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EFFECTS OF ENVIRONMENTAL CONTAMINANTS ON THE MALE GAMETE OF AMERICAN PLAICE.

by

Daniel G. Cyr and James J. Nagler

Marine Environmental Sciences Division, Maurice Lamontagne Institute, Fisheries and Oceans of Canada, Mont-Joli, Québec G5H 3Z4, Canada.

ABSTRACT

There has been increasing concern over the effects of environmental contaminants on male vertebrate reproduction. The Baie des Anglais region on the St. Lawrence Estuary receives inputs from both industrial and municipal sources. Bottom sediments contain elevated levels of polycyclic aromatic hydrocarbons, polychlorinated biphenyls and dibenzofurans. The objective of this study was to determine the effects of long-term exposure of male American plaice (*Hippoglossides platessoides*) to bottom sediments collected at two sites inside the bay and from a third control site outside the bay. Chemical analyses of the sediments indicated that site 1 contained ten fold higher levels of organic contaminants than site 2 and 100-fold more than site 3 (control). Sexually maturing male plaice were exposed in the laboratory to the bottom sediments for 5 months prior to spawning. At the end of the exposure, hepatic cytochrome P 450 1A1 mRNA levels, used as a bioindicator of exposure to organic contaminants, were almost three fold higher in plaice exposed to site 1 sediments and two fold higher in plaice exposed to site 2 relative to plaice exposed to site 3 sediments. Males exposed to site 1 sediments had a significantly lower fertilization capacity (15%) than plaice exposed to either site 2 or site 3 sediments, despite there being no differences in sperm number. The total number of larvae hatched from eggs fertilized by males exposed to sediments from site 1 and 2 were 50 and 38% lower than the number of larvae obtained from males exposed to site 3 sediments. Together these results suggest that sediments contaminated by organic chemicals may alter sperm DNA and consequently affect the progeny of the male.

INTRODUCTION

Reproduction in fish is highly sensitive to chemical toxicants, and the environmental contamination of aquatic ecosystems has been linked to deleterious reproductive effects in fish [1,2]. Although early life history stages of fish can be very susceptible to contamination [3,4], exposure of the adults during the period of early gonadal development and gametogenesis is also important in terms of the quality and quantity of offspring produced [5]. The majority of studies reporting the effects of

contaminant exposure on gametogenesis involve the female [6-8]. The effects of contaminant exposure on males prior to spawning and the possible influence this could have on the quality of sperm and resulting larvae, exclusive of maternal influence, has received little attention.

Amongst fish species that inhabit contaminated marine environments, those living on or near the bottom are most at risk. This is due to the bulk of contaminants in marine habitats being associated with the bottom sediments. Flatfish are often studied because of their benthic lifestyle and economic importance in commercial fisheries. Some studies have documented biochemical and physiological alterations in flatfish living in contaminated areas [9-11], but it is difficult to interpret these changes in terms of eventual reproductive output. Reproduction is best assessed by the quantity and quality of offspring generated, since these features will have the greatest impact on future fish populations. Far fewer reports exist concerning the reproductive output of flatfish living in contaminated habitats [12,13].

The present study was undertaken to determine whether chronic exposure to environmentally contaminated sediments, during the period leading up to spawning, would impair the reproductive performance of male American plaice. This was assessed primarly by fertilization and hatching success of eggs, from a single non-exposed female, that were fertilized with sperm from males exposed to sediments containing different degrees of organic contamination. Sediments were obtained from three sites on a marine estuary (Baie des Anglais, Québec) that have been previously characterized in terms of their toxicity and contaminant levels [14]. The level of liver cytochrome P450 1A1 mRNA was used as a biomarker to indicate exposure to organic compounds [15].

MATERIALS AND METHODS

Animals

American plaice (*Hippoglossoides platessoides*) were captured by trawl on the St. Lawrence Estuary near Matane, Québec, approximately seven months before exposure. The fish were maintained in holding facilities as previously described [14]. Fish were fed chopped capelin twice weekly and every sixth feeding fed in-house prepared capelin-based food pellets supplemented with vitamins [16].

Experimental Protocol

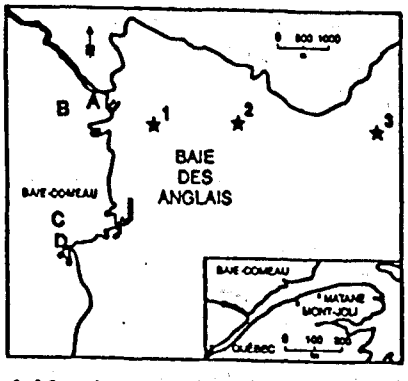

Fig. 1. Map showing locations (Sites 1,2 and 3) in and near Baie des Anglais, Québec, Canada, from which the sediments were collected. The letters denote the location of the grain storage facility (A), aluminum smelter (B), pulp and paper mill (C) and municipal sewage outfall (D).

Three sites (designated Sites 1, 2 and 3) in and near Baie des Anglais, Québec, previously characterized for geographical location, and sediment chemical concentrations and toxicity were used [14]. These sites represent a contamination gradient with Site 1 the most contaminated, Site 2 about 10-fold less so, and Site 3 100-fold less contaminated (i.e. control). The sediments from these sites contain, predominantly, organic compounds such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and

dibenzofurans (PCDFs). Metal levels are low and representative of background levels found throughout the St. Lawrence River and Estuary [14].

Mature male plaice were exposed for five months, prior to spawning, to sediments collected at the three sites in 0.9 x1.2 x 0.6 m tanks containing 15 cm of premixed sediments. At the end of the exposure period semen was collected from all fish with motile sperm and used to fertilize eggs (250) from a non-exposed female. Fertilized eggs were maintained until hatching. One week after spawning the anesthetized males were killed, weighed, and measured. Their livers were removed, frozen in liquid nitrogen, and stored at -80°C. The number of sperm per ml semen was determined by appropriately diluting the semen and counting the sperm with a hemocytometer. A subsample of semen was separated from the sperm by centrifugation, and the concentration of seminal Na^+ and K^+ determined by atomic absorption spectrophotometry using appropriate Na^+ and K^+ standards.

The number of fertilized eggs (FE) was determined, 24 hr after fertilization, by counting the number of floating and hydrated eggs in one jar from each male. Plaice eggs upon successful fertilization undergo dramatic hydration and are buoyant [17], allowing easy separation of fertilized and non-fertilized eggs. The total number of eggs (TE) was determined by summing the FE and the non-fertilized eggs, and percent fertilization calculated as (FE/TE)x100. The remaining two jars of fertilized eggs were maintained until the larvae hatched, between 16-18 days post-fertilization. Each day all dead eggs were removed from the jars and 75% of the seawater was replaced. The number of larvae that hatched each day were recorded and removed from the jar; a mean was calculated from the total number hatched from each of the two jars for each male. The total hatch (TH) was calculated as the mean number of larvae for all males within a given treatment.

Northern blot analysis was done on total cellular RNA was isolated from liver using a full length cDNA probe from European plaice (*Pleuronectes platessa*) cytochrome P450 1A1 (CYP 1A1) [18], which was generously provided by Dr. S.G. George (NERC, University of Stirling, Stirling, Scotland). Quantification of the intensity of the CYP1A1 mRNA signal from each sample was measured by densitometry and corrected for sample loading using the intensity of the 18S rRNA signal [14].

All data are presented as the mean ± the standard error of the mean. Percent fertilization values were arc sine transformed before statistical tests were applied. The data for each parameter studied were compared by ANOVA ($p<0.05$) to determine if significant differences existed. A multiple comparisons test (Tukey's Test) was used to identify significant differences ($p<0.05$) between treatment groups.

RESULTS AND DISCUSSION

The exposure of male plaice to bottom sediments contaminated with organic compounds, prior to the spawning season, and subsequently using their sperm to fertilize "clean" eggs has been shown to significantly reduce the number of larvae that hatch (Fig. 2). Most studies in the past, which have examined the effect of pollutant exposure on sexually developing fish, involve exposure of both sexes. This approach makes it difficult to ascribe any anomalies observed in the resulting progeny to one sex or the other. Generally, it has been presumed that the female is the most pollution sensitive of the two sexes, with respect to the probability of affecting the number or quality of the offspring

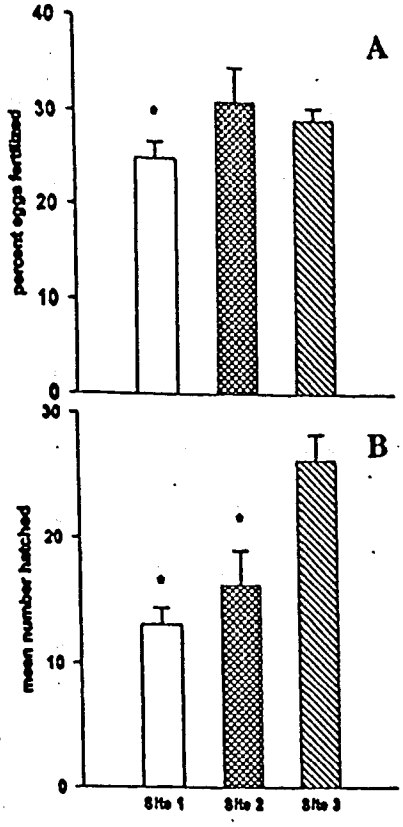

Fig. 2. A. Percent fertilization by sperm from male American plaice exposed for five months to contaminatedsediments from three different sites. B Mean number of larvae hatched from eggs fertilized by sperm from maleAmerican plaice exposed to contaminated sediments from three different sites. * = significantly different from Site 3 ($p<0.01$).

produced. There is good reason to accept this presumption and it derives from two features of oviparous fish reproductive physiology; 1) the much greater energetic investment required to produce eggs versus sperm, and 2) the opportunity for transfer of maternally bioaccumulated toxins to the yolk of developing oocytes and eventual eggs. A significant body of evidence exists to support the detrimental effects of exposure of female fish to toxic chemicals during oogenesis and the resulting impact on the offspring [20-23]. Previous studies in fish, in which the effect of parental exposure on early life history stages were dealt with in a way that separated effects arising from each sex, indicated that males exposed to copper [24] or mercury [25] had either no negative effect or significantly reduced hatch, respectively. This study shows, for the first time, that exposure of male fish to organic contaminants before spawning can specifically affect the number of offspring produced.

The significant reduction in the number of larvae hatched from eggs fertilized by males exposed to the most contaminated sediments indicates a problem with the quality of the sperm, since sperm numbers and other quantitative reproductive parameters measured were not significantly different between any treatment group (Table 1). The quality of sperm can be broadly assessed by the motility of the sperm, sperm-egg recognition, and successful transfer of the haploid complement of sperm DNA to the egg. All these features of sperm quality, if they are significantly impaired, are most easily detected by a reduction in the number of eggs fertilized. Fertilization success was marginally, but significantly, lower in fish exposed to the most contaminated sediments (i.e. Site 1), relative to the other treatment groups. This suggests that the process of fertilization could have contributed to the reduced hatch observed in eggs fertilized by males exposed to Site 1 sediments. However, the capacity for fertilization in Site 1 exposed males was reduced by about 14%, compared to that of the Site 3 exposed group (Fig. 2), while TH was reduced by 50% between these two treatments (Fig. 2). Therefore, the reduction in percent fertilization of Site 1 exposed males can not account for the ultimate reduction in TH observed in this treatment (Fig. 2). In addition, there were no differences in fertilization success between males exposed to Site 2 and Site 3 sediments (Fig. 2), yet TH was significantly reduced by 38% in the Site 2 exposed group, relative to the Site 3 exposed group. The significant reductions in TH which occurred in eggs fertilized by males exposed to contaminated sediments from Site 1 and Site 2 probably results from a problem after fertilization was assessed (i.e. early embryological development; >24 hours).

The mechanism by which contaminants in the sediments from Sites 1 and 2 alter the sperm and

Table 1. Reproductive parameters from male American plaice exposed for five months to contaminated sediments from three different sites.

Parameter	Site 1	2	3
Sperm number (x 10^9/ml)	4.06 +/- 2.3	4.42 +/- 1.7	3.71 +/- 2.4
Seminal plasma sodium (μg/ml)	3100 +/- 556	2856 +/- 526	4080 +/- 367
Seminal plasma potassium (μg/ml)	136 +/- 20	263 +/- 141	150 +/- 54
GSI	1.57 +/- 0.4	0.98 +/- 0.4	1.70 +/- 0.26
n	6	4	7

cause the resulting decrease in hatched larvae is unknown. While it is possible that contaminants adduct to proteins on the sperm and are transported into the egg, the most likely explanation is an alteration of the sperm DNA. Chemically induced DNA alterations in fish can take the form of adduction and secondary modifications, such as strand breaks, changes in minor nucleoside composition and unscheduled DNA synthesis [26]. A flatfish, English sole (*Parophrys vetulus*), exposed to contaminated sediments experienced measurable levels of liver DNA adduction with compounds such as PAHs [27]. Similarly, male English sole fed benzo(*a*)pyrene (BP) had BP metabolites adducted to testicular DNA [28]. The level of PAH combustion products found in sediments from Site 1 was 39.8 μg/g dry sediment [14], comparable to sediment PAH levels in studies which report English sole liver DNA-PAH adduction [27]. It is possible that sperm DNA could become adducted following exposure to these levels of sediment associated PAHs, similar to liver DNA. Sediments from Site 1 also contain high levels of PCBs and PCDFs. The effect of these compounds on DNA adduction or secondary modifications in fish is unknown. In mammals, several DNA alkylating chemicals such as cyclophosphamide, triethylenemelamine [29], methyl methanesulfonate [30], and acrylamide [31] have been shown to alter sperm DNA. Studies in which cyclophosphamide was administered to male rats caused subsequent post-implantation loss [32]. This may be the result of a decrease in spermatozoal decondensation which occurs following fertilization. Further studies will be needed in order to determine if a similar mechanism exists in plaice exposed to sediments containing organic contaminants.

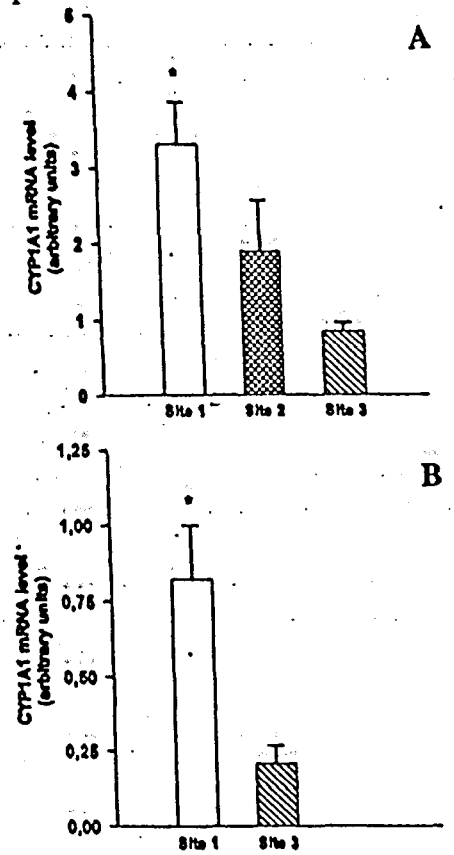

Fig. 3. A. Northern blot analysis of hepatic CYP1A1 mRNA levels in male American plaice exposed for five months to contaminated sediments from three different sites B. Northern blot analysis of hepatic CYP1A1 mRNA levels in juvenile American plaice exposed for two weeks to Site 1 and Site 3 sediments The amount of CYP1A1 mRNA is expressed as arbitrary units; * = significantly different from Site 3 ($p<0.05$).

Chronic exposure of male plaice to sediments contaminated with organic compounds resulted in elevated levels of hepatic CYP1A1 mRNA (Fig. 3). This is the conventional response of fish cytochrome P450 enzymes of the 1A1 subfamily to organics, which are

finding wide usage as sensitive biomarkers of exposure to PAHs, PCBs, and PCDDs [15,33-35]. Considering that the sediments from Sites 1 and 2 have been shown to contain higher concentrations of organic contaminants, relative to Site 3 [14], it is not surprising that hepatic CYP1A1 mRNA levels increased in plaice following exposure to these sediments. At the end of the exposure of mature male plaice, immature plaice were exposed to sediments for two weeks. Similarly, significantly elevated levels of hepatic CYP1A1 mRNA were observed in juvenile plaice exposed to sediments from Site 1 (Fig. 3), relative to Site 3.

In conclusion, the sperm quality of male American plaice exposed to sediments collected from an environmentally contaminated location is severely impaired, resulting in a 50% reduction in the number of larvae hatched, relative to a control group. This is the first report that exposure of maturing male fish to organic contaminants, prior to spawning, can significantly affect the number of resulting offspring, exclusive of any female derived effects. The major effect of reduced sperm quality appears to be manifest during early embryological development (i.e. >24 hr), and may result from DNA alterations. The impairment in sperm quality documented is attributed to organic compounds (PAHs, PCBs, and PCDFs) which are known to be present at high levels in the sediments and bioavailable to the fish as indicated by elevated CYP1A1 mRNA levels. The treatment group with the highest level of CYP1A1 mRNA induction displayed the greatest decrease in the number of embryos which hatched. *This study was supported by the St. Lawerence Vision 2000 and Fisheries and Oceans Canada Toxic Chemicals Program.*

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