10 min. We added 5 μL of the *rTth* enzyme (diluted to 1 unit/μL) after 90 s of the 75°C incubation at the beginning of the reaction to initiate the amplification with "hot start." PicoGreen dye (Invitrogen Corporation, Carlsbad, CA, USA) was used to quantify the template and PCR product. DNA concentrations were then converted to lesion frequencies per 10kB DNA by application of the Poisson distribution, as described by Ayala-Torres et al. (2000). This approach defines the control samples as undamaged, and generates a lesion frequency in experimental samples relative to the control samples, based on alterations in amplification efficiency and an assumption of random distribution of damage. With each PCR reaction, we included 5 ng of one of the control DNAs to monitor amplification quality. Only PCR products in which the amplification of 5 ng DNA was 40-60% of the control DNA (10 ng), indicating that the PCR reaction was quantitative, were used in the analysis.

2.7. Statistics

Statistical analyses were performed using SPSS, version 15.0 for Windows (SPSS Inc., Chigago, IL, USA). The assumption of normality was tested for all data sets using the Shapiro-Wilk's test. Analysis of Variance (ANOVA) and Fisher's Protected Least-Significant Differences (LSD) were used to test for differences among groups ($\alpha = 0.05$).

3. Results

3.1. Adaptation of the LA-QPCR assay

To confirm the success of primer selection and condition optimization for this assay, we exposed purified total DNA in buffer from adult male killifish liver and brain to various doses of UVC and assessed damage (Figure 1). A dose-dependent increase in damage to nDNA and mtDNA exposed to various doses of UVC radiation was detected ($p < 0.001$), but no differences in damage were observed between mtDNA and nDNA at a given dose ($p = 0.775$). The increase in DNA damage fit the linear regression with r^2 values of 0.84 for mtDNA and 0.81 for nDNA respectively, and the lesion frequencies detected were comparable to those obtained previously using DNA purified from human cells in culture or *Caenorhabditis elegans* at the same UV doses (Eischeid et al., 2008).

3.2. DNA damage in response to BaP

As expected, adult male killifish dosed with intraperitoneal (i.p.) injection of 10 mg/kg BaP showed increased levels of DNA damage relative to killifish dosed with corn oil for both mtDNA and nDNA (Figure 2) in all three tissues examined. Three-way ANOVA showed that there was a significant effect of treatment ($p < 0.001$). However, neither DNA source (mitochondria versus nucleus) nor tissue type significantly affected the result ($p = 0.177$ and $p = 0.493$ respectively). In addition, there was no interaction among any of the independent variables.

3.3. Comparison of Elizabeth River and reference site killifish populations

Killifish from the Atlantic Wood Superfund site and King's Creek (reference site) were sacrificed within 24h of capture, and lesion frequencies in mtDNA and nDNA from muscle and liver were examined (Figure 3). In this case, brain was not examined due to difficulties in acquiring sufficient amount of tissue. Three-way ANOVA showed that there was a significant effect of site ($p < 0.001$) and tissue type ($p = 0.047$). However, DNA source (nuclear or mitochondrial genome) was not significant ($p = 0.839$). There was also a significant interaction between tissue type and population ($p = 0.033$), reflecting the fact that the Elizabeth River killifish seemed to show more sensitivity to nDNA damage in muscle, but to mtDNA damage in liver. However, while statistically significant, this difference does not seem large enough to be of clear biological relevance.

4. Discussion

We have shown for the first time that the LA-QPCR assay can be adapted to a widely studied environmental model. We detected significant increases in the frequency of DNA lesions after exposure to an environmentally relevant dose (10 mg/kg) of BaP as well as contaminants present at a Superfund site.

In our experiments with BaP-treated fish as well as with the field-caught fish, we detected no significant differences in damage in response to BaP treatment between mtDNA and nDNA. This was a surprise since previous data in mammalian cell culture studies indicated a much greater susceptibility of the mitochondrial genome to polycyclic aromatic hydrocarbon exposure (Allen and Coombs 1980, Backer and Weinstein 1980). We do not know the reason for this difference. It seems unlikely to be related to the requirement for metabolic activation: Backer and Weinsten used the reactive metabolite benzo[*a*]pyrene diol epoxide, rather than the parent benzo[*a*]pyrene, but Allen and Coombs used parent compounds that require activation, as we did. More likely candidates include *in vitro* vs *in vivo* differences (the studies cited above used cell culture systems), DNA damage detection methodology, or species differences. With aflatoxin B_1, another lipophilic chemical that is activated to a DNA-reactive form by CYP proteins, Niranjan et al (1982) saw ~3-fold higher binding to mtDNA than nDNA *in vivo*. While this difference was still significant, it is much less than the *in vitro* differences reported by Allen and Coombs and Backer and Weinstein (40- to 500-fold). Furthermore, the vulnerability of mtDNA to this chemical showed species variation (Niranjan et al 1986), which may be in part related to mitochondrial enzyme differences leading to differential activation (Niranjan et al (1985). It will be interesting to further explore the relative vulnerabilities of the mitochondrial and nuclear genomes in fish; mtDNA has been shown to be more sensitive to various genotoxins than nDNA in mammalian studies (Backer and Weinstein, 1980; Balansky et al., 1996; Yakes and Van Houten, 1997; Sawyer and Van Houten, 1999).

However, another important implication of the equal or greater sensitivity of the mitochondrial genome to at least many pollutants is that unless a specific nuclear-coded gene needs to be targeted to assess DNA damage to specific genes, using the LA-QPCR assay with just mtDNA would be sufficient in many field studies. This is advantageous for many environmental models, such as the Atlantic killifish, as they generally do not have significant nuclear genome sequence data available, particularly the 10 kb or more of contiguous sequence needed to design primers for the LA-QPCR assay. In such models, it is much easier to design primers for mtDNA, since there is a tremendous amount of mtDNA sequence data available and "universal" primers have already been designed in conserved regions that will amplify the mtDNA of most vertebrates (Kocher et al., 1989). In fact, the primers that we used for *Fundulus* were initially designed for the javeline goby (*Acanthogobius hasta*) (Kim et al., 2004).

Interestingly, liver DNA showed more DNA damage in Superfund site killifish than did muscle DNA, although this difference was not observed in the acute exposure. This may be explained by the fact that the killifish population at the Superfund site has been chronically exposed to a complex mixture of chemicals including primarily several different PAHs, as well as PCP and metals (EPA, 2007). Therefore, this population is exposed continuously to a variety of genotoxic agents through their diet. In this case, liver would be one of the primary targets of the toxic effect. The differences in contaminant mixture, and/or time course and route of exposure, may be why we see differences in the DNA damage profile of Elizabeth River *Fundulus* relative to fish acutely exposed to BaP via i.p. injection. Thus, our data suggest that data from muscle tissue alone might not be as informative as other tissues such as liver. Therefore, an examination of several tissues may be necessary to correctly assess the genotoxic effects of pollutants.

Currently, there are several assays that measure DNA damage. DNA-adduct analysis by ^{32}P-postlabelling method can be used to measure chemical-specific adducts. The method is considered the most sensitive in detecting PAH-adducts, but is expensive and time consuming (van der Oost et al., 2003). Flow cytometry (Barbee et al.,; Theodorakis, 2001; Goanvec et al., 2004), single-strand break assays (McFarland et al., 1999; Bolognesi et al., 2006) and the micronucleus test (Al-Sabti and Metcalfe, 1995; Hayashi et al., 1998; Arkhipchuk and Garanko, 2005; Cavas and Ergene-Gozukara, 2005; Bolognesi et al., 2006) are also used in laboratory and field studies as genotoxic indicators, but these assays detect gross chromosomal damage or abnormalities from clastogenic and aneugenic effects. The comet assay is one of the most widely used biomarkers of DNA damage in laboratory (Pandrangi et al., 1995; Belpaeme et al., 1996; Nacci et al., 1996; Devaux et al., 1997; Devaux et al., 1998) and field studies (Pandrangi et al., 1995; Devaux et al., 1998; Steinert et al., 2002; Lemos et al., 2005; Yang et al., 2006). However, there is still high study-to-study variation, and standardization of measurement is necessary to overcome this issue (Cotelle and Férard, 1999; Siu et al., 2004; Lemos et al., 2005).

Considering the issues concerning the assays described above, the adaptation of LA-QPCR assay will be an important and effective means to measure general DNA damage in the environment. With this assay, one can detect general lesions caused by a variety of pollution sources or complex mixtures. At the same time, one can target a specific gene or the entire mitochondrial genome for damage assessment. This ability to easily distinguish mtDNA damage from nDNA damage or total DNA damage is an important advantage of this assay, since there is increasing concern for the vulnerability of mitochondria to various pollutants (Backer and Weinstein, 1980; Sawyer and Van Houten, 1999), and for the lower DNA repair capability of the mitochondria for certain kinds of damage (Yakes and Van Houten, 1997; Larsen et al., 2005).

In conclusion, we have successfully adapted the LA-QPCR assay in an environmental model. This assay can be utilized as a sensitive method of detecting general nuclear and mitochondrial DNA damage, and has significant potential as a tool for biomonitoring. Therefore, we propose the use of the LA-QPCR assay for DNA damage for use in environmental assessments.

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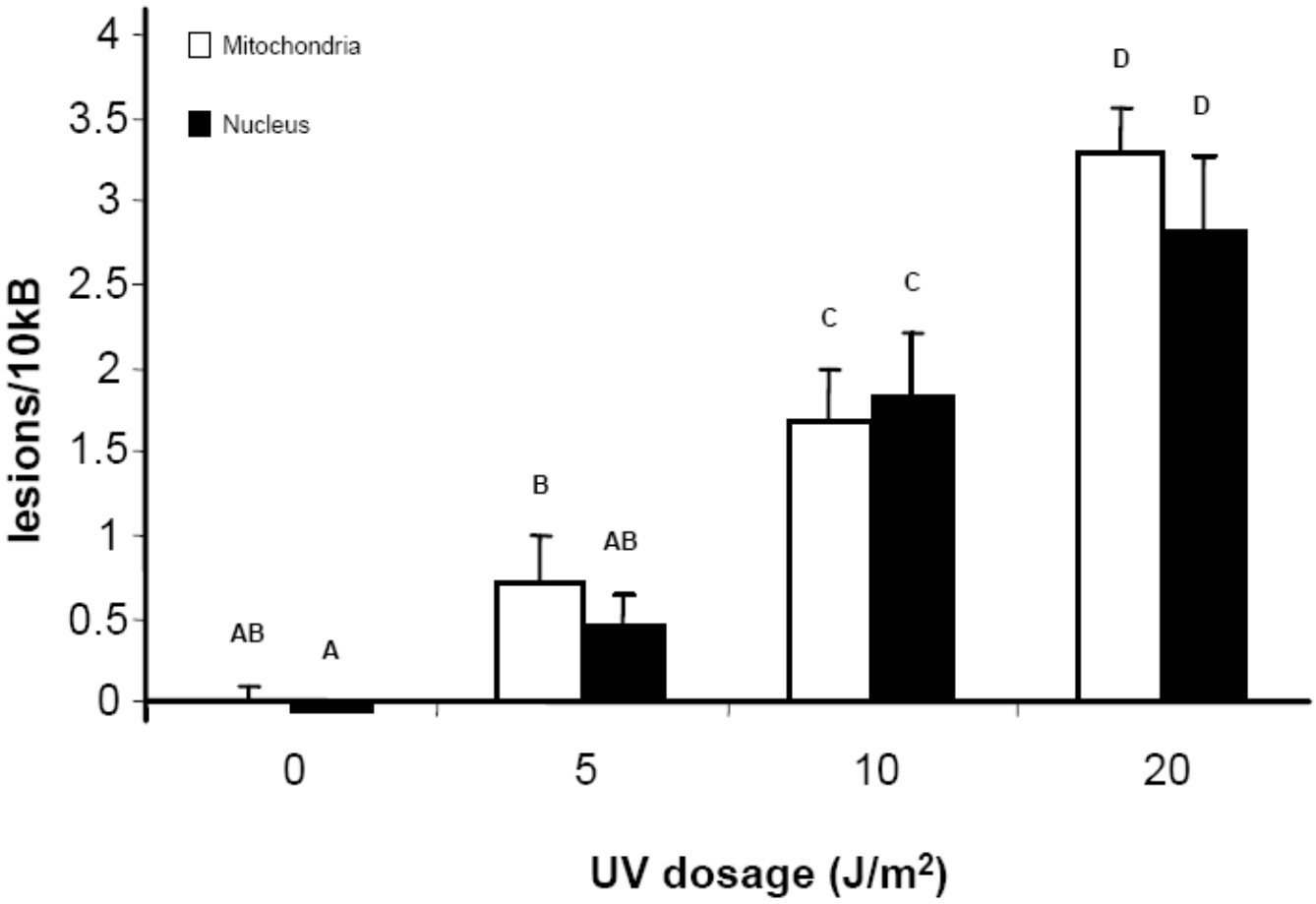

Figure 1. DNA damage in UVC-treated killifish DNA

DNA isolated from adult killifish liver and brain was exposed to different doses of UVC. There are significant dose-dependent increases in both mitochondrial and nuclear DNA ($p < 0.001$), but no differences between mtDNA and nDNA at a given dose ($p = 0.775$). Letters indicate significant groupings ($p < 0.05$) according to Fisher's LSD. n = 6 per treatment group. Error bars indicate standard error of means.

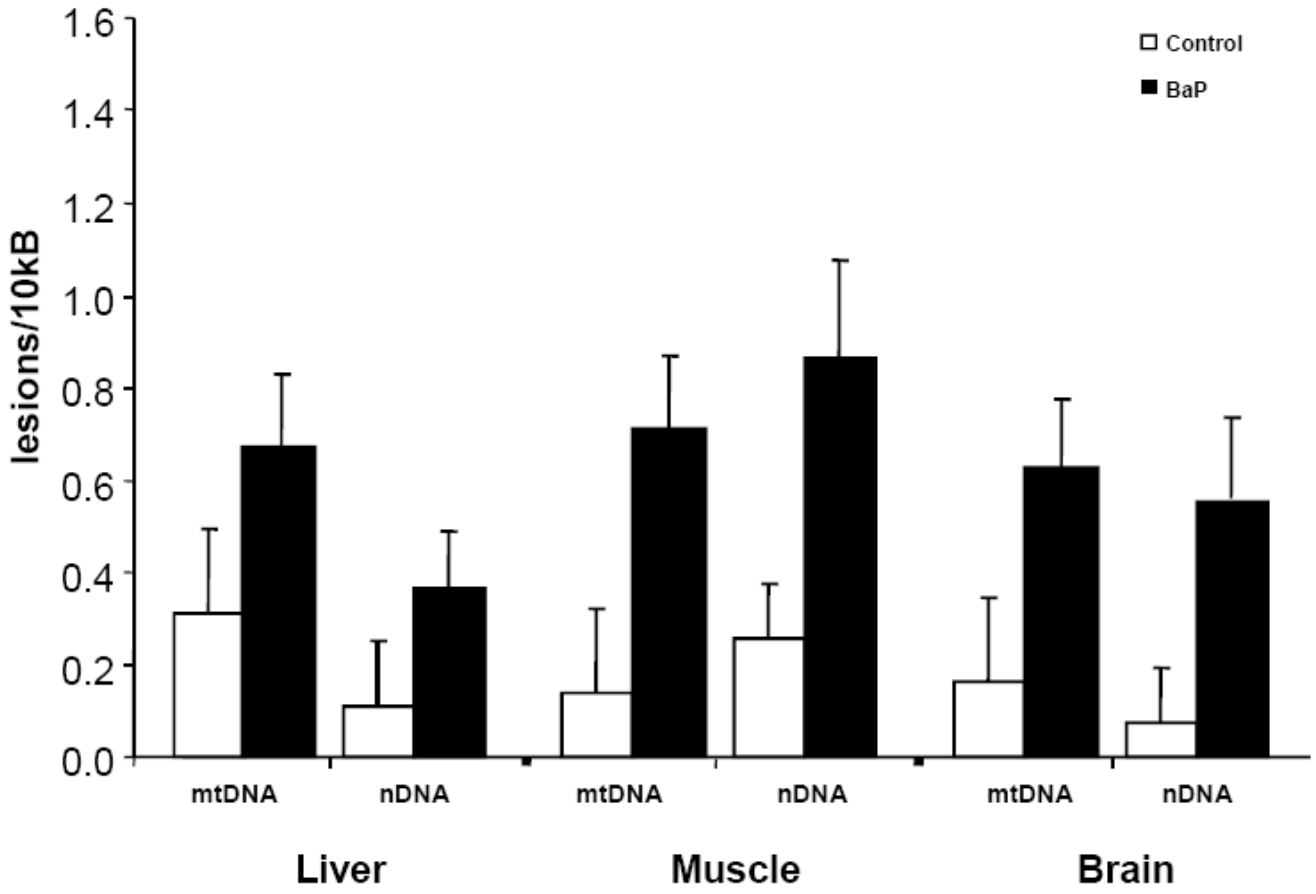

Figure 2. Levels of DNA damage in BaP treated adults

DNA lesion frequencies were measured from adult male fish dosed with 10 mg/kg BaP. BaP treatment was the only significant factor according to three-way ANOVA ($p < 0.001$). Neither DNA source nor tissue significantly affected the result ($p = 0.177$ and $p = 0.493$ respectively). n = 10 per treatment group. Error bars indicate standard error of means.

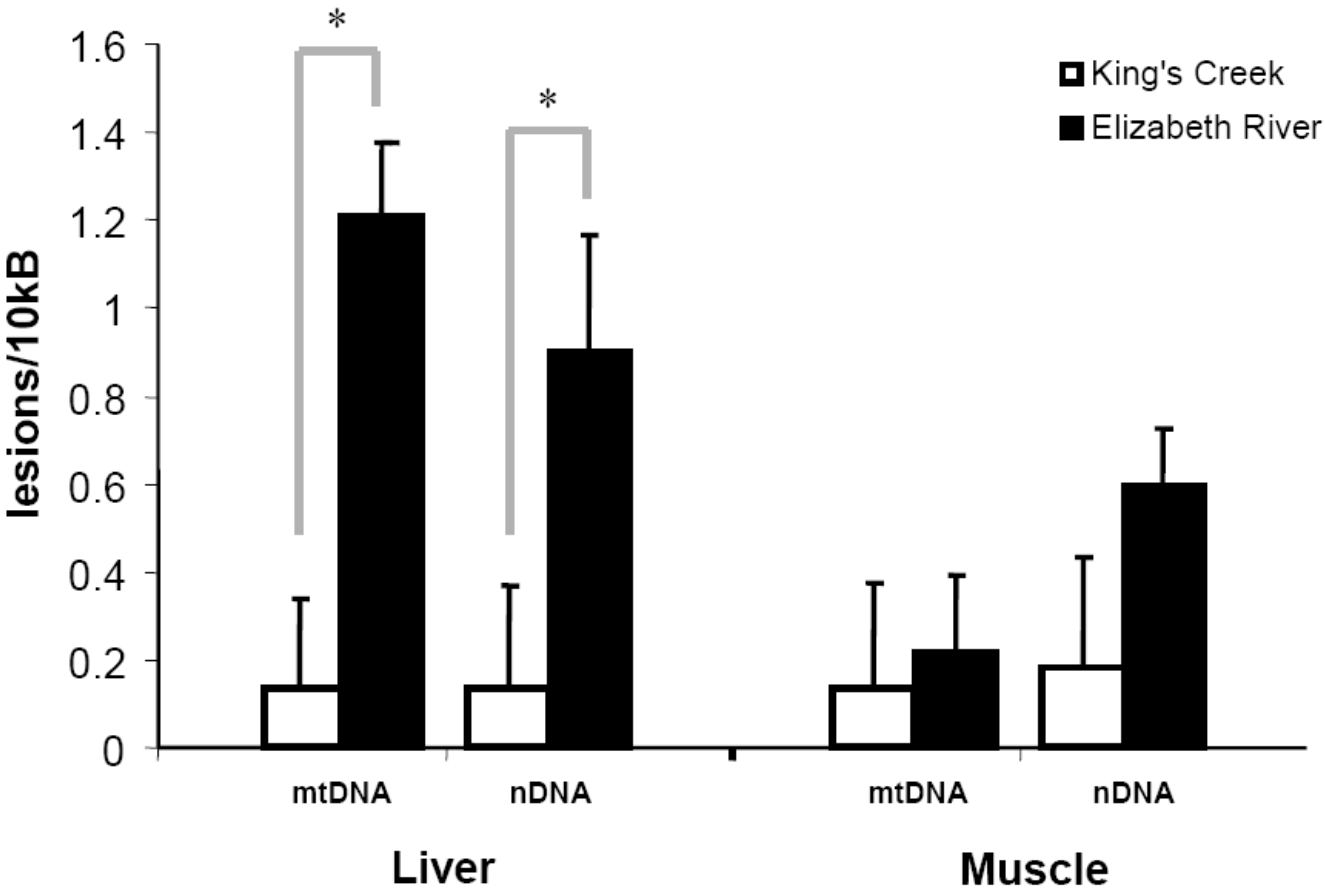

Figure 3. Levels of DNA damage in Superfund site and reference *Fundulus* populations

DNA lesion frequency was measured from liver and muscle of adult fish captured from a Superfund site and a reference site. Population and tissue type significantly affected the results ($p < 0.001$ and $p = 0.047$ respectively) according to three-way ANOVA. However, there was no difference between mtDNA and nDNA ($p = 0.839$). * denotes significant difference ($p < 0.05$) according to Fisher's LSD. n = 5 per treatment. Error bars indicate standard error of means.

Table 1

Primers used for *Fundulus heteroclitus* LA-QPCR assay.

Target	Primer sequences
Large nuclear target 11459 bp	F: 5'- CAGCCGCCCGCAAATTCTCA -3' R: 5'- CAGAATGCGGGCCTTGCTGA -3'
Small nuclear target 234 bp	F: 5'- GCCGCTGCCTTCATTGCTGT -3' R: 5'- ATGAGCTGGGTGTGCGCTGA -3'
Long mitochondrial target [a] 9416 bp	F: 5'- TTGCACCAAGAGTTTTTGGTTCCTAAGACC -3' R: 5'- GATGTTGGATCAGGACATCCCAATGGTGCA -3'
Small mitochondrial target 264 bp	F: 5'- ATCTGCATGGCCAACGCCTA -3' R: 5'- GGCGGTGCCAGTTTCCTTTT –3'

[a] Adapted from (Kim et al., 2004)