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Comparison of DNA Damage in the Early Life Stages of Cod, *Gadus morhua*, Originating From the Barents Sea and Baltic Sea

Gunilla Ericson, Gun Åkerman, Birgitta Liewenborg & Lennart Balk

Institute of Applied Environmental Research, Laboratory for Aquatic Ecotoxicology, University of Stockholm, Studsvik, S-611 82, Nyköping, Sweden

ABSTRACT

*DNA adducts in cod embryos and larvae were analysed by ^{32}P-postlabeling to test the hypothesis that anthropogenic substances, which could form reactive intermediates, are involved in the reproductive failure of cod (*Gadus morhua*) from the Baltic Sea. A comparison with cod from the Barents Sea was performed. The mean value of DNA adducts in cod embryos/larvae from the Baltic Sea was 2.3 nmol of adducts/mol nucleotides, compared to 0.12 nmol of adducts/mol nucleotides in the embryos/larvae from the Barents Sea. Copyright © 1996 Elsevier Science Ltd*

Several teleost fish species from the Baltic Sea reproduce at a level which can be assumed to be far below their normal rate. The most well-known reproductive problem is the so-called M-74 syndrome in Baltic salmon, (*Salmo salar*; Bengtsson *et al.*, 1994), but other species also seem to be affected. For instance, in burbot (*Lota lota*) from the inner part of The Gulf of Bothnia, a high frequency of sterile individuals (with retarded gonad development) has been reported (Pulliainen *et al.*, 1992). In the western Baltic, high rates of malformed embryos have been registered among pelagic fish eggs (Westernhagen *et al.*, 1988). Reared cod embryos/larvae from the Baltic Sea show a high frequency of abnormalities such as precipitates in the yolk-sac, slight bending of the vertebrae, heavy vertebral deformation in combination with precipitated material and malformed embryos and larvae (Åkerman *et al.*, 1994). Recently, high mortality has been reported in reared eggs and embryos of Baltic cod (*Gadus morhua*), when compared with cod from Lofoten, an island area located close to the main land in the northern part of Norway (Åkerman *et al.*, 1995). The underlying reason/s for this is at present unknown, but an environmental pollution factor can not be excluded.

To test the hypothesis that anthropogenic substances which can form reactive intermediates, are involved in the reproductive failure of cod, a comparison with NE Arctic Cod was performed. This population has its feeding area in a relatively unpolluted part of the Barents Sea, and migrate to the Lofoten area in the northern part of Norway to

spawn. The nuclease P1 version of the ^{32}P-postlabeling assay for analysis of DNA adducts (Reddy & Randerath, 1986) was used as a sensitive method to detect exposure of the embryos/larvae to DNA damaging substances and as an instrument to detect reactive intermediates in general.

Cod were collected during the spawning season by trawling. Stripping and fertilization were performed directly on the boat deck. The fertilized egg batches were transported to the laboratory, where they were held under optimal conditions for cod embryo/larval development (Åkerman *et al.*, 1995), until they were sacrified for DNA purification and adduct analysis. Each egg batch contained eggs from one female, fertilized with sperm from one male. Embryos/larvae from seven individual females caught at Lofoten, and from eleven individual females caught close to the Bornholm Basin in the Baltic Sea were analysed. A sub-sample of 90 individuals was taken from several egg/larvae batches at three different stages of embryo/larval development, i.e. when the blastopores were closed and the embryos slightly pigmented, shortly after hatching and when the yolk was almost completely consumed. These samples were immediately frozen in liquid nitrogen and stored at −120°C until DNA purification could be carried out.

Samples were minced and incubated in 50 mM Tris-buffer pH 8.0 containing 100 mM NaCl, 20 mM EDTA, 0.5% sodium dodecyl sulphate and 1 mg/ml proteinase K for 3 h at 37°C. DNA was purified using sequential extractions with phenol, phenol/chloroform:isoamylalcohol and chloroform:isoamylalcohol, and precipitated with ethanol as described by Dunn *et al.* (1987). The dissolved DNA was incubated with RNase A and RNase T_1, to remove any co-precipitated RNA; α-amylase treatment was omitted. Purified DNA was redissolved in distilled water, and quantified spectrophotometrically using 1 $A_{260} = 50$ μg/ml.

An aliquot (12.5 μg) of isolated DNA was digested enzymatically to deoxynucleoside-3′-monophosphates by treatment with micrococcal nuclease (4.2 μg) and spleen phosphodiesterase (2.4 μg) for 4 h, in a total volume of 34 μl. This digest was then enriched for aromatic/hydrophobic adducts by selective dephosphorylation of normal nucleotides by the addition of 5 μg nuclease P1 and continued incubation for 30 min. The pH was adjusted to 7.6 by adding 0.5 M Tris-base. The mixture was then evaporated to dryness and thereafter reconstituted in 4 μl distilled water. Remaining nucleotides were labeled with ^{32}P, using 1.2 μM γ-^{32}P-ATP (5000 Ci/mmol) and 8 units of 3′-phosphatase free polynucleotidekinase in a total volume of 14 μl. Adducted nucleotides were then separated from the remaining normal nucleotides and excess ATP by selective chromatography on polyethyleneimin (PEI) cellulose thin layer plates, and resolved in two dimensions by further chromatography, as in the legend to Fig. 1. Adducts were located by screen enhanced autoradiography. The areas corresponding to ^{32}P-labeled adducts were excised from the TLC-plates, and the radioactivity was determined by liquid scintillation counting. Adduct levels were calculated using the net amount of radioactivity, the specific activity of the [γ-^{32}P]ATP used and the relationship of 1 μg DNA = 3240 pmol nucleotides.

In all the autoradiograms derived from the DNA of embryos/larvae caught in the Baltic Sea, several adduct spots were observed. The adduct pattern was relatively similar for different egg batches, but a variation in the intensity of individual spots was registered. In contrast, autoradiograms of DNA from the Lofoten embryos/larvae had very faint or undetectable adduct spots. Figure 1 shows representative autoradiograms derived from embryos/larvae from the Lofoten area and the Baltic Sea.

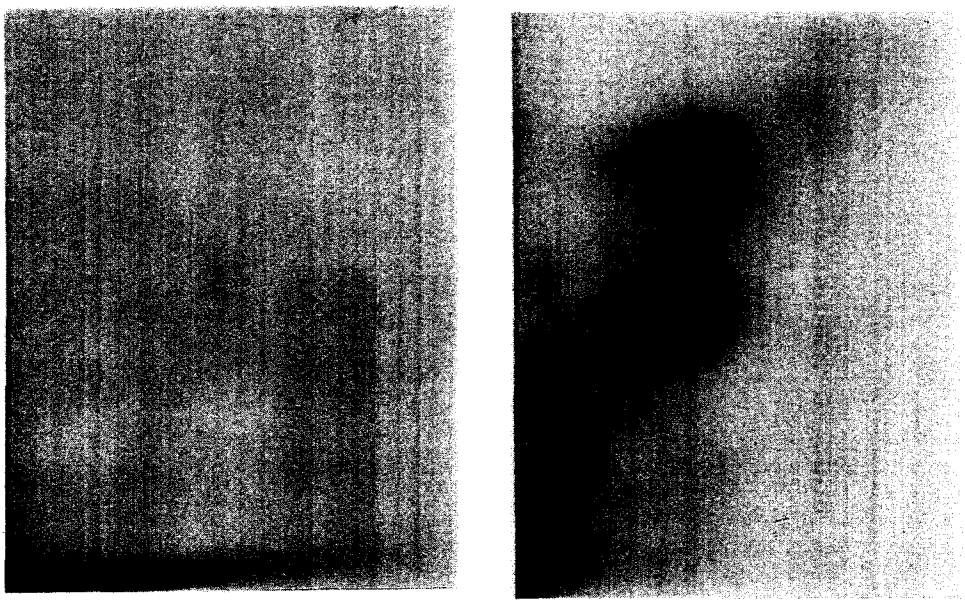

Fig. 1. Representative autoradiograms of digested, nuclease P1 treated and ^{32}P-labeled DNA from cod larvae derived from Lofoten and the Baltic Sea. Samples were spotted on polyethyleneimin TLC plates which were developed with 1 M sodium phosphate, pH = 6.8 (D1). The origins were transferred to fresh plates and further developed in two-dimensions with 4.5 M lithium formate, 9 M urea, pH 3.5 (D3) and 0.8 M lithium chloride, 0.5 M Tris-HCl, 9 M urea, pH = 8.0 (D4). A final development using 1.7 M sodium phosphate was undertaken in the same direction as D4 onto a stapled Whatman chromatography paper. Autoradiography was carried out at −120°C for 66 h.

Total DNA adducts measured in individual subsamples was between 0.7 and 7.2 nmol of adducts/mol of nucleotides for cod embryos/larvae from the Baltic Sea, and between $\leqslant$0.2 and 0.4 nmol of adducts/mol nucleotides in the embryos/larvae from the Lofoten area. Average adduct levels analysed are summarized in Fig. 2. No trend in adduct levels or adduct pattern was observed due to sampling at different times from the same egg batch during embryo/larval development, which indicates that DNA reactive substances are continuously produced throughout this period. DNA adduct levels would otherwise have been reduced with time, by dilution with unaffected DNA during growth of the larvae. Only floating eggs and swimming larvae were sampled for DNA adduct analysis, which means that embryos/larvae with the most severe developmental disorders were not selected. Furthermore, DNA was extracted from whole body tissue and if the main part of the adducts originates from DNA in specific target tissues or cells (e.g. liver and kidney), the level of adducts in these tissues was diluted by non adducted DNA. The whole body consists of only a small percent of target tissues, which means that adduct levels in, for instance the liver, may be 50–100 times higher than the ones observed in DNA from whole animals.

The Baltic Sea has a very slow turnover of the water mass, about 3–4 decades, due to the very narrow openings in the SW to the North Sea. It is surrounded by heavily industrialized nations, inhabitated by almost 80 million people, and many rivers originating in these areas have their outlets into the Baltic Sea. Anthropogenic substances of different origin such as DDT and different polychlorinated biphenyls are present in much higher levels in cod and

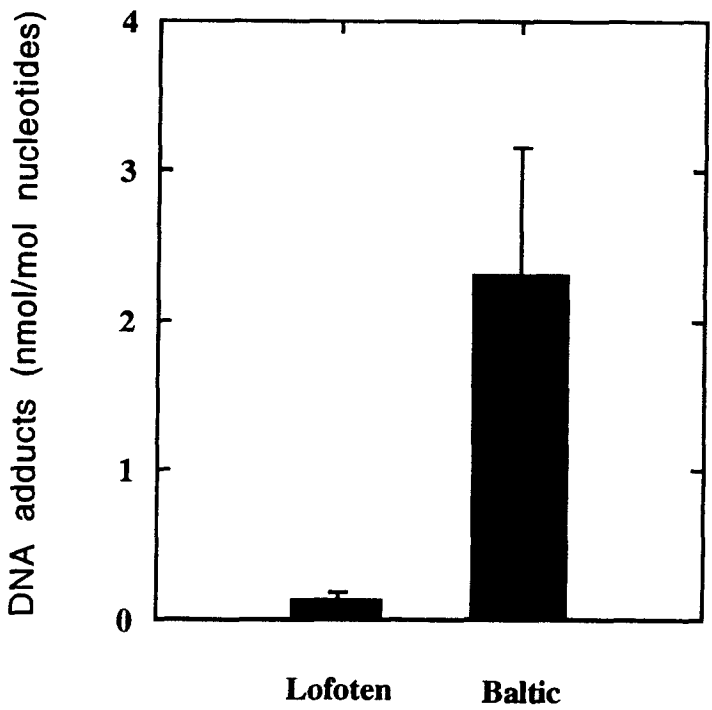

Fig. 2. DNA adduct levels (mean and 95% confidence intervals) in cod embryos/larvae subsampled from seven egg batches from Lofoten and 11 egg batches from the Baltic Sea.

other fish species from the Baltic Sea compared to the Swedish west coast and the northern North Sea (De Boer *et al.*, 1993; Falandysz *et al.*, 1992; Koistinen, 1990; Jensen *et al.*, 1972). Concentrations of chlorinated hydrocarbons in cod from the Baltic Sea are approximately 10 times higher than in cod from the Barents Sea (Savinova *et al.*, 1994/95).

Damage to the genetic material, in the form of DNA adducts, can in itself be a very significant observation with respect to the abnormalities described in Baltic cod embryos/larvae (Åkerman *et al.*, 1995). However, DNA adducts analyzed with the ^{32}P-postlabeling method, may also be used to detect reactive metabolites formed in the cells of the embryos/larvae that may exert toxicity through other mechanisms. Further studies are needed to elucidate the identity of the adduct spots and possibly correlate them to the abnormalities observed. The rather specific and distinct 'fingerprint' obtained on the chromatograms, may facilitate such studies.

This study is a part of a larger investigation aimed at elucidating the underlying mechanisms for cod reproduction failure in the Baltic Sea, and possibly correlating them to disturbances from anthropogenic substances. Measurements of DNA adducts might be a valuable tool in this work.

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