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Genetic ecotoxicology IV: survival and DNA strand breakage is dependent on genotype in radionuclide-exposed mosquitofish

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Abstract

Western mosquitofish (Gambusia affinis) were caged in situ in a radioactively-contaminated pond in order to determine if survival and amount of DNA strand breakage were dependent on genotype. Genotypes of fish were determined using the randomly amplified polymorphic (RAPD) technique, and DNA strand breakage was determined using agarose gel electrophoresis. This study is a continuation of research undertaken at the Oak Ridge National Laboratory, which examined the effects of radionuclide contamination on the population genetic structure of mosquitofish. The previous research found 17 RAPD markers that were present at a higher frequency in contaminated than in reference populations (‘contaminant-indicative bands’), and fish from contaminated sites which possessed these markers had higher fecundity and fewer strand breaks than fish which did not. One of the contaminated populations (Pond 3513) was colonized from one of the reference populations (Crystal Springs) in 1977. In the present study, fish were obtained from Crystal Springs and an additional reference site, and caged in Pond 3513. The percent survival and amount of DNA strand breakage were then determined for fish with and without the contaminant-indicative markers. When Crystal Springs fish were caged in Pond 3513, it was found that the genotypic distribution of the survivors was more similar to the native Pond 3513 population than to the Crystal Springs population. Furthermore, for nine of the contaminant-indicative markers, the percent survival was greater for fish which possessed these markers than for fish which did not. For five of these markers, fish which possessed them had higher DNA integrity (fewer strand breaks) than fish which did not. These data indicate that probability of survival and degree of DNA strand breakage in radionuclide-exposed mosquitofish are dependent on RAPD genotype, and are consistent with the hypothesis that the contaminant-indicative RAPD bands are markers of loci which impart a selective advantage in radionuclide-contaminated environments. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Gambusia; Population genetics; Radiation; Survival; DNA damage

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1. Introduction

The effect of environmental contamination on population genetic structure is a well documented phenomenon (for example, see Mulvey and Diamond (1991) and Guttman (1994) for reviews). Pollution exposure can alter survival and/or reproductive success of individuals in affected populations, leading to perturbation of population genetic structure by two possible mechanisms. First, contamination may lead to reductions in population size, with a resultant decrease in genetic diversity (Anderson et al., 1994; Bickham and Smolen, 1994; Guttman, 1994). Secondly, contaminant-mediated natural selection (hereafter referred to as ‘contaminant selection’) and subsequent adaptation to toxicants may lead to shifts in genotype frequencies. Effects of contamination on population genetic structure most probably develop by a combination of these two mechanisms.

One method of documenting the occurrence of contaminant selection and adaptation is by comparing genotype or allele frequencies between contaminated and reference populations. An elevated frequency of a particular genotype or allele in polluted sites may indicate that these genotypes are at a selective advantage in contaminated environments. However, there are other environmental variables besides contamination, and other genetic processes besides natural selection (e.g. genetic drift, gene flow) which could affect genotype frequencies. Consequently, it is necessary to demonstrate differential fitness between genotypes in order to verify that differences in population genetic composition are affected by contaminant exposure. In addition, it is often possible to identify a biochemical response that is indicative of the mechanism of toxicity for the contaminant in question (for example, see McCarthy and Shugart, 1990). If the magnitude of this response is dependent on genotype, this would further implicate contaminant exposure as a selective pressure. Hence, in order to provide conclusive evidence of contaminant-mediated natural selection, gene frequency differences between populations need to be supplemented with data showing direct evidence for differential fitness and, perhaps, biochemical effects that are indicative of the mode of action for the contaminant in question.

In a recent study (Theodorakis and Shugart, 1997), radionuclide-contaminated and reference populations of western mosquitofish (Gambusia affinis) at the Oak Ridge National Laboratory (ORNL) were examined to determine if exposure to genotoxicants (in this case, radionuclides) could alter population genetic structure. This endeavor was pursued in an attempt to determine the utility of population genetics in biomonitoring, and to examine the molecular basis of genetic adaptation to genotoxicants. This research used the randomly amplified polymorphic DNA (RAPD) assay, a polymerase chain reaction (PCR)-based DNA fingerprinting technique that uses 10 base-pair oligonucleotide primers to amplify short fragments from genomic DNA. When these fragments are analyzed via agarose gel electrophoresis, they are visualized as bands similar to other types of DNA fingerprints, with polymorphisms being identified on the basis of band presence or absence. Two of the ORNL mosquitofish populations were contaminated with radionuclides (Pond 3513 and White Oak Lake) and two were reference sites (Crystal Springs and Wolf Creek). Pond 3513 is contaminated with radionuclides such as \(^{137}\)Cs, \(^{90}\)Sr and various other nuclear fission products (Tamura et al., 1977); White Oak Lake is contaminated with radionuclides as well as polycyclic aromatic hydrocarbons (PCBs), heavy metals, aromatic hydrocarbons, and numerous other organic and inorganic contaminants (Blaylock et al., 1991). In 1977, Pond 3513 was colonized with individuals from Crystal Springs. Genetic distances were calculated using the frequencies of these bands in each population, and revealed that the Pond 3513 population was most genetically similar to White Oak Lake, instead of Crystal Springs as expected. This implied that the population genetic composition of Pond 3513 may have been affected by contaminant selection. A total of 142 RAPD bands were amplified using 15 different primers, and 17 of these bands were present at a higher frequency in the contaminated sites than in the reference sites. For the sake of discussion, these bands will hereafter be referred to as ‘contaminant-indicative bands’ (CIBs), not...
to suggest that they are indicative of any toxic mode of action, or that they are specific to any one contaminant, or that they are indicative of all contaminants in general, but merely as a matter of convenience. These particular bands are listed in Table 1 (nomenclature of RAPD primers and bands explained therein). In many cases, the fecundity of fish from contaminated populations was greater for fish with than without the CIBs. Such patterns were not seen in reference populations. These data imply that, in these radionuclide-contaminated environments, fish that possess these markers have greater fitness over those that do not.

A follow-up investigation (Theodorakis and Shugart, 1998) set out to determine if the magnitude of DNA damage (in this case, strand breakage) was dependent on RAPD genotype. DNA strand breakage was examined because it is indicative of a mode of action for radiation toxicity (Roots and Okada, 1975; Kampf and Eichhorn, 1983; Kraft et al., 1989), and because it has been shown previously that mosquitofish living in the contaminated sites at ORNL exhibited more strand breaks than fish from the reference sites (Theodorakis et al., 1997). The subjects of this study included fish that were native to the contaminated ponds or were collected from reference ponds and exposed to X-rays in the laboratory (Theodorakis and Shugart, 1998). For many of the CIBs, fish that displayed the band had fewer strand breaks than fish that did not. Because the relative amount of DNA strand breakage in radiation-exposed organisms has been correlated with relative radioresistance (Radford, 1985), this suggests that fish with the CIBs are more radioresistant than fish without the bands.

However, these hypotheses are still tentative, and more evidence is needed to further substantiate these findings. For example, a higher frequency of CIBs in the contaminated populations (Theodorakis and Shugart, 1997) could be due to higher survival of fish with than without the CIBs. An alternative scenario would be that exposure to radiation causes appearance of these bands in fish which previously did not display them, although, for reasons to be expounded (see Section 4), this is a less likely scenario. Caging fish from non-contaminated ponds in Pond 3513 and examining CIB frequencies before and after caging and survival of CIB genotypes would be a test of the hypothesis that the differences between contaminated and reference populations seen in Theodorakis and Shugart (1997) are due to chronic contaminant exposure.

Additionally, the level of contamination in Pond 3513 sediments is spatially heterogeneous (Tamura et al., 1977), which could lead to differential dose rates and, ultimately, to variation in DNA strand breakage, independent of genotype. This could have affected the strand breakage/genotype relationships discussed. Although these effects were controlled by exposing the fish in the laboratory to X-rays (Theodorakis and Shugart, 1998), this scenario is not completely analogous to

<table>
<thead>
<tr>
<th>Operon Technologies primers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>University of British Columbia primers&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>OPD&lt;sub&gt;2,590&lt;/sub&gt;</td>
<td>UBC&lt;sub&gt;2,110&lt;/sub&gt;</td>
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<td>UBC&lt;sub&gt;4,2060&lt;/sub&gt;</td>
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<td>UBC&lt;sub&gt;4,1810&lt;/sub&gt;</td>
</tr>
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<td>UBC&lt;sub&gt;4,1810&lt;/sub&gt;</td>
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<tr>
<td>OPD&lt;sub&gt;13,430&lt;/sub&gt;</td>
<td>UBC&lt;sub&gt;6,880&lt;/sub&gt;</td>
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<td>UBC&lt;sub&gt;16,120&lt;/sub&gt;</td>
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<td>UBC&lt;sub&gt;16,1020&lt;/sub&gt;</td>
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<sup>a</sup> These markers were present at a higher frequency in two radioactively-contaminated populations than in two reference populations.

<sup>b</sup> The RAPD assay uses the polymerase chain reaction to amplify DNA fragments of various sizes. The name of each RAPD marker (DNA fragment) is derived from the name of the primer used to amplify it (designated by the manufacturer, e.g. OPD2) followed by the molecular length of the fragment (number of nucleotides) as a subscript.

<sup>c</sup> Markers amplified using primers manufactured by Operon Technologies (Alameda, CA).

<sup>d</sup> Markers amplified using primers manufactured by the Nucleic Acid Research Unit, University of British Columbia (Vancouver, BC).
Pond 3513. First, laboratory exposures cannot simulate the numerous environmental variables which could modulate genotoxic response. Second, in Pond 3513, fish are exposed to $\alpha$, $\beta$, and $\gamma$-rays, all of which have different physical characteristics to X-rays. Hence, further experiments are needed which expose fish to Pond 3513 sediments in situ, but within a small confined area, where effects of spatial variability of contamination are minimized.

Therefore, the objective of this study is to further substantiate the findings of Theodorakis and Shugart (1997, 1998) by caging fish in Pond 3513 and examining changes in frequency of CIBs and the relationship between RAPD genotype and strand breakage. It is predicted that the frequency of the CIBs will be higher in the survivors of such exposure than in the original sample of fish, and that fish with the CIBs will have fewer strand breaks than fish without such bands.

2. Methods and materials

2.1. Caging design

Adult female western mosquitofish (*Gambusia affinis*; 30–35 mm, standard length) were collected by seine or dip-net from Crystal Springs or an unnamed, non-contaminated pond located on the Oak Ridge National Laboratory grounds (hereafter referred to as Pond X). Cages were constructed of commercially available hardware cloth (1.27-cm mesh) and fashioned into a cylinder 1-m high \times 1-m diameter. The inside of the cages were lined, and the top and bottom were enclosed, with black polyethylene plastic netting (0.32-cm mesh) and fastened together with hot glue. These experiments were carried out during the 1995 and 1996 field seasons. After exposure, fish were collected from the cages and placed on ice to slow the metabolism and inhibit DNA repair processes. Exposed and control fish were collected within 0.5 h of each other, and both groups were dissected concurrently.

2.1.1. 1995 experiment

Fish were collected from Crystal Springs and enclosed in cages located in either Pond 3513 or in Pond X for 6 weeks (30 fish per cage). After the exposure, the fish were removed from the cages, and liver tissues were collected from the survivors for DNA extraction (see later).

2.1.2. 1996 experiment

Based on the results of the 1995 experiment (see later), another experiment was conducted in 1996 to calculate the percent survival of each genotype. This was done by collecting caudal fin tissue before and after caging for RAPD genotype analysis. However, it was not possible to collect any fish from Crystal Springs during the 1996 field season. Therefore, fish were collected from Pond X and allowed to acclimate to laboratory conditions for at least 1 week. They were then anesthetized with tricane methanosulfonate (MS222; 0.5 g l$^{-1}$), and a small piece of caudal fin was excised and frozen in liquid nitrogen. The fish were then allowed to recover for 1 week before transfer into a cage. Forty fish were placed in a cage, either in Pond 3513 or in Pond X. After 2 weeks, survivors were recovered from the cages, sacrificed by overdose of anesthetic and cervical scission, and the liver and caudal fin tissues were collected and frozen in liquid nitrogen.

2.2. DNA analysis

2.2.1. DNA extraction

Because DNA repair can be initiated relatively rapidly after radiation exposure, the liver was chosen for DNA strand break analysis because it can be quickly located and removed from the fish. Also, it is the largest organ in these fish, and so provides a large amount of DNA (a factor which is important in small fish such as *Gambusia*). Finally, it is a very soft organ, so it can be disrupted with a minimal amount of homogenization (a process which can lead to shearing of the DNA). All fish were kept on ice prior to dissection, and the livers were immediately frozen in liquid nitrogen upon removal.

DNA was extracted from liver by homogenizing the tissue (approximately 20–50 mg) in 500 μl
TEN (50 mM Tris, 25 mM EDTA, 100 mM NaCl, pH 8.0) in polypropylene microcentrifuge tubes with a Teflon pestle. Care was taken to insure that each sample was homogenized with the same number of strokes of the pestle (five), in order to minimize any variation in DNA integrity which might be introduced by homogenization. Fifty microliters of 10% sarcosyl (sodium lauryl sarcosine) was added and the samples were allowed to incubate on ice for 10 min. The homogenates were then extracted twice with 0.5 ml buffered phenol/chloroform/isoamyl alcohol (PCI) (25:24:1, v:v:v, pH 8.0) and once with 0.5 ml chloroform. The DNA was precipitated with two volumes (1 ml) ice-cold ethanol, redissolved in 500 µl TEN, and digested at 55°C for 2 h with RNase A and proteinase K (final concentration, 500 µg ml⁻¹ for each). The digestate was extracted once with PCI and once with chloroform (as described). The DNA was then precipitated with ethanol (as described), and the DNA was redissolved in 10 mM Tris, 0.1 mM EDTA, pH 8.0 (TE).

DNA was extracted from fin tissue by digesting the tissue (approximately 5–10 mg) in 500 µl TEN plus 50 µl 10% sarcosyl at 55°C for 2 h with RNase A and proteinase K (final concentration, 500 µg ml⁻¹ for each). The digestate was extracted once with PCI and once with chloroform (as described). The DNA was then precipitated with ethanol (as described), and the DNA was redissolved in 10 mM Tris, 0.1 mM EDTA, pH 8.0 (TE).

Because manipulations during extraction and processing of the DNA may cause shearing, care was taken to minimize and control for this source of variation. By delaying the addition of detergent (sarcosyl) until after homogenization, nuclei were kept intact during the homogenization process (verified by fluorescence microscopy). Secondly, all mixing of the samples (e.g. after addition of the sarcosyl, after addition of PCI, etc.) were done on a rotary mixer to minimize variation in DNA shearing which may have been introduced if the samples were mixed by hand. Samples were mixed at a rate of 1 rpm for 10 s (when mixing with sarcosyl) or 10 min (when mixing with PCI). Finally, after the samples were mixed with PCI or chloroform, they were centrifuged in microcentrifuge tubes containing a gel which forms a barrier between the aqueous (DNA) and organic layers (5 Prime to 3 Prime Corporation, Boulder, CO). This allows the aqueous layer to be gently poured off the organic layer after centrifugation, eliminated the need for pipetting the DNA (another potential source of shearing).

2.2.2. RAPD analysis

DNA was used for RAPD analysis as described previously (Theodorakis and Shugart, 1997). Reactions were run on an Idaho Technology Air Thermo Cycler (Idaho Falls, ID), with 20 ng of DNA per reaction. Primers were obtained from Operon Technologies (Alameda, CA) or University of British Columbia (Vancouver, CA). Amplification products were separated via agarose gel electrophoresis (2% agarose) in 90 mM Tris, 90 mM Na borate, 2 mM EDTA, pH 8.0 (TBE), stained with ethidium bromide, and photographed under ultraviolet (UV) light. Band presence or absence was determined visually. Band frequency was calculated as (number of fish with the band)/(total number of fish). Because the RAPD technique can amplify non-reproducible bands, only bands which were reproducible were used in this study. Reproducibility was determined by comparing banding patterns from two or more duplicate PCR runs. The RAPD technique is sensitive to changes in reaction conditions (e.g. Mg, primer, and salt concentrations), so the exact same reaction conditions were used for all samples.

Using data from the 1995 field season, Roger’s genetic distance was calculated between the caged survivors and the populations of Pond 3513 or Crystal Springs. The distances were calculated as described in Theodorakis and Shugart (1997) using band frequencies. Basically, this metric measures similarity in the distribution of band frequencies between two samples according to the equation (Nei, 1987):

\[ D_{ij} = \frac{\sum_{x=1}^{n} (x_i - x_j)^2}{k} \]

where \( x_i \) and \( x_j \) are the frequencies of band \( x \) in populations \( i \) and \( j \), respectively, and \( k \) is the total number of bands examined. It was not possible to...
calculate the percent survival of each genotype because tissues were not collected before and after caging, as was done in the subsequent field season.

Data from the 1996 field season were used to calculate the percent survival for fish with or without each band as \[ \frac{\text{number of surviving fish with (or without) band}}{\text{initial number of fish with (or without) band}} \]. The percent survival of fish with versus without bands was tested with the \( \chi^2 \) test.

### 2.2.3. Strand break analysis

DNA from liver tissues was used for determination of DNA strand breakage. Only tissues from the 1996 experiment were used for this assay. Strand breakage was analyzed via agarose gel electrophoresis under alkaline or neutral conditions, as described previously (Theodorakis et al., 1997). Electrophoresis performed under alkaline (pH 12.0) conditions separates the DNA into single strands. If electrophoresis was performed under neutral conditions (pH 8.0), the DNA remained in its double-stranded state. Briefly, 0.5 µg DNA was subjected to electrophoresis in alkaline solution (30 mM NaOH, 2 mM EDTA, pH 12.0) or TBE (pH 8.0), stained with ethidium bromide, and photographed under UV light. In order to minimize the effect of ohmic heating, and to ensure a homogeneous pH throughout the buffer tanks, the buffer was continuously recirculated (approximately 100 ml min\(^{-1}\)) through coiled Tygon\textsuperscript{	extregistered} tubing immersed in an ice-water bath throughout the electrophoresis. The average molecular length \( (L_n) \) of each DNA sample was determined from the photographs (Freeman and Thompson, 1990) with a scanning laser densitometer (BioRad Corp., Richmond, VA). Coliphage T4 DNA, and \( \lambda \) Hind III and X173 Hae III digestes were used as molecular length standards. The number of DNA strand breaks is inversely proportional to the \( L_n \) (i.e. a lower \( L_n \) value reflects more strand breakage). The \( L_n \) determined from alkaline electrophoresis is affected by both single- and double-strand breaks, while the \( L_n \) determined with neutral electrophoresis is affected by double-strand breaks only. The difference in \( L_n \) between fish with and without each band was tested with the Wilcoxon Rank sum test. This was done for the 1996 study only.

### 3. Results

#### 3.1. Survival

Upon dissection, it was observed that the stomach and intestines of the fish were filled with organic matter. Also, none of the fish appeared to be emaciated. Therefore, it was assumed that the fish had an adequate food supply.

Photographic examples of all bands discussed are shown in Fig. 1.

##### 3.1.1. 1995 Experiment

After a 6-week exposure, nine of the original 30 fish survived in the Pond 3513 cage, and 27 survived in the Pond X cage. The distribution of band frequencies of the survivors caged in Pond 3513 were most similar (smallest genetic distance) to the feral Pond 3513 population. The distribution of band frequencies for survivors caged in Pond X was most similar to the Crystal Springs population (Table 2).

##### 3.1.2. 1996 Experiment

After a 2-week exposure, 24 of the fish in the Pond 3513 cage survived, while all of the fish caged in the Pond X cage survived. For nine of the CIBs listed in Table 1, the survival of the fish with the bands was higher than for fish without the bands (Fig. 1; \( P < 0.05 \), \( \chi^2 \) test). For bands UBC2810 and UBC121270, none of the fish without the band survived, and for band UBC121270, all of the fish with the band survived (Fig. 2).

#### 3.2. DNA integrity

The DNA integrity of fish caged in Pond 3513 was lower (i.e. more strand breaks) than for those fish caged in Pond X. This was true for both single- and double-strand breaks (\( P < 0.05 \), Wilcoxon Rank sum test; Fig. 3).

For five of the CIBs, the DNA integrity was higher for fish with the bands than without (higher \( L_n \)). This was true for both single- and
double-strand $L_n$ (Fig. 4; $P < 0.05$, Wilcoxon Rank sum test). This pattern was found only for the fish caged in Pond 3513; there were no significant differences between fish with and without the CIBs for the fish caged in the reference pond (Pond X).

4. Discussion

The results presented here are consistent with the hypothesis that these bands are markers of loci which impart a selective advantage over others in these radionuclide-contaminated habitats.
Previous research (Theodorakis and Shugart, 1997) has shown that these RAPD bands were present at a higher frequency in radionuclide-contaminated ponds than in reference ponds. In the present study, when fish from Crystal Springs were caged in Pond 3513, many of them died, and the distribution of band frequencies in the survivors was more similar to the Pond 3513 than to the Crystal springs population. The results from this experiment indicated that, for many of the contaminant-indicative bands, the survival of fish with these bands was higher than fish without these bands. When the founding population was introduced into Pond 3513 from Crystal Springs in 1977, the fish exhibited signs of stress and mortality was high (B.G. Blaylock, Oak Ridge National Laboratory, personal communication). These past observations, coupled with the current findings, suggest that differences between the present-day Pond 3513 and Crystal Springs populations may have been due, at least in part, to differential survival between genotypes after introduction into Pond 3513.

The fact that mortality was high in the Pond 3513 cages, and was low or non-existent in the reference cages, suggests that differential mortality can be attributed to toxic affects in Pond 3513. Because Pond 3513 is heavily contaminated with radionuclides, and other types of toxic contaminants are lacking (Tamura et al., 1977), the mortality of the fish caged in Pond 3513 was most probably due to radionuclide exposure.

These findings of genotype-dependent survival in the present study are consistent with previous studies that have found similar results when *Gam-

Table 2
Roger’s genetic distance\(^a\) between fish caged in a reference pond (Pond X) or a radionuclide-contaminated pond (Pond 3513) and feral populations living in Crystal Springs\(^b\) or Pond 3513

<table>
<thead>
<tr>
<th>Caged fish</th>
<th>Feral population</th>
<th>Pond 3513</th>
<th>Crystal Springs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caged in Pond X</td>
<td>0.392</td>
<td>0.281</td>
<td></td>
</tr>
<tr>
<td>Caged in Pond 3513</td>
<td>0.263</td>
<td>0.467</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Roger’s genetic distance was calculated using RAPD band frequencies.
\(^b\)Crystal Springs was the source of the caged fish as well as the feral Pond 3513 population.
The results presented in this study also suggest that the amount of strand breakage is dependent on genotype. These results are consistent with previous findings which showed that Pond 3513 and White Oak Lake Gambusia which possessed many of the contaminant-indicative bands had higher DNA integrity (fewer strand breaks) than fish which did not (Theodorakis and Shugart, 1998). It was mentioned earlier that the spatial heterogeneity in amount of sediment contamination may have affected the genotype/strand break correlation. However, in the present study, the fish were confined in a small area (1-m diameter), so the effects of spatial heterogeneity in sediment contamination (and presumably, radiation dose) were minimized. This further supports the hypothesis that fish with the contaminant-indicative bands are more radioresistant than fish without these bands.
It is also possible that the relationship between strand breakage and genotype, as well as the differences in band pattern frequencies between fish before and after exposure, could be due to appearance or disappearance of RAPD bands as a result of induced mutations, DNA damage, or other DNA alterations as a result of radiation exposure. However, this is highly unlikely for a number of reasons. First, mutations are relatively rare events, so it is unlikely that they would be detectable in the small number of fish used in this study. Second, the RAPD amplification sites represent only a very small percentage of the entire genome, so the probability that they would be affected by multiple rare events, such as mutations, would be very low. Furthermore, because the RAPD technique relies on PCR amplification, a band could be amplified even if many of the amplification sites are damaged. Thus, in order for DNA damage or mutations to affect the amplification of a RAPD band, a large proportion of the DNA molecules in a tissue sample would have to be damaged in the same small region. In order for this to affect band frequencies, this would have to occur in multiple individuals. Given the stochastic nature of radiation-induced DNA damage, this is not very probable.

Nonetheless, the main objectives of the present study are to further substantiate the findings of Theodorakis and Shugart (1997, 1998). Whether these patterns stem from genotype-dependent survival and relative radioresistance, or from radiation-induced changes in RAPD banding patterns, the most important conclusion is that results from field-collected fish parallel those from caged fish. This is consistent with the hypothesis that the

![Fig. 3. DNA integrity (average molecular length, $L_n$, in kilobases) of mosquitofish caged in a radionuclide-contaminated pond (Pond 3513) or a reference pond (Pond X). Bars represent medians, error bars represent first and third quartiles. Data are presented for both single-strand (A) and double-strand $L_n$ (B).](image-url)
The authors acknowledge the fact that this experiment was only performed with one cage per pond, so that the results may be viewed as pseudoreplication, rather than true replication. However, because of logistical constraints on gaining access to the highly contaminated Pond 3513 and on the amount of contaminated waste (i.e. used cages) generated, this could not be avoided. Also, one of the objectives of the experimental design was to confine the fish in a small area so as to minimize the effects of environmental heterogeneity. This would have been more difficult to accomplish if multiple cages had been used.

Finally, it should be noted that single-strand breaks, measured under alkaline conditions, could be produced in vivo or in vitro. In vivo strand breaks are produced when DNA repair enzymes remove sections for DNA containing base modifications (e.g. 8-hydroxy guanosine; Sancar and Sancar, 1988). In vitro strand breakage occurs when DNA containing certain base modifications, such as 8-OH guanine and abasic sites, is exposed to alkaline conditions (Schneider et al., 1990). Such modifications are termed ‘alkaline labile sites’, because DNA single-strand breaks can form at these sites at alkaline pH. However, like in vivo single-strand breaks, the 8-hydroxy guanosine and abasic sites are induced as a result of oxidative stress (reviewed in Drouin et al., 1996), and oxidative stress is one effect of radiation exposure (Shulte-Frohlinde and Von Sonntag, 1985). Consequently, an increased incidence of alkaline labile sites could also signify greater radiosensitivity. Thus, any reference to ‘single-strand breaks’ in the preceding discussion refers to those produced both in vivo and in vitro.

In conclusion, the loci from which these bands are amplified could be useful as genetic markers for the study of contamination-mediated natural selection and environmental monitoring of genotoxic contaminants. It is possible that these RAPD markers are amplified from, or closely linked to, loci which are involved in mitigation or repair of cellular or molecular lesions induced by radiation exposure. However, the molecular characteristics of these markers (e.g. sequence, copy number) are, as yet, unknown, so more research is needed toward this end. Current efforts are under
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