GREAT LAKES FISHERY COMMISSION

2004 Project Completion Report[1]

Studies on Sea Lamprey Reproduction: Fertility Assessment in Males and Females

by:

Dr. Konrad Dabrowski[2], Andrzej Ciereszko[3], Kyeong-Jun Lee[4], Mary Ann G. Abiado[2], and Tobie Wolfe[2]

[2]School of Natural Resources
The Ohio State University
Columbus, Ohio 43210, U.S.A.

[3]Institute for Animal Reproduction and Food Research
Polish Academy of Sciences
Olsztyn 10-747, Poland

[4]Faculty of Applied Marine Science
Cheju National University
Jeju-Do, Jeju 690-756, Korea

May 2004

[1]Project completion reports of Commission-sponsored research are made available to the Commission's cooperators in the interest of rapid dissemination of information that may be useful in Great Lakes fishery management, research, or administration. The reader should be aware that project completion reports have not been through a peer review process and that sponsorship by the Commission does not necessarily imply that the findings or conclusions are endorsed by the Commission.

Final Report to the Great Lakes Fishery Commission

Project Title: **Studies on sea lamprey reproduction: fertility assessment in males and females**

Principal Investigator: Konrad Dabrowski

School of Natural Resources, The Ohio State University,

Columbus, Ohio 43210

Co-investigators: Andrzej Ciereszko

Institute of Animal Reproduction and Food Research

Polish Academy of Sciences, 10-747, Olsztyn, Poland

Tobie Wolfe, Mary Ann G. Abiado, Kyeong-Jun Lee*

School of Natural Resources, The Ohio State University,

2021 Coffey Road, Columbus, Ohio 43210, USA

(*Present address: Faculty of Applied Marine Science, Cheju National University, Jeju-Do, Jeju 690-756, Korea)

1. Introduction

With the opening of the St. Lawrence Seaway, the non-indigenous sea lamprey *Petromyzon marinus* gained access to the Great Lakes in 1932. The invasion of the sea lamprey in the Great Lakes had a devastating impact not only on lake trout, *Salvelinus namaycush*, but on other fish assemblages (Coble *et al*., 1990). Current attempts to control sea lamprey are mainly (1) treatment of streams with lampricides to kill larvae, (2) production and release of sterile males (sterilized with bisazir) to decrease spawning success, and (3) provision of barriers. Lampricide treatments frequently result in reduced growth and biomass of salmonids inhabiting small streams (DuBois and Blust, 1994). Assessment of sterilization success and efficacy of bisazir (especially in relation to state of sea lamprey spermiation/ovulation) may increase the likelihood of achieving the goals of the program. Unfortunately, short-term biological fertilization tests cannot be used for evaluation of bisazir effects because its mutagenic action is observed only during later stages of embryonic development (Ciereszko *et al*., 2000). Attractive alternatives to biological tests are assays directed to estimate the extent of damage to DNA. Due to the addition of females to the sterilization program, studies on bisazir effect on female reproduction are necessary. We developed procedures by which the success of fertilization at the 2-cell stage can be extrapolated to determine hatching percentages (Ciereszko *et al.*, 2000) and set criteria for evaluation of normal (control) and bisazir-treated sperm of sea lamprey (Ciereszko *et al.*, 2002). Based on these procedures, we attempted to evaluate the mutagenic actions of bisazir on sea lamprey erythrocytes as an index to its general physiological condition using single cell-electrophoresis (Comet assay). Additionally, to optimize the effectiveness of bisazir treatment in sea lamprey, in terms of timing and dosage, we will estimate the level of

bisazir in sea lamprey tissues right after injection, three to four days after treatment, and at the time of ovulation or spermiation.

2. Objectives

Year 1

1. To optimize comet and 8-hydroxy-deoxyguanosine assays
2. To validate bisazir assay in sea lamprey tissues using HPLC

Year 2

3. To evaluate the mutagenic action of bisazir *in vitro* and *in vivo* using comet assay of sea lamprey erythrocytes

4 To evaluate the effect of bisazir on sea lamprey spermatozoa and ova;

5. To determine bisazir concentration in blood, seminal plasma and ova of sea lamprey

Year 3

6. To evaluate the effectiveness of bisazir treatment in terms of timing and dosage.
7. To optimize of 8-hydroxy-deoxyguanosine assay of sea lamprey sperm and ova and measure its concentration in urine

3. Summary of Research Activities

3.1. Year 1

3.1.1. Source of experimental animals

Sea lampreys were obtained on four separate occasions from Hammond Bay Biological Station. Two air shipments of non treated (May 5) and bisazir treated lamprey (May 15), were obtained (males and females). Three additional shipments were executed by direct road travel from Hammond Bay to Columbus (June 12, July 4 and July 14, 2001). Fish were separated at the time of arrival by sex and into those injected with bisazir or controls. We attempted to accelerate maturation in the case of the first batch of lamprey males (n = 15) with two subsequent luteinizing hormone releasing hormone analogue (LHRHa) (Sigma Chem. Co.) injections. The effect was significant. On June 6, 5 of 6 males in the LHRHa injected group and 2 of 7 in the control group provided semen. However, mortality, particularly in the case of bisazir-injected males, prevented further comparison. Significant loss in body weight was noticed in lamprey males (Figure 1) that was related to unseasonably high water temperatures in our facility (18°C). Female lamprey were also experiencing significant loss of body weight during 4-5 weeks maintenance in our facility. Mortality was higher in bisazir injected than control fish, however, ovulation was uniformly distributed among year 2001 fish. We upgraded our facility following the 2001 season to maintain live lamprey. Present technical features include cooling and partial recirculation system for 6 400L volume tanks.

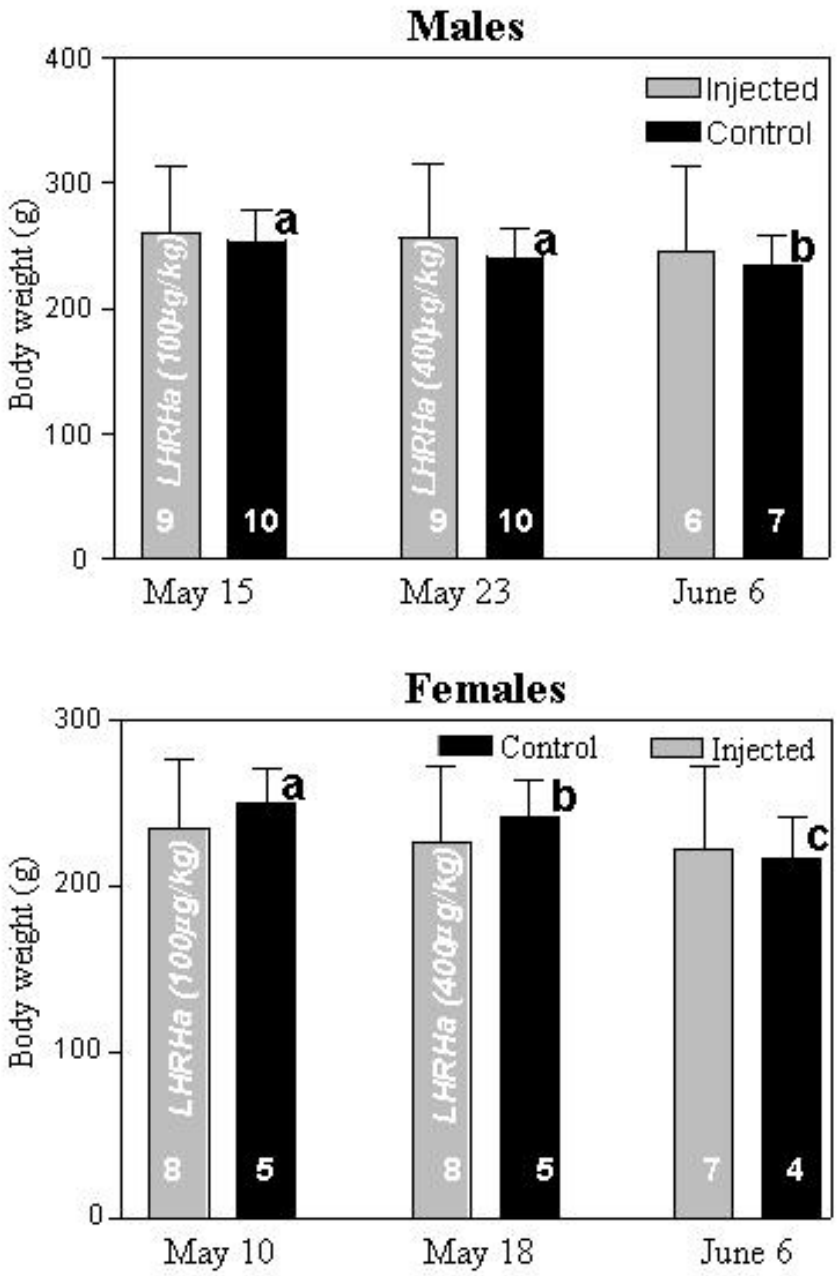

Figure 1. Change in body weight of sea lamprey males (from May 15 to June 6) and females (from May 10 to June 6). Bars having different letters are significantly different ($P<0.05$).

3.1.2. Objective 1. To optimize comet and 8-hydroxydeoxyguanosine assays

Experiment 1-1. Effect of H_2O_2 concentrations on DNA fragmentation of sea lamprey spermatozoa measured by neutral and alkali comet assay

Methods

We evaluated the effects of hydrogen peroxide (H_2O_2) concentrations on DNA fragmentation of sea lamprey spermatozoa measured by neutral and alkali comet assay. Hydrogen peroxide is an oxidizing substance causing fragmentation of DNA. For this reason it can be used as a positive control for Comet assay. Spermatozoa differ from somatic cells in having more compactly packed DNA and different nuclear proteins. This may modulate the yield of comet production and their shape. Hence, we determined the usefulness of neutral and alkali comet assays for determination of damage to DNA of sea lamprey spermatozoa. Semen of three males was used. It was stored on ice for one day. Sperm concentrations amounted to 0.906, 0.634, and 0.797 x 10^9 mL^{-1}, respectively. Spermatozoa were suspended in PBS containing 0, 1, 10, and 100μM of H_20_2. Suspensions were incubated on ice for 30 minutes and after this period the comet assay was performed. The methodology of comet assay as described in Trevigen instructions was used. The procedure of Green et al. (1996) was used for visual scoring of comets.

Results

Neutral comet assay

A dose-dependent increase in damage to DNA of sea lamprey sperm in relation to concentration (range 0 – 100 μM) was observed (Figure 2A). Control spermatozoa had minimal

damage (0.58 ± 0.14). In parallel experiments, sperm exposed to 100 μM H_20_2 had a mean score of 3.26 ± 0.09. Comet shape was typical for neutral electrophoresis of other cells, i.e. exhibiting round heads and long tails with a width close to the diameter of heads. Damage extent ranged from undamaged nuclei to maximal damage seen as a comet with a long tail. Tails were not clearly visible under normal light microscopy (Figure 3A). The quality of subjective scoring was significantly improved when negative-like microscopic pictures were employed (Figure 3B). We identified two peculiar characteristics of the neutral comet assay of sea lamprey spermatozoa. First, especially in the least damaged nuclei we found the presence of remnants of sperm structures, including tails. Second, it seems that the first damage to sperm is visualized as a significant increase in diameter of the nucleus.

Alkali comet assay

A dose-dependent increase in damage to DNA in relation to concentration range (0 – 100 μM) was observed (Figure 2B). Control spermatozoa had rather high levels of damage (2.19 ± 0.24), and sperm exposed to 10 and 100 μM H_20_2 had mean scores close to the maximum possible (3.71 ± 0.07 and 3.94 ± 0.01, for 10 and 100 μM H_20_2, respectively). Comet shape was characterized by rather oval shape of heads and clearly visible tails (Figure 3C). With increased damage the shape of tails tended to have an oval figure as well (Figure 3). Maximal damage to sperm DNA was characterized by a lack of comet heads (Figure 3C). No sperm remnants were observed.

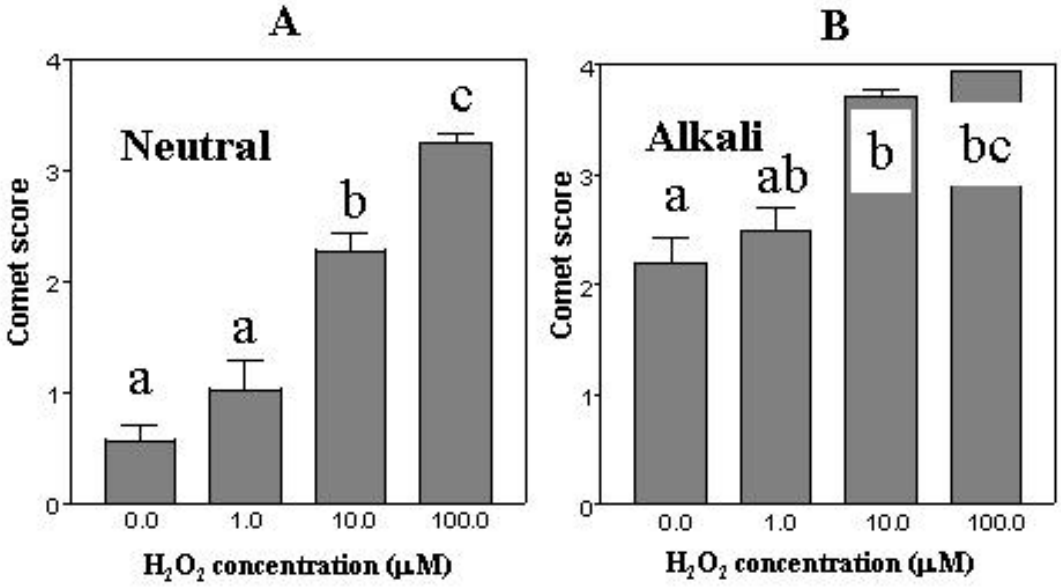

Figure 2. Effect of hydrogen peroxide (H_2O_2) concentrations on damage to sea lamprey spermatozoa in neutral (A) and alkali (B) Comet assay.

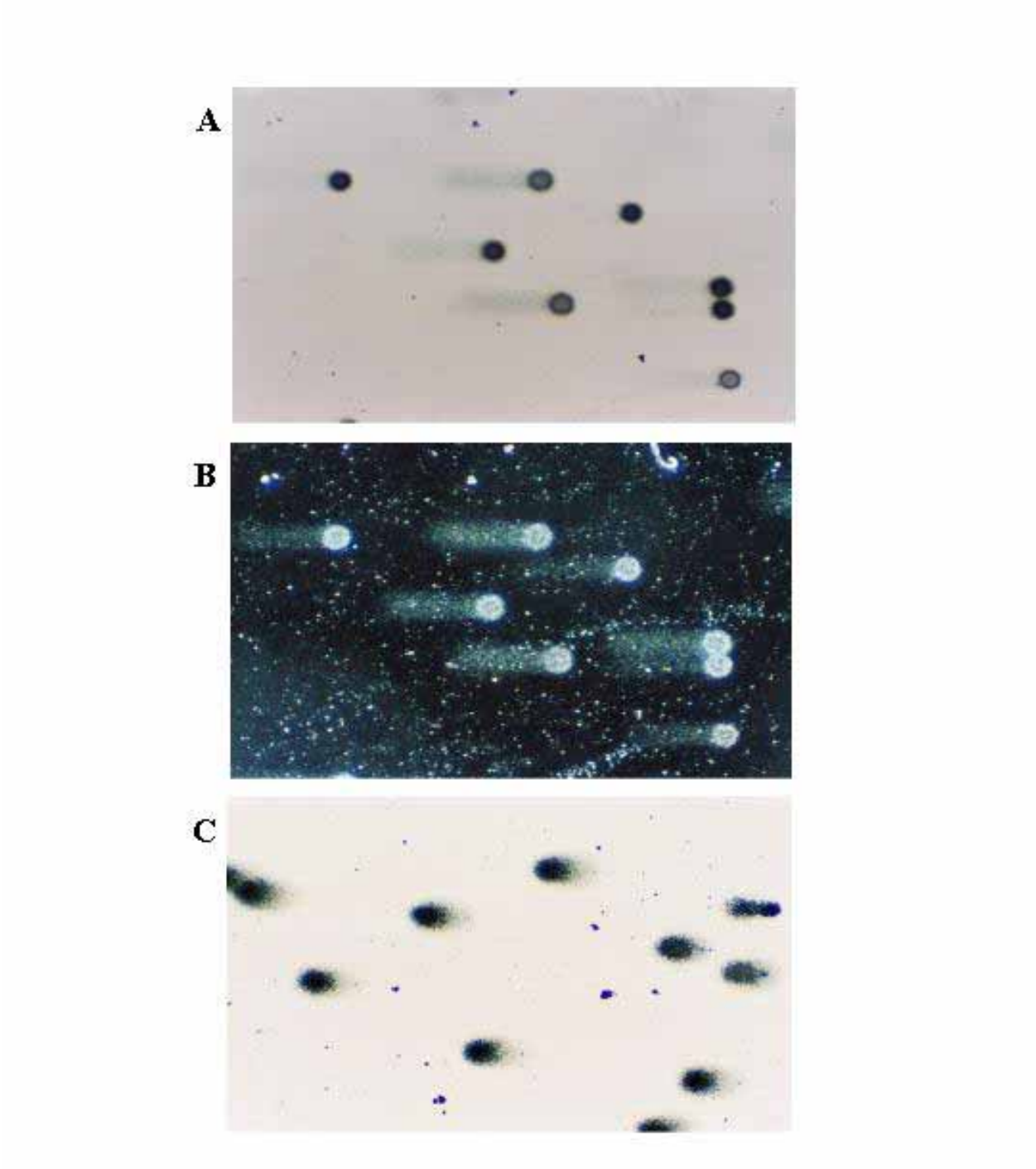

Figure 3. Comet assay pictures of sea lamprey spermatozoa showing a positive control under normal light microscopy (A) and negative-like microscopy (B) by neutral and by alkali comet assay.

Conclusions

These results indicate that both negative and alkali comet assays are able to detect damage to sea lamprey spermatozoa caused by an oxidizing agent. Induction of comets in sea lamprey spermatozoa was possible without special procedures used for mammalian sperm (due to the presence of protamines in the mammalian nucleus). This agrees with data indicating that in lampreys histones are the major protein component of the sperm of agnathans (Saperas et al., 1997). An optimal concentration range for the alkali comet assay is between 0 and 10 μM of H_2O_2. On the other hand, a range for the neutral assay seems to be wider. Both analyses provide a different kind of information; comets produced differ in shape, scores for negative controls are different, and efficiency of lysis differs as well. It needs to be evaluated how information obtained may be used for better understanding of sperm physiology. A very efficient genotoxic action of hydrogen peroxide (100 μM = 0.11%) makes this compound (or similar substances) a good potential candidate as a sterilizing agent for male sea lampreys.

Experiment 1-2. Neutral and alkali comet assay of sperm and erythrocytes of control and bisazir-treated sea lamprey

As part of an ongoing sterile-male-release program to control sea lamprey in the Great Lakes, males are sterilized with injections of bisazir. Sterility of these lampreys was confirmed in our previous *in vitro* studies. In these experiments we sought to find if sterility of sperm may be indicated by neutral and alkali comet assays. We also sought to find if the action of bisazir is specific or if its action is at the level of somatic cells. We tested sperm motility, concentrations (Ciereszko *et al.*, 2002) and DNA fragmentation of sperm and red blood cells.

Methods

In 2000, we evaluated the extent of DNA damage of sperm and erythrocytes of treated and control sea lamprey using the alkali comet assay. Semen of 5 (13 for semen characteristics) bisazir-treated males and 5 (11 for semen characteristics) control males was used. Blood of 8 bisazir-treated females and 8 control females was used. In 2001, we evaluated the extent of DNA damage of sperm and erythrocytes of treated and control sea lamprey using the neutral comet assay. Semen of 3 bisazir-treated males and 5 control males was used. Blood of 4 bisazir-treated males and 4 control males was used.

Results

Semen characteristics and neutral comet assay of spermatozoa

Sperm motility did not significantly differ between bisazir-treated and control males (Figure 4A). The significant difference was found in sperm concentration and comet assay scores between these groups (Figure 4A). Bisazir-treated sea lampreys had sperm of lower concentration and higher damage to DNA than control males (Figure 4B). The levels of damage, however, were far lower as compared to the maximal damage of the positive control, established previously (Figure 5).

Neutral comet assay of erythrocytes

Erythrocytes of bisazir treated sea lampreys showed higher levels of DNA damage than those of control males. The comet scores were similar to values recorded for spermatozoa.

Alkali comet assay of erythrocytes

Erythrocytes of bisazir treated sea lampreys showed higher levels of DNA damage than those of control males (Figure 5B).

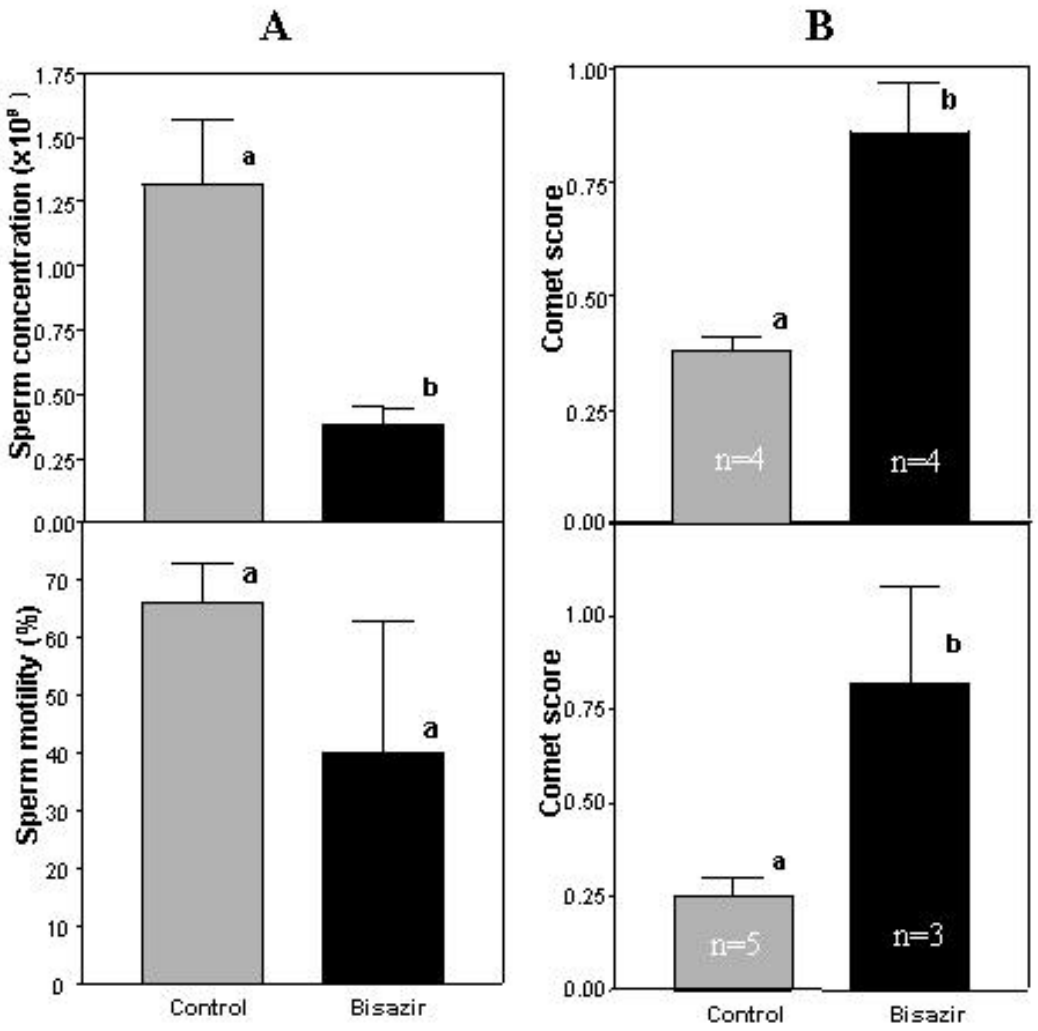

Figure 4. A) Sperm concentrations and motility of control and bisazir-injected sea lamprey (2001), B) Neutral assay of erythrocytes and sperm of control and bisazir-injected sea lampreys (2001). Bars having different letters are significantly different ($P<0.05$).

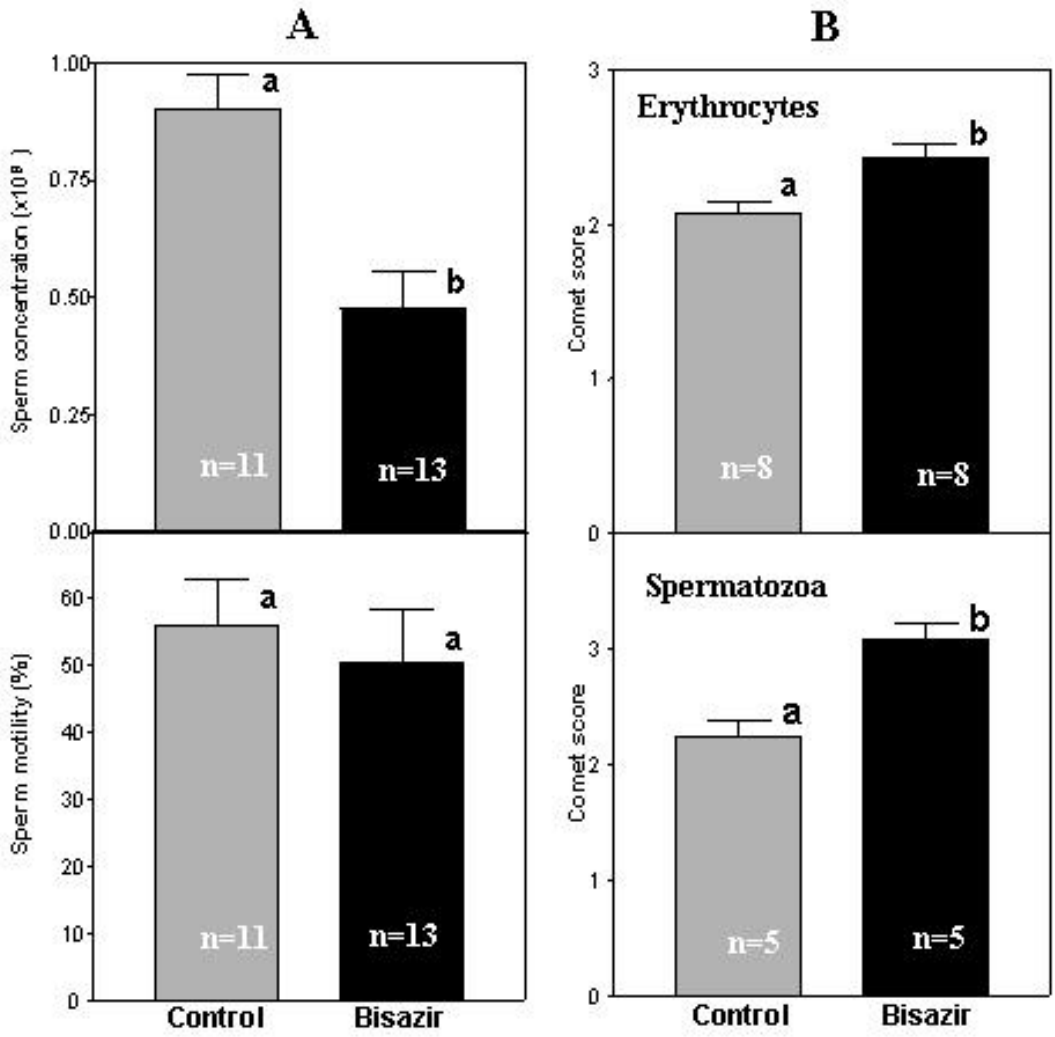

Figure 5. A) Sperm concentration and motility, and B) Comet assay score for erythrocytes and spermatozoa of control and bisazir-injected sea lampreys (2000). Bars having different letters are significantly different ($P<0.05$).

Conclusions

Our data confirmed, for the second year in a row, the lower quality of bisazir-treated animals. This may be related to a different quality of bisazir used. Sterility of males is not associated with maximal damage to sperm, when compared to the positive control (100 μM H_2O_2). This may suggest that total fragmentation of DNA is not part of the mechanism related to the genotoxic effects of bisazir and points to rather restricted action of this compound towards the sperm genome. It is possible that the lower sperm concentration of bisazir-treated animals may indicate that spermatozoa (or their precursors) with higher levels of damage died due to apoptosis and were removed from the reproductive tract. This hypothesis needs to be tested by performing comet assays during different times after injection of bisazir. Damage to erythrocytes identified by the comet assay suggests that action of bisazir is not restricted to germ cells but acts also on somatic cells. It needs to be estimated if these damages may affect the condition of sterilized males and decrease their ability to compete with normal males.

Experiment 1-3. Standardization of 8-hydroxy-deoxyguanosine (8-OHdG) analysis by HPLC

Methods

For the 8-OHdG standardization, the procedure described by Foksinski et al. (1999) was used. The standard of 8-OhdG was purchased from Sigma (Sigma Chemicals Co., St. Louis, MO). The standard of 8-OhdG was run using the HPLC system with simultaneous operation of UV and EC detectors. The stock solution of 8-OHdG standard was prepared and diluted with the mobile phase solution, which was a major solvent for sample extractions. The mobile phase

consisted of 20 mM ammonium acetate (pH 5.3) and methanol (85:15, v/v) and the wavelength for UV detection was 290 nm. For electrochemical detection, we adopted 850 mV of applied potential with the range value of 20 nA. The flow rate was 1.0 ml/min.

Results

The standard curve showed a linear response from 0.14 to 7.0 ppm concentrations (r = 0.999) for both UV and EC detectors. The chromatograms of 8-OHdG standards are provided in Figure 6.

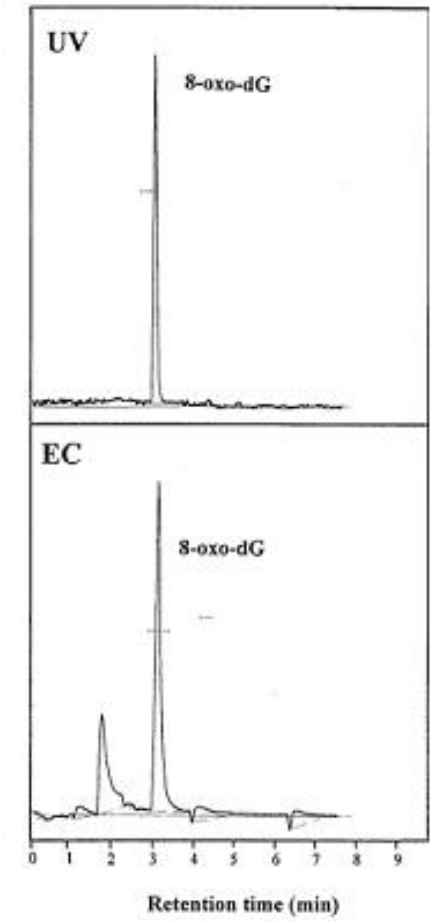

Figure 6. Chromatogram of 8-hydroxydeoxyguanosine (8OHdG: standard of 1.4 mg/ml) by ultraviolet [UV] detection, top; the same standard by electrochemical [EC] detection, bottom)

3.1.3. Objective 2. To validate bisazir assay in sea lamprey tissues using HPLC

Experiment 2-1. Standardization of bisazir analysis by HPLC

Bisazir (p,p-bis 1-azirridinyl-N-methylphosphoinothionic amide) was provided by CCR Inc. (USA). For validation of chromatographic techniques, the HPLC system with UV and electrochemical (EC) detectors was adopted (see Lee and Dabrowski, 2002). The procedure used for bisazir determination was based on methods described by Scholefield *et al.* (1997). The mobile phase consisted of water and methanol (1:1, v/v) and the wavelength for the UV detector was 210 nm. The stock solution of bisazir standard was first prepared in deionized water and diluted with the mobile phase. Methanol/water was the main solvent for sample extractions. The standard curve showed a linear response from 0.4 to 40 ppm concentrations (r = 0.999). The electrochemical detector cannot be used for bisazir molecule detection. The effect of storage of bisazir standard in room temperature and illumination or refrigerated (and in dark) was compared after several days (Figure 7). No significant losses were recorded (~4%).

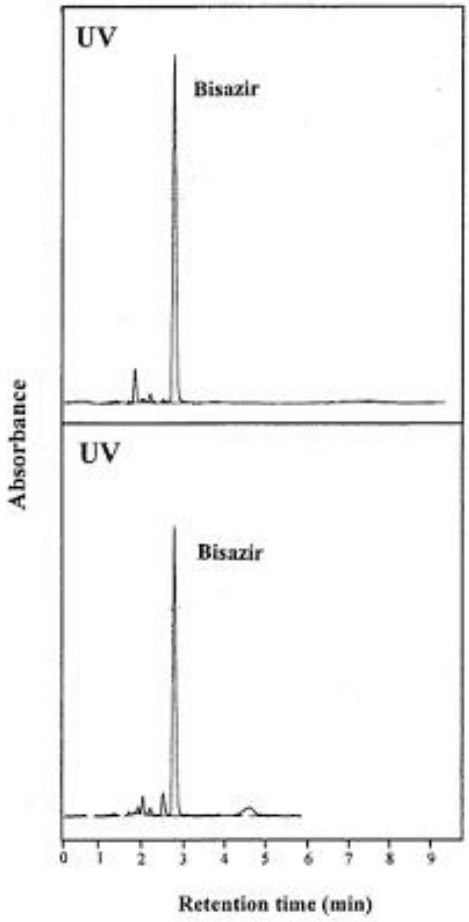

Figure 7. Chromatogram of bisazir (A:standard of 40 μg/ml), top; the same standard after 65 hours kept under light and room temperature, bottom).

3.2. Year 2

3.2.1. Source of experimental animals

Sea lampreys were obtained on four separate occasions from Hammond Bay Biological Station, Millersburg, Michigan. Three shipments were transported by ground from Hammond Bay to Columbus (June 1, July 12 and August 9, 2002). Fish were separated at the station by sex and into control and bisazir-injected groups. We attempted to accelerate the maturation in the second batch of animals (n=6 male and 5 female controls) with prime and resolving doses of luteinizing hormone releasing hormone analogue (LHRHa) (Sigma Chemical Company, St. Louis, Missouri). Sperm was obtained from only 1 of 6 injected males 8 days following the initial injection. Sperm was obtained from 6 non-injected males 5 and 8 days post injection. Eggs were obtained from 1 of 5 injected females 8 days after injection. Eggs were obtained from 4 non-injected females 11 and 14 days post injection. Animals were maintained at 16°C for 25-40 days.

During maintenance of animals at the Columbus Aquaculture facility, skin lesions were observed and sloughing mucus contributed to increased water turbidity. The poor response to hormone injection could be attributed to poor water quality and separation of males and females in both control and bisazir-treated groups. In order to reduce water turbidity, we constructed an activated carbon filter to fit in the chilling tank. The filter significantly decreased water turbidity. For the 2003 season, we will routinely utilize this filter to maintain good water quality and improve survival. We will also administer LHRHa injections soon after arrival of males and females to re-evaluate the response, spermiation and/or ovulation.

Semen was stripped from spermiating males and sperm was stored on ice. Concentrations of spermatozoa were measured using a Double Neubauer Counting Chamber.

Sperm cells were diluted with cold phosphate buffered saline (PBS) to a final concentration of 250,000 per ml and used for comet assay on the day of collection.

3.2.2. Objective 3: To evaluate the mutagenic action of bisazir *in vitro* and *in vivo* using comet assay on sea lamprey erythrocytes

Experiment 3-1. Comet assay of spermatozoa and erythrocytes treated with bisazir

We hypothesize that the sterilizing effect of bisazir may be better estimated if it can be evaluated *in vitro.*

Methods

Semen or blood from 3 – 4 lampreys was used. After resuspension to a concentration of 150 000 cells/ml, cells were exposed to bisazir (final concentration 2 mg/ml; stock 200 mg/ml in ethanol). Control suspensions received 1% ethanol. After 60 min incubation on ice, alkali or neutral comet assays were performed.

Results

We did not observe any significant effect of bisazir on fragmentation of DNA by neutral and alkali comet assays (Figure 8).

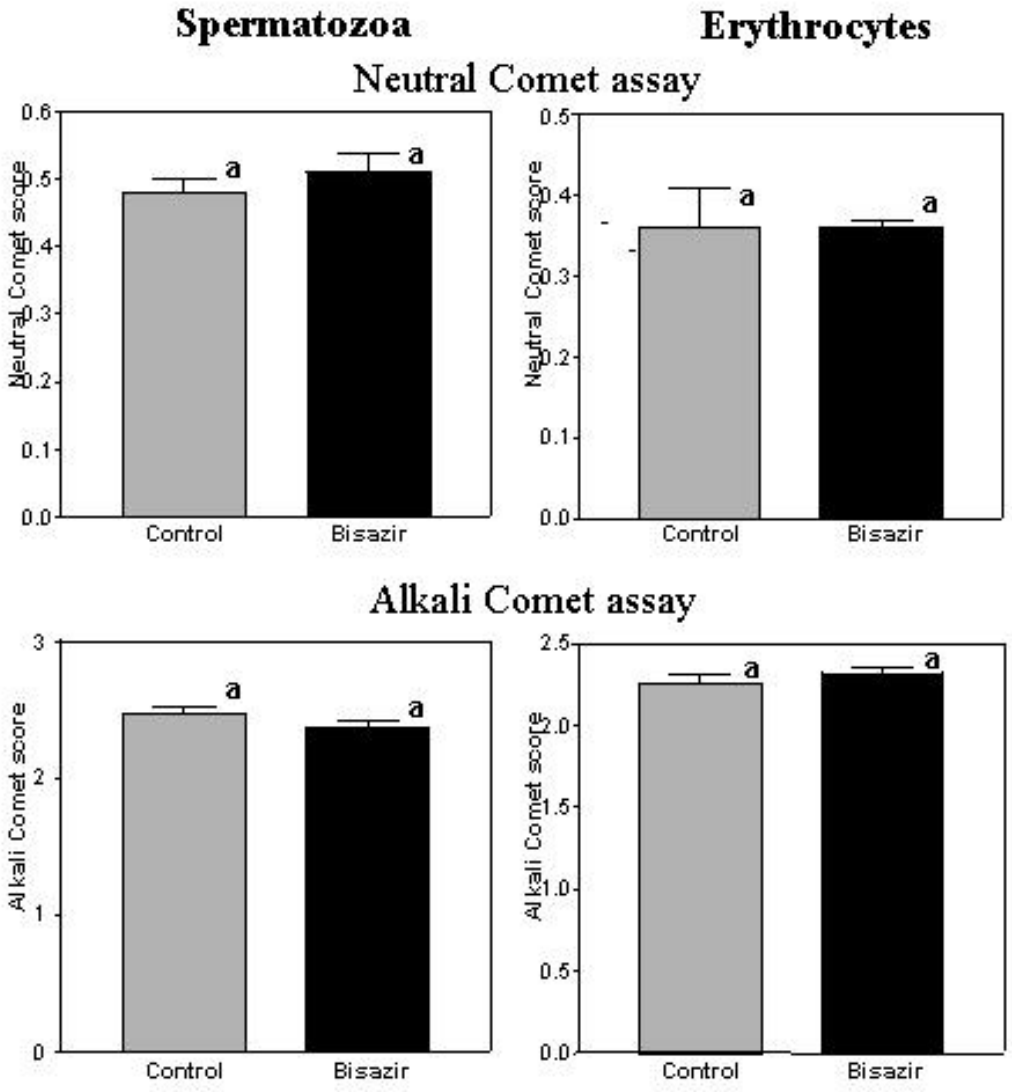

Figure 8. *In vitro* effect of bisazir (2 mg/ml) on comet assay score in sea lamprey spermatozoa and erythrocytes. Bars having different letters are significantly different ($P<0.05$)

Conclusions

These data confirmed the conclusion of the previous experiment suggesting that the mechanism of bisazir action on sea lamprey spermatozoa and erythrocytes does not involve dramatic fragmentation of DNA. It needs to be tested if some extent of DNA fragmentation (as observed in bisazir-injected sea lamprey sperm) may be induced by prolonged in vitro exposure of spermatozoa to bisazir.

Objective 4. To evaluate the effect of bisazir in sea lamprey spermatozoa and ova

Comet assay

A comet assay reagent kit for single cell gel electrophoresis (SCGE) (Trevigen, Inc., Gaithersburg, MD) was used. Cells were combined with molten LM Agarose (at 42°C) at a ratio of 1 to 10 (v/v) and immediately pipetted onto one of two sample cavities on Comet Slide microscope slides. Slides were incubated at 4°C in the dark for 30 minutes in order to solidify and adhere the agarose to the slide. The slides were then immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% sodium lauryl sarcosinate, 1% Triton X-100, and 1% DMSO) for 60 minutes (4°C) followed by immersion in alkali solution (300 mM NaOH, 1 mM EDTA) at room temperature in the dark. After DNA unwinding, slides were subjected to alkali electrophoresis for 30 minutes (300 mM NaOH – 1 mM EDTA, pH > 13). Electrophoresis was performed in a Sub-Cell BT cell, 15 x 15 cm gel (Bio-Rad, Hercules, California). Current parameters were 25 V, 300 mM. By using this apparatus, we were able to run up to 12 slides concurrently. After electrophoresis, samples were immersed in ethanol and air-dried. DNA was stained with diluted SYBR Green (Trevigen, Inc., Gaithersburg, MD) for epifluorescence

microscopy. The excess dye was tapped off the slide and an anti-fade solution (Trevigen, Inc., Gaithersburg, MD) was applied to each circle. The slides were mounted, sealed with nail polish and viewed immediately.

Comets were scored using Komet6 (Kinetic Imaging, Bromborough, Wirral, United Kingdom) software. Approximately 25-50 comets were measured per sample (i.e., slide circle).

According to the manufacturer's instructions, alkali electrophoresis will detect single-stranded DNA breaks, double-stranded DNA breaks and the majority of apurinic, apirymidinic sites as well as alkali labile DNA addicts (e.g., phosphoglycols, phosphotriesters). A representative comet of fish spermatozoa is shown in Figures 9-13.

Previous comet measurements (Annual Report, 2002) have been performed by manually scoring comets using a 5-type scale (0-4) related to the extent of DNA damage (Green et al., 1996). Zero damage was scored as 0 and maximal damage (induced by 100 μM H_2O_2) was scored as 4. This method is limited by the parameters that can be measured, but it is also subject to a "scorer bias". Furthermore, previous studies (Annual Report, 2002) revealed no detectable damage to DNA by visual scoring of comets. Komet6 software (Kinetic Imaging, Wirral, United Kingdom) was purchased and tested as an unbiased tool to measure various parameters of the comet and to detect a traceable damage that may be missed by visual scoring. Emphasis was placed on learning the capabilities of the software in providing quantitative measures of comet parameters for direct evaluation between treatments. Following is a list of parameters that Komet6 measures (Kinetic Imaging, Wirral, United Kingdom):

Comet intensity profile: (mode, mean, std. dev., extent, distributed moment, inertia, skew, coefficient of variation, optical intensity)

Head parameters: (mode, mean, std. dev., extent, distributed moment,

inertia, skew, coefficient of variation, optical intensity)

Tail parameters: (mode, mean, std. dev., extent, distributed moment, inertia, skew, coefficient of variation, optical intensity)

Global Comet Parameters: (head % DNA, Tail % DNA, comet optical intensity, tail length, tail from center, tail/head, Olive tail moment = (tail mean- head mean)*tail %DNA/100, extent tail moment = tail length * tail % DNA/100

Experiment 4-1. Alkali comet assay of sperm from negative control and bisazir-treated sea lamprey

As part of an ongoing sterile-male-release program to control sea lamprey in the Great Lakes, males are sterilized with injections of bisazir. Sterility of these lampreys was confirmed in our previous *in vitro* studies. In these experiments, we sought to find if sterility of sperm might be indicated by alkali comet assay and measured by Komet6. Manual scoring has previously shown very low levels of damage compared to the maximal damage of the positive control.

Methods

Prior to the assay, sperm characteristics were evaluated from eleven control and eight bisazir-treated males. Comet assay was conducted from four of the eleven control males and four of the eight bisazir-injected males. Semen was stored on ice for use the same day. Results were compared to experiments from 2000 and 2001 (data of all years including 2002 were evaluated by different investigators). We tested sperm motility, concentrations (Ciereszko et al., 2002) and DNA fragmentation of sperm cells.

Results

Sperm concentration and motility of fish from each treatment group are shown in Table 1. Sperm concentrations for the control males selected for comet assay were: 1.64, 0.88, 1.08, and 0.96 x 10^9 ml^{-1}. Likewise, sperm concentrations for the bisazir-treated males were: 1.3, 0.93, 0.45, and 1.14 x 10^9 ml^{-1}.

Negative control demonstrated no DNA damage. Comet appearance was illustrated as an intact head with no trailing DNA fragments. These comets were the brightest, as all the DNA was still within the "head" and thus fluorescence was concentrated in this region. Bisazir treated spermatozoa demonstrated no visibly detectable DNA damage. Heads were intact and bright with no or small tails (Figure 10 A,B) and the heads tended to have a high diameter.

Table 1. Mean (± SD) characteristics of sperm from control and bisazir-treated males.

Treatment group	Sperm concentration (x 10^9 sperm/ml)	Sperm motility (%)
Control males (n=11)	1.02 ± 0.47	36.0 ± 28.1
Bisazir-treated males (n=8)	0.70 ± 0.41	46.9 ± 24.9

Results of the computerized analyses confirmed our results obtained earlier by visual scoring. A small but significant differences in percentage head and tail DNA were found between control and bisazir-injected males (Figure 14). On the other hand, no differences in the other comet measurements were found presumably due to a high variability within bisazir-injected males.

We performed the same analyses in 2003. Although the same trends in differences between the control and bisazir-injected males were revealed, no statistically significant differences were found, likely due to a high variability in the comet parameters in the bisazir-injected males (Figure 14A). These data suggest that the response of individual males to bisazir

may vary. Interestingly, the fertilizing ability of bisazir-injected animals in 2003 was unusually high when compared to previous years. Perhaps lower DNA fragmentation revealed by comet assay may substantiate the lower efficacy of bisazir for sterilization in 2003.

Experiment 4-2. Effect of hydrogen peroxide (H_2O_2) concentrations on DNA fragmentation of sea lamprey spermatozoa (Figure 15)

Hydrogen peroxide is an oxidizing substance causing fragmentation of DNA in fish cells (Mitchelmore and Chipman, 1998). For this reason, it can be used as a positive control for comet assay. Spermatozoa differ from somatic cells in having more compactly packed DNA and different nuclear proteins. This may modulate the yield of comet production and their shape. The objective of this experiment is to to determine the usefulness of epifluorescence microscopy and Komet6 software for determination of damage to DNA of sea lamprey spermatozoa.

Methods

Semen from eight males was collected and stored on ice for use the same day. Sperm concentrations were: 0.66, 1.17, 1.59, 1.08, 0.96, 1.57, 0.25, and 2.635 x 10^9 ml^{-1}, respectively. Spermatozoa were suspended in ice-cold PBS containing 0, 100 and 1000 μM H_2O_2. Suspensions were incubated on ice for 30 minutes immediately followed by the comet assay procedure.

Results

A dose-dependent increase in damage to DNA of sea lamprey sperm in relation to concentration range (0-1000 μM) was observed (Figure 15). Control spermatozoa had minimal damage. Sperm exposed to 100 and 1000 μM H_2O_2 demonstrated high and maximal damage,

respectively. Comet shape was characterized by round heads with a few trailing DNA fragments (Figure 2). With increased damage, the shape of the tails became elongated and oval in appearance, with some separation between head and tail observable in some samples. Maximal damage to sperm DNA was characterized by the absence of a comet head (Figure 10C). All or most DNA was concentrated in the tail. No sperm remnants were observed.

Increased concentrations of H_2O_2 caused a significant decrease of sperm DNA in the head and an increase in the tail as well as in tail length (Figure 15). Both extent tail moment and Olive tail moment increased with an increase of H_2O_2 concentrations. This data indicate that all major comet measurements are useful for evaluation of H_2O_2 effects on sea lamprey spermatozoa.

Experiment 4-3. Short-term effects of ice storage on sperm motility and DNA fragmentation of sea lamprey spermatozoa

In our laboratory, we routinely store sperm of sea lamprey on ice prior to fertilization of eggs. This experiment was performed to test whether progressive DNA damage occurs as storage of sperm on ice is prolonged.

Methods

Sperm was collected by stripping and stored on ice for 0, 1, 2 and 4 days. Following each respective incubation period, cells were counted using a hematocytometer and diluted to 250,000 cells ml^{-1} for comet assay.

Results

A significant decrease in head and an increase in tail DNA were found during a short-term storage of sea lamprey milt. Those differences were parallel to the decrease in sperm motility at the same time (60 ± 4, 37 ± 12, and 14 ± 9%, for 0, 2, and 4-day storage, respectively). These results indicate for the first time that an increase in DNA fragmentation may be characteristic for sea lamprey sperm storage on ice.

Experiment 4-4. Effect of UV irradiation on DNA fragmentation of sea lamprey spermatozoa

UV irradiation is a known DNA mutagen. To further test the efficacy of the comet assay and Komet6 program, we performed additional positive controls. This was deemed necessary to further evaluate the Komet software since the observed damage to bisazir treated spermatozoa is very low.

Sperm was collected by stripping and stored on ice. 150 µl of sperm was diluted with 850µl of sea lamprey Ringer's solution. 500 µl of this dilution was placed on a glass dish sitting on ice, and irradiated using the Stratalinker (Stratagene, La Jolla, California). Sperm was irradiated for 0, 0.1, 0.2, 0.3 or 0.6 minutes. The dish was covered with "black-painted" cover to prevent photoreactivation. Eggs (0.3 g) were immediately inseminated with 200µl irradiated

sperm and covered with 5 ml well water. Irradiated sperm (10 μl) was pipetted into 10ml of ice cold PBS, counted with a hematocytometer and diluted to final concentrations of 250,000 cells ml^{-1} for comet assay.

Results

Comet assay appears to be a useful tool for monitoring of UV effects of sea lamprey sperm DNA. We are able to indicate dose-dependent effects of UV on DNA fragmentation for most comet characteristics (Figure 16). Those changes were parallel to dose-dependent decrease in sperm fertilizing ability measured at pre-hatching stage (71.4 ± 5.2, 29.6 ± 2.3, and 15.3 ± 1.6%, for 0, 0.1, and 0.2 min exposure, respectively).

Experiment 4-5

Effect of potassium permanganate ($KMnO_4$) on DNA fragmentation of sea lamprey spermatozoa

$KMnO_4$ was tested as an additional positive control, although we suspected that this could not induce a severe treatment as ultraviolet light radiation or H_2O_2. To develop accurate procedures for measuring bisazir treated spermatozoa, it is necessary to test a wide range of positive controls.

Methods

Sperm was collected by stripping and stored on ice for use the same day. Stock solutions of $KMnO_4$ were prepared by mixing 25 μl of PBS (negative control), 25 or 250 μM KMnO4 with 1.325 ml ice cold PBS. 10 μl of each stock solution was added to 90 ul of sperm and

incubated on ice for 30 minutes. Sperm motility was then checked. 0.3 g of eggs were fertilized with 200ul of sperm and covered with 5ml of well water. Sperm was counted on a hematocytometer and diluted to 250,000 cells ml^{-1} for comet assay.

Results

Representative comets from these experiments and quantitative measurements of the comets are shown in Figures 9-17.

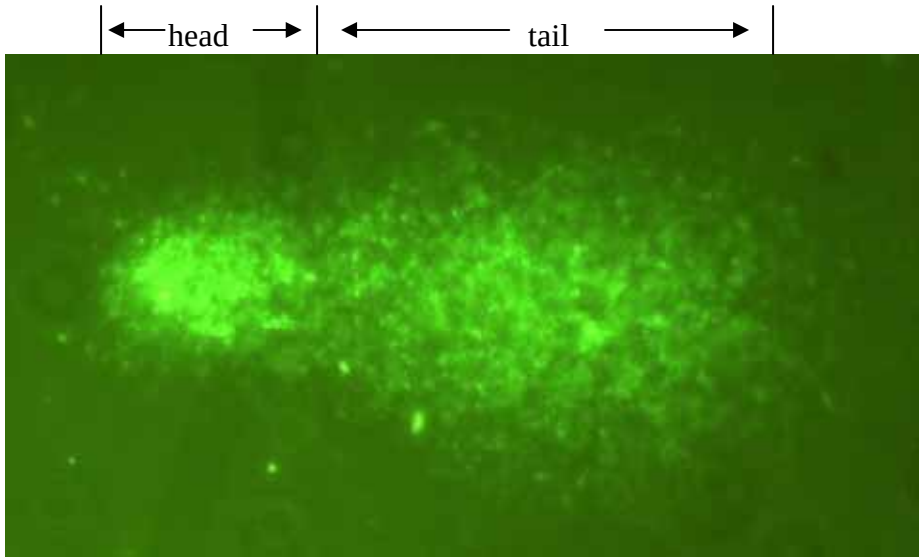

Figure 9. A typical comet showing DNA migrating away from the sperm cell (head), forming a "tail" of DNA fragments. The tail length is positively correlated with the extent of DNA damage. Likewise, high fluorescence intensity indicates high concentration of DNA in a specific region.

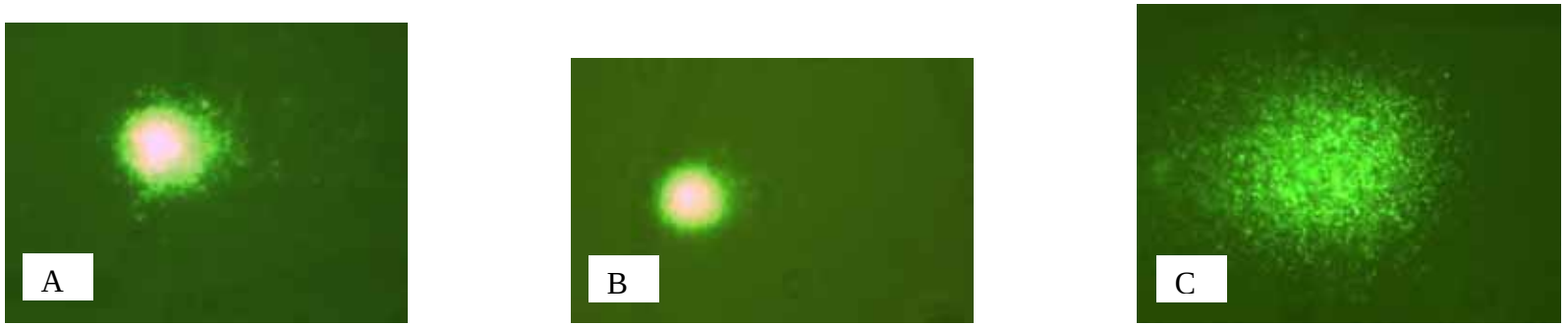

Figure 10. Comets of sperm of bisazir-treated fish **(A)**, non-bisazir-treated fish (**B**, negative control), and sperm treated with hydrogen peroxide (**C**, H_2O_2, positive control).

Figure 11. Comets of sperm after two **(A)** and four days **(B)** storage on ice.

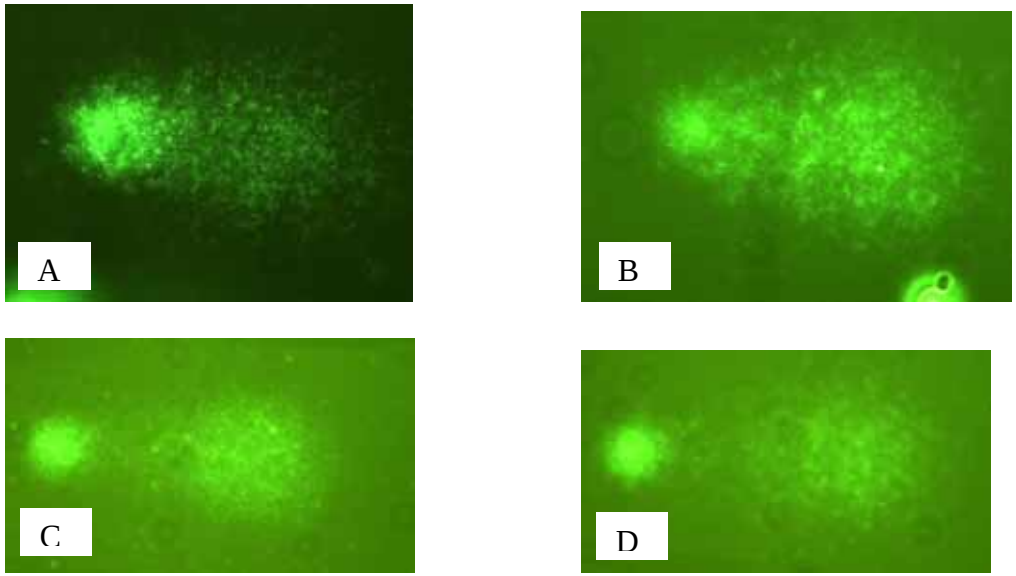

Figure 12. Comets of sperm exposed to increasing duration of ultraviolet light radiation, 0.1 min **(A)**, 0.2 min **(B)**, 0.3 min **(C)**, and 0.6 min **(D)**.

Figure 13. Comets of sperm exposed to increasing concentrations of potassium permanaganate ($KMnO_4$), 25 μM **(A)**, and 250 μM **(B)**.

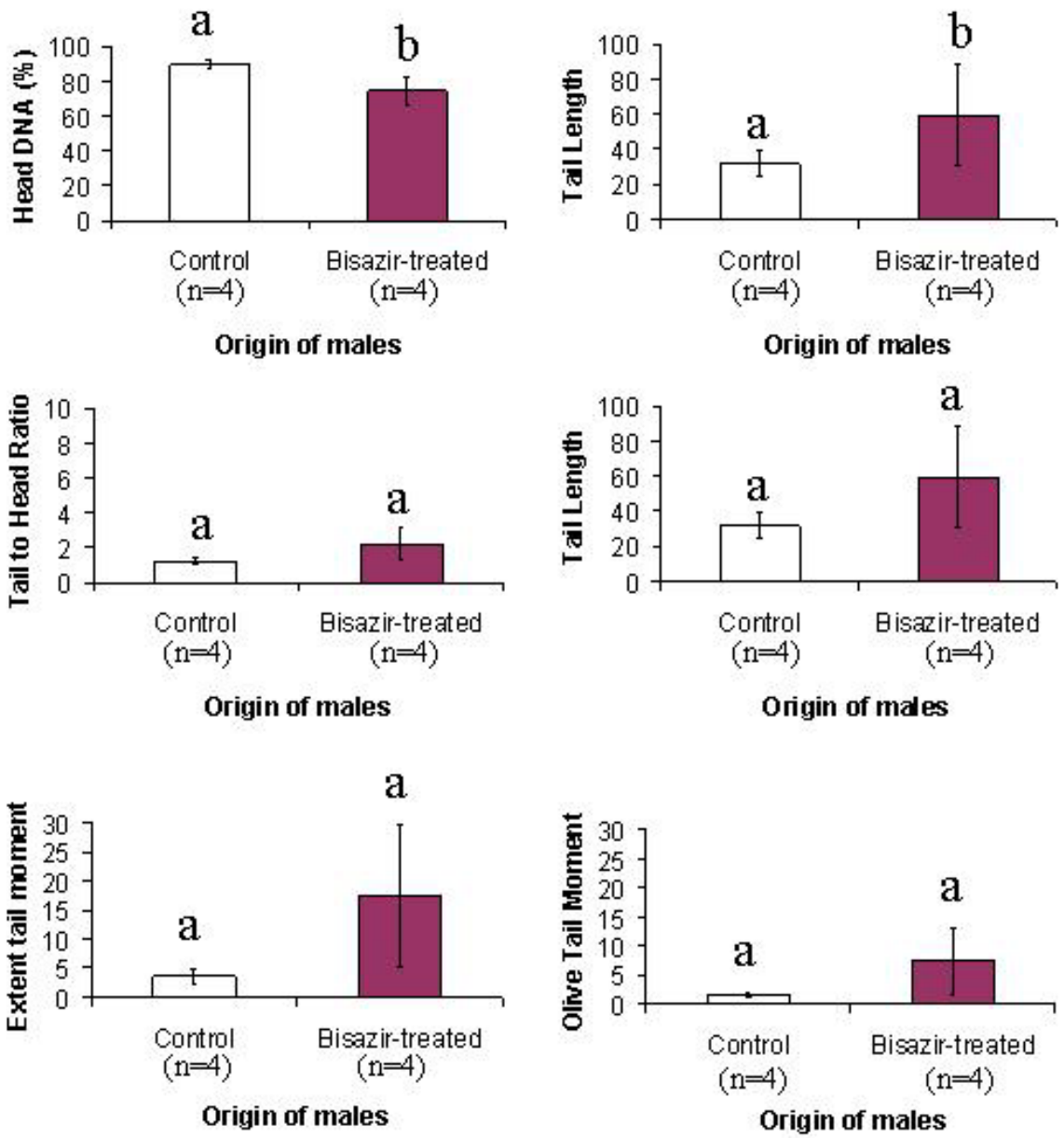

Figure 14. Sea lamprey sperm comet measurements of the control and bisazir-treated males (Data from 2002).

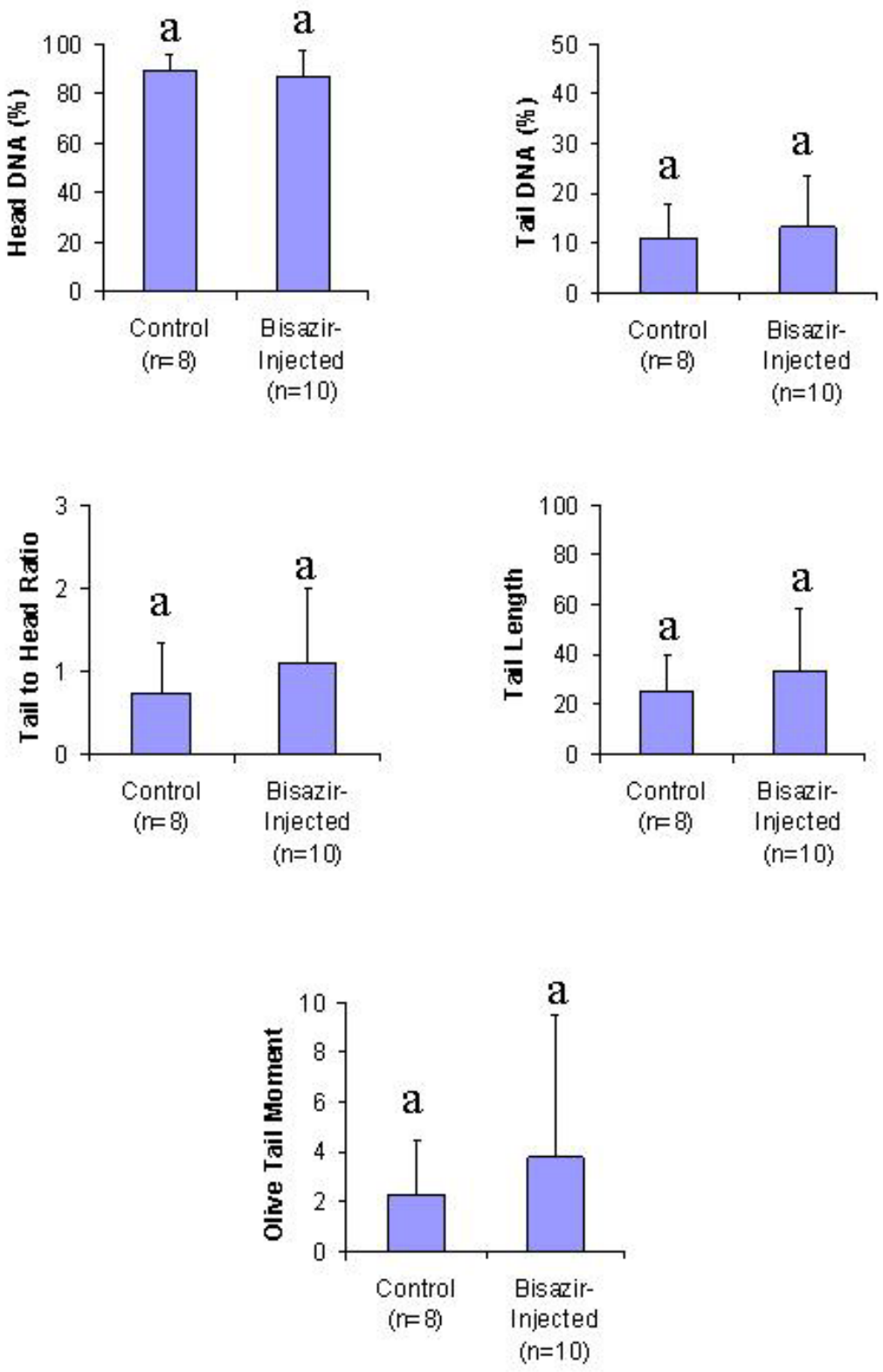

Figure 14a. Sea lamprey sperm comet measurements of the control and bisazir-treated males (Data from 2003)

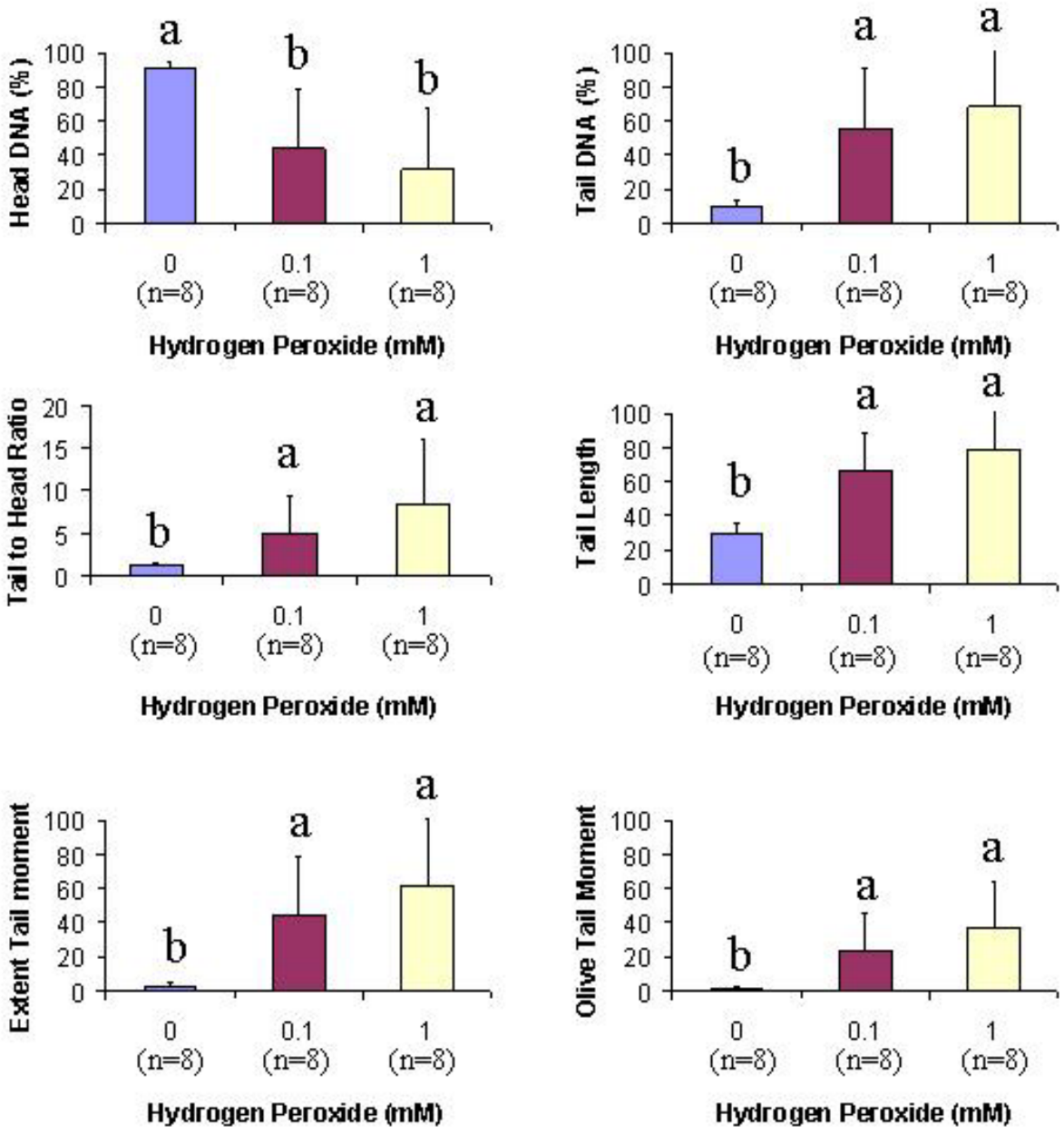

Figure 15. Effect of hydrogen peroxide (H_2O_2) on sea lamprey sperm measurements.

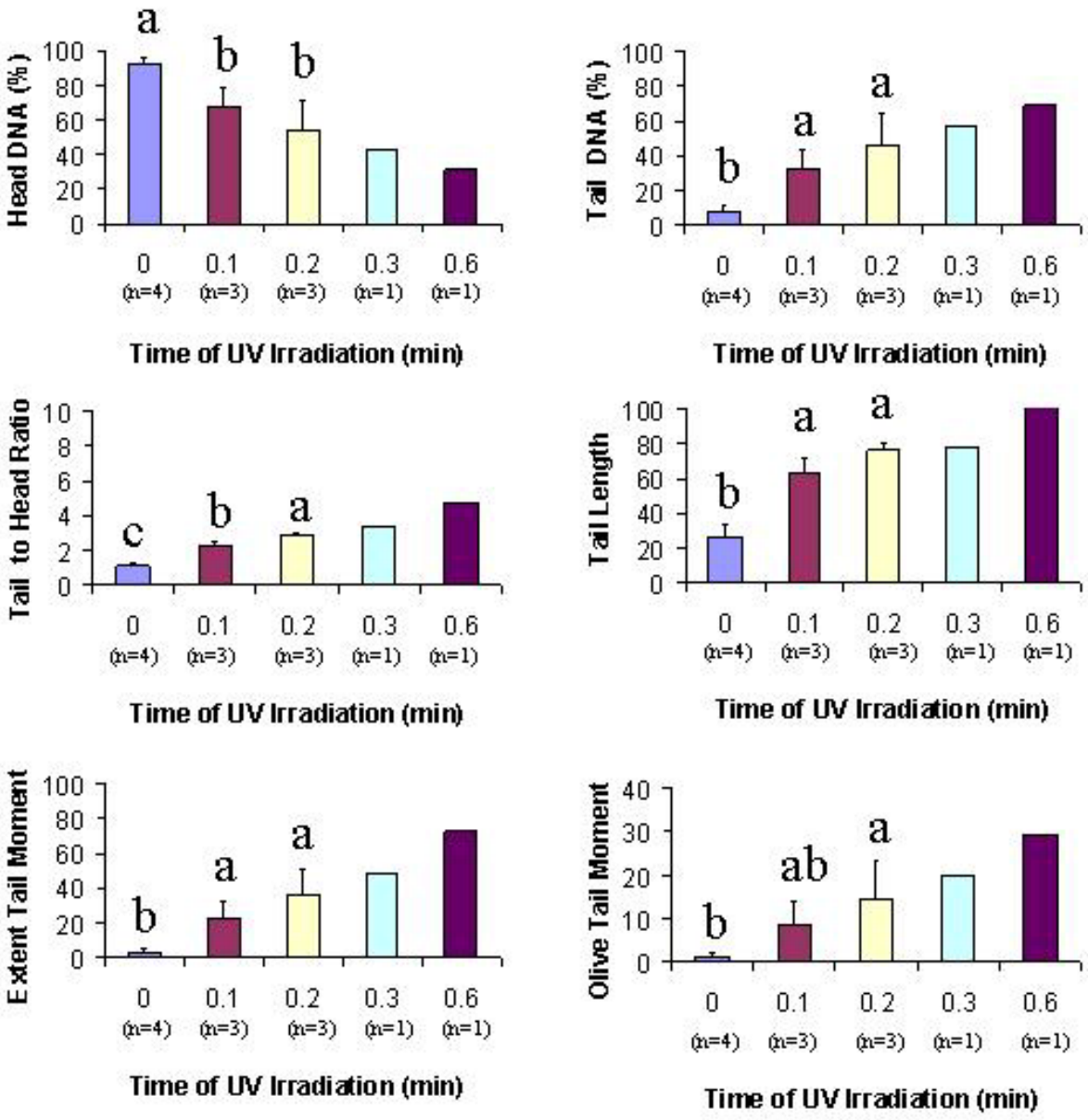

Figure 16. Effect of UV irradiation on sea lamprey sperm comet measurements.

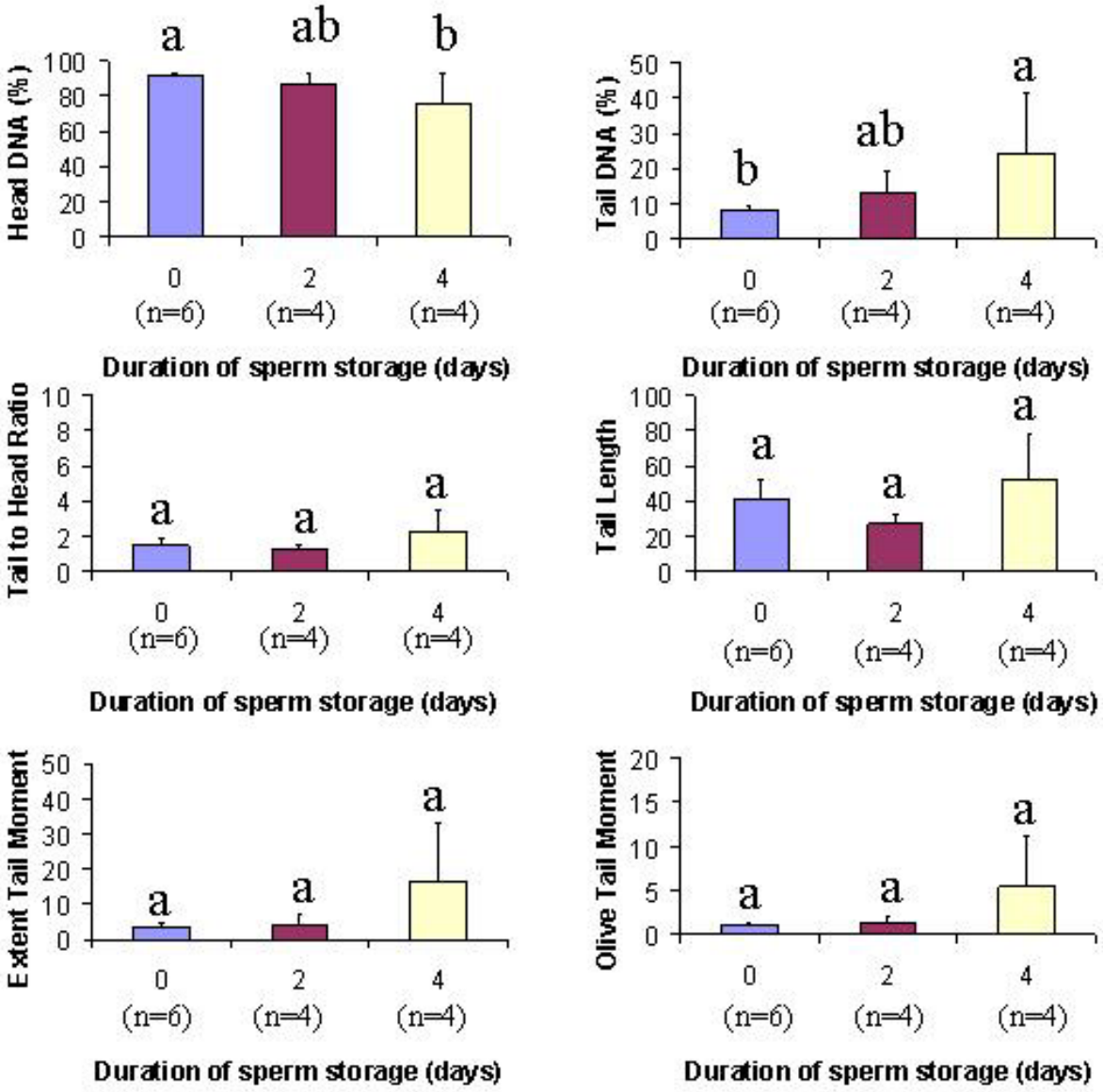

Figure 17. Effect of storage time on sea lamprey sperm comet measurements.

Objective 5. To determine bisazir concentration in blood, seminal plasma and ova of sea lamprey

Experiment 5-1. Analysis of bisazir in fish tissues using high performance liquid chromatography

Blood and other tissues (liver, kidney, and gonads) were collected from male and female, bisazir-treated sea lamprey within three to four days after injection. The main objective is to detect the difference in concentrations of bisazir injected and the remaining amount accumulated in the blood and other tissues to have a measure of the bisazir concentration released into the water, effects of sex, maturation and environmental conditions on depuration rates.

Materials and Methods

Bisazir in holding water and liver tissues of sea lamprey was analyzed by HPLC with modification of the method described by Scholefield et al. (1997). No extraction steps were applied for the water sample. The water sample was directly filtered through a syringe filter (0.45 μm, Whatman Inc., Clifton, NJ, USA) and injected into HPLC system. The liver sample of approximately 0.5 g was weighed and 1.5 ml of an extraction solvent was added. The sample mixture with the extraction solvent (50% methanol in water) was homogenized (Model: Omni 5000, Omni International, Inc., Marietta, GA, USA) for 30 s and centrifuged for 30 min at 10,000 rpm at 4^{o}C. After centrifugation, an aliquot of the supernatant was diluted with demineralized distilled water to obtain a desirable concentration for detection. The diluted sample extract was filtered with the syringe filter and injected into the HPLC system. The HPLC system consisted of two delivery system pumps (Model 506A, Beckman Instruments Inc., San

Ramon, CA, USA) equipped with a 20 μl injection loop connected to a 4.6 mm × 150 mm Phenomenex Discovery C-18 column (Phenomenex USA, Torrance, CA, USA) packed with octadecyl-bonded porous silica gel (5 μm). The UV detector (Programmable detector module 166) was purchased from Beckman Instruments Inc., San Ramon, CA, USA. The UV wavelength was set to 210 nm. The flow-rate was set to 1.0 ml/min. The mobile phase was composed of water and methanol (1:1, v/v). External standard curve of bisazir was made for the calculations. Detection levels for bisazir was < 1.0 ng/20 μl with a signal-to-noise ratio of 3 for UV detection at 210 nm.

Results

The HPLC system was effectively separating bisazir based on chromatograms produced (Figure 18). All bisazir peaks were distinct, occurring at the same retention time.

Bisazir treated animals were segregated into male and female groups and held in a closed water system for twelve hours during transport from Millersburg, Michigan to Columbus, Ohio. Ice in plastic bags was used for cooling. Upon arrival at The Ohio State University, Columbus, Ohio, water and tissue samples were collected. Liver samples from four bisazir treated males contained an average of 174.7 μg/g of bisazir and liver samples from four bisazir treated females contained an average of 129.2 μg/g of bisazir (Figure 19). The water in which the male animals were held contained an average of 3.21 ± 1.77 μg/ml of bisazir 12 hours after the lamprey were injected. The water housing the females contained an average of 2.90 ± 1.24 μg/ml of bisazir.

Bisazir concentrations in both liver and water samples of male lamprey were 26% and 10%, respectively, higher than those found in females. The average whole body weights of males exceeded females by only 0.05g, or 1%. The females appear to be eliminating bisazir

from the liver more rapidly than the males but are not excreting the chemical from their bodies as quickly.

In 2003, we determined bisazir concentration in liver, kidney, gonad, and muscle tissues (Figure 20; Table 2-3). The highest concentrations were found in gonads. These results suggest that bisazir can be preferentially accumulated in the gonads. However, due to a high variability, more data needs to be obtained.

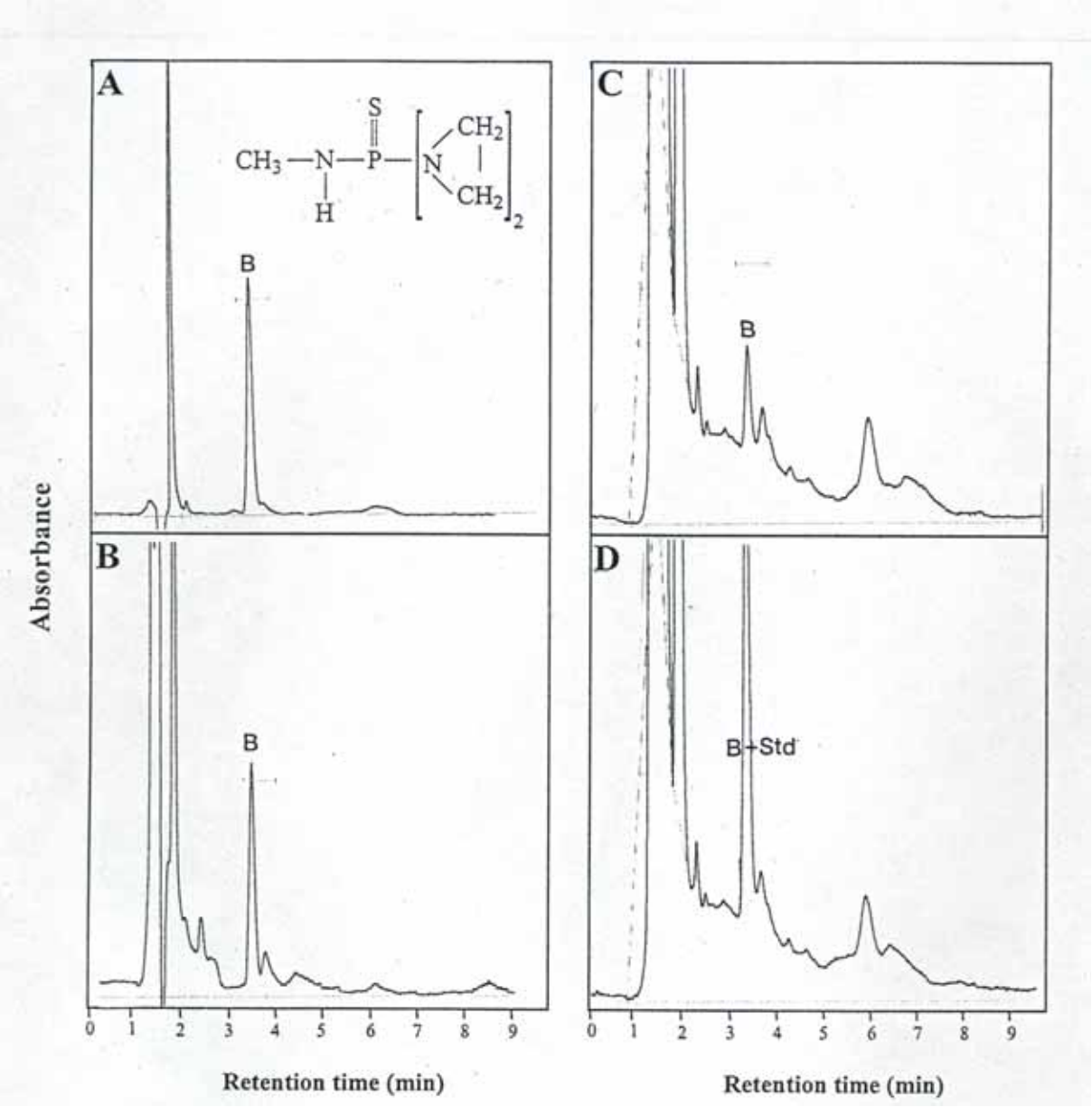

Figure 18. Chromatogram of bisazir; Bisazir standard **(A)**, bisazir in holding water of treated male sea lamprey 12 h post-injection **(B)**, bisazir in liver of treated sea lamprey female 12 h post-injection **(C)**, and spiked with bisazir standard **(D)**. "B" designates bisazir.

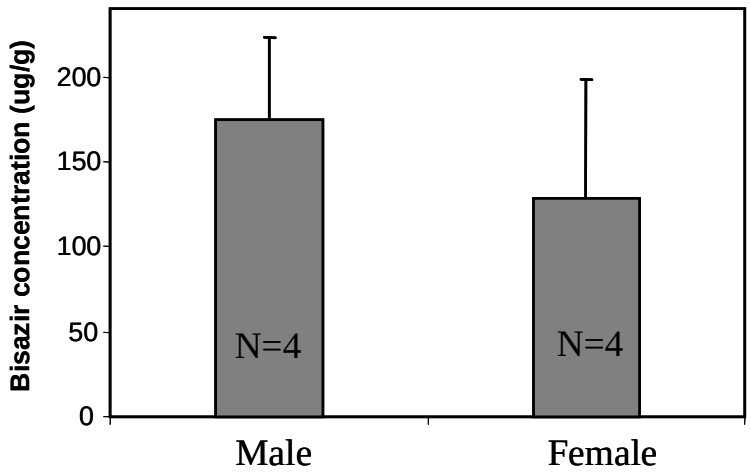

Figure 19. Bisazir concentration in liver of sea lamprey 12 h post-injection.

Table 2. Bisazir concentration in tissues at 10 hours post-injection (6-17-03).

	Bisazir Concentration					
Sex	Code	Liver (μg/g)	Kidney (μg/g)	Gonad (μg/g)	Muscle (μg/g)	Blood plasma (μl/l)
Female (252.5 ± 89.2 g)	9	17.26	10.87	43.76	6.34	-
	11	17.17	15.95	61.40	11.25	-
	12	23.89	16.79	8.62	15.43	-
	Mean ± SD (n=3)	19.44 ± 3.85	14.54 ± 3.20	37.93 ± 26.87	11.01± 4.55	-
Male (288.13 ± 42.7 g)	7	15.31	11.49	26.38	9.15	19.87
	8	57.14	37.68	41.27	34.27	66.00
	10	3.30	3.213	5.47	2.42	8.00
	Mean ± SD (n=3)	25.25 ± 28.26	17.46 ± 17.99	24.37 ± 17.98	15.28 ± 16.79	31.29 ± 30.64

One way analysis of variance revealed no significant differences in bisazir concentration of various muscles between females and males.

Table 3. Bisazir concentration 9 hours post-injection (7-10-03)

	Bisazir Concentration (μg/g)				
Sex	Code	Liver	Kidney	Gonad	Muscle
Female (242.3 ± 9.6 g)	20	12.54	6.44	111.55	2.19
	21	17.47	9.27	1.04	5.54
	22	13.97	6.89	2.83	5.35
	Mean ± SD (n=3)	14.66 ± 2.54	7.53 ± 1.52	38.47 ± 63.30	4.36 ± 1.88
Male (317.3 ± 32.0 g)	17	67.84	42.94	198.66	22.02
	18	25.02	12.05	14.85	11.60
	19	21.06	18.56	45.37	9.11
	Mean ± SD (n=3)	37.97 ± 25.94	24.52 ± 16.28	86.29 ± 98.50	14.24 ± 6.85

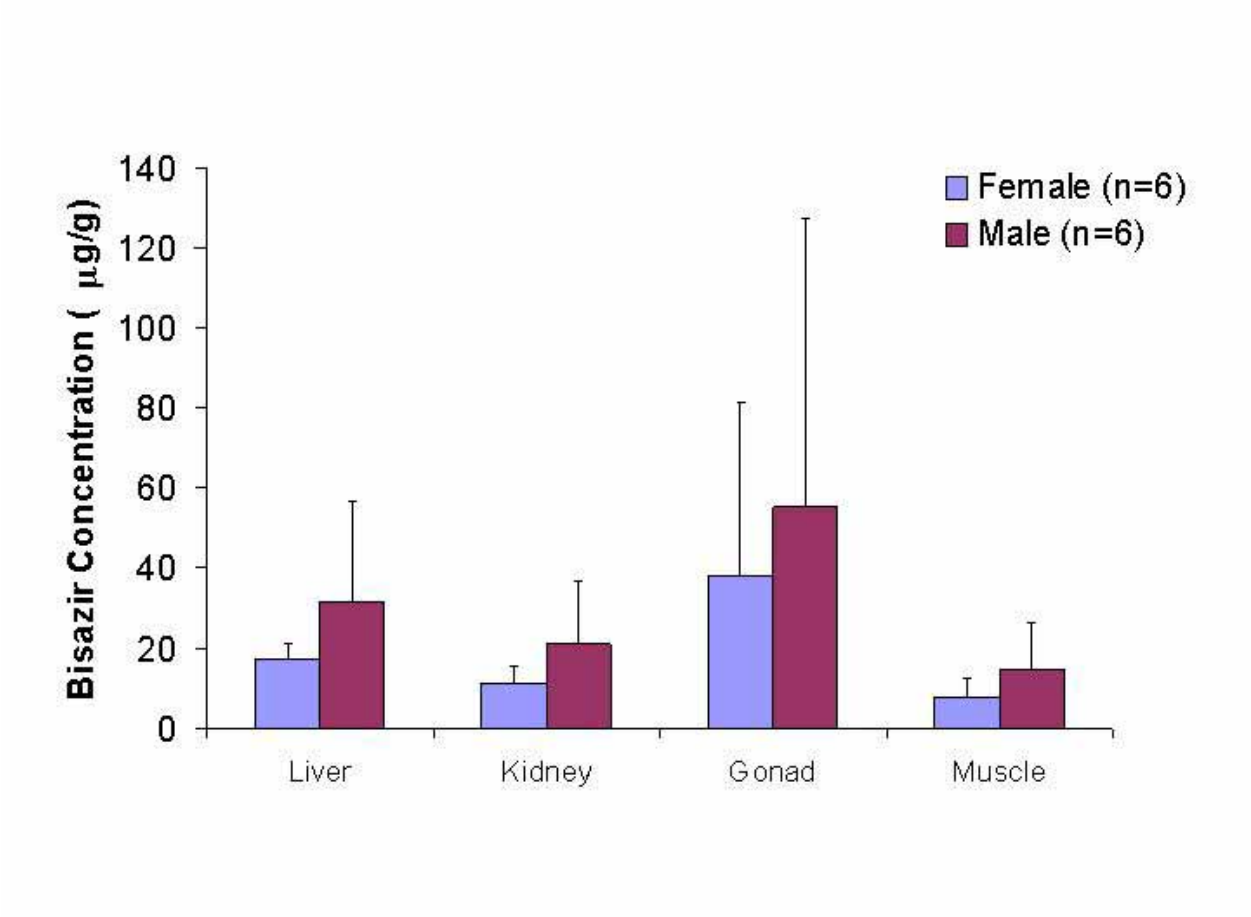

Figure 20. Bisazir concentration in various tissues of sea lamprey at 9-10 hours post-injection. No detectable bisazir levels were observed seven days after injection. Four control sea lampreys (non-bisazir injected had no detectable levels of bisazir. One way analysis of variance revealed no significant differences between sexes and between dates of sampling (6/17/03, 7/10/03).

3.3. Year 3.

3.3.1. Objective 6. To evaluate the effectiveness of bisazir treatment in terms of timing and dosage

Experiment 6-1. Effects of bisazir injection on gonadal maturation, hatching and larval viability of progenies

Male and female sea lampreys were obtained from Hammond Bay Biological Station, Millersburg, Michigan. Some fish were injected intraperitonneally with bisazir (100mg/kg) and some were kept as controls (non-injected). Fish were then transported to Columbus, Ohio in "ice" water for 8-9 hours and acclimated to 18°C upon arrival. Some fish were injected intraperitonneally with bisazir (100mg/kg) and some were kept as controls (non-injected). Fish were then maintained in 4 circular 400-L tanks, with bisazir and control animals separated, in the aquaculture facility at the School of Natural Resources, The Ohio State University.

Both genders, on average 15 males and 15 females per treatment, were injected with luteinizing hormone releasing hormone analog (LHRHa) (Sigma, St. Louis, Missouri) at a dose of 200 μg/kg as a primary dose. Females were then administered a resolving dose of 500 μg/kg. Females were checked for signs of ovulation by palpitating the abdomen and observing any release of eggs, or by anesthetizing and attempting to strip the eggs. When the first females were ovulating, males were checked for sperm release by gentle massage of the abdomen.

Fertilization trials were performed in Petri dishes using 0.3 g of eggs (~200 eggs) and an excess of spermatozoa corresponding to ~50,000 spermatozoa per egg or 30 μl of milt per dish. Sperm samples were composed of 3-5 control males (non-bisazir). Bisazir-injected males were examined individually for fertility. Gametes in all experiments were activated with water (10

ml) and incubated first at room temperature for 5 h (2-cell embryo stage), then transferred to California-type hatching trays for 9-10 days at 19^{o}C (pre-hatching stage).

The results indicate that both genders injected with bisazir and LHRHa reached reproductive maturity sooner than those injected solely with LHRHa. Bisazir females ovulated on average 4.3 ± 0.58 days after the initial injection with LHRHa while control females ovulated on average 7.5 ± 1.87 days after the initial LHRHa injection. The bisazir appears to have accelerated ovulation by 3.2 ± 2.19 days. Five of the 11 control females were injected a second time, 4 days after the initial LHRHa injection. This may have accelerated ovulation of these control females over those injected only once (Figs. 21 and 22). However, until this observation was made, we did not intend to compare the effect of LHRHa, but solely targeted "synchronized" ovulation in lamprey.

All males received only one LHRHa injection and were checked for spermiation when the first ovulating females were detected. The first ovulating females (bisazir/LHRHa) were found in both experiments (July and August) at 4 days after the initial LHRHa injection. When males were checked at this time, 100% of the bisazir/LHRHa injected males were spermiating compared to 50% of control/LHRHa and bisazir/saline injected males. Males were checked the second time at 6 days after the initial LHRHa injection. All of the Bisazir/LHRHa injected males were spermiating as well as 100% of the bisazir/saline-injected males. The percentage of control/LHRHa spermiating males increased by 16% (from 50% to 66%) and the percentage of control/saline injected males spermiating remained the same at 44% on both sampling days (Fig. 23).

Fertilization experiments were performed to test the viability of eggs obtained from bisazir treated females. Milt from control males was used to test the effectiveness of bisazir to prevent

egg fertilization. Likewise, milt from individual bisazir treated males was tested against control females. Fertilization rates were assessed at the 2-cell and pre-hatching stages. The fertilization rate for bisazir males with control females at the 2-cell stage decreased significantly in the pre-hatching stage. The fertilization rate for bisazir females with control males was 66.3 ± 34.0 at the 2-cell stage and also decreased significantly in the pre-hatching stage (Figure 23, Table 4).

Preliminary data suggests the possibility that bisazir may act to accelerate the reproductive maturation of sea lamprey. The mechanism by which this may be occurring is as yet unknown. However, it is clear that bisazir is not as effective a sterilant in females as it is in males and may actually be enhancing the reproductive performance of these females and the probability of producing viable larvae. Treating both males and females with bisazir sets the stage for early maturation of both genders and, therefore, early fertilization of bisazir females by control (normal) males. This selection theoretically enhances fertilization rates with less competition from control (normal) females. Conversely, bisazir treated lamprey males may spermiate earlier and die before control (normal) females become ready to spawn (ovulate). This may compromise the competitiveness of bisazir treated males against control (normal) males for fertilization opportunities of control (normal) female eggs.

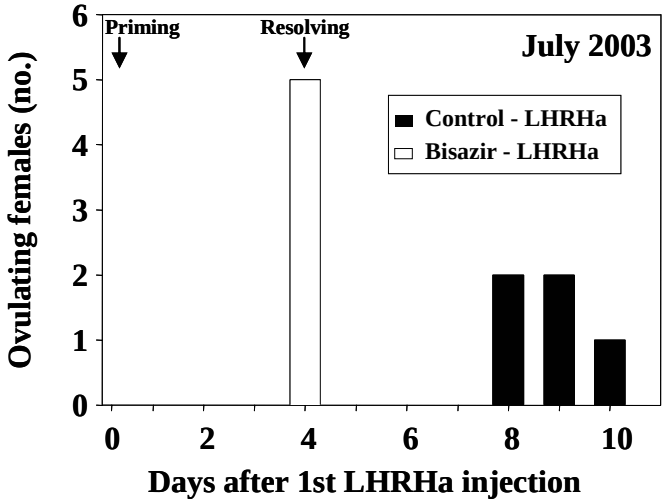

Figure 21. Number of ovulating sea lamprey females, injected with or without bisazir, following LHRHa injection on July 10, 2003. Ovulating bisazir females received only the priming dose of LHRHa, whereas controls were injected with the resolving dose on the 4th day after the priming dose. First injection: n=18 control/LHRHa injected; n=17 bisazir/LHRHa injected. Second injection: n=16 control/LHRHa injected; n=4 bisazir/LHRHa injected. None of the control females ovulated before the resolving dose was administered and none of the bisazir females ovulated after receiving a resolving dose.

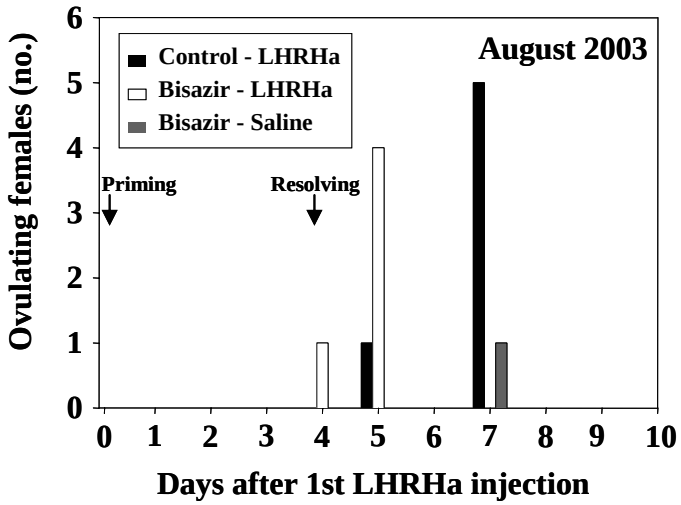

Figure 22: Number of ovulating sea lamprey females, treated with or without bisazir, following LHRHa or saline injection on July 28, 2003. Only the animals ovulating on day 7 received the resolving dose on the 4th day after the priming dose. First injection: n=19 control/LHRHa injected; n=16 bisazir/LHRHa injected; n=13 bisazir/saline injected. Second injection: n=10 control/LHRHa injected; n=1 bisazir/LHRHa injected; n=6 bisazir/saline injected.

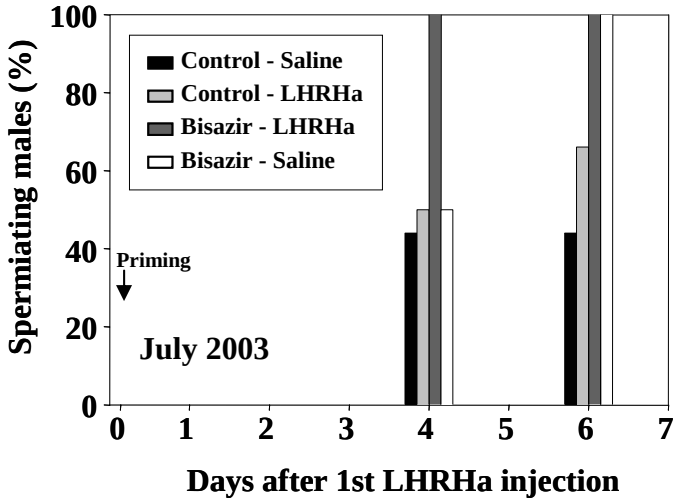

Figure 22A: Number of spermiating sea lamprey, treated with or without bisazir, following LHRHa or saline injection in July 2003. All males received only a priming dose on day 0.
n=10 control/saline injected; n=7 control/LHRHa injected; n=7 bisazir/LHRHa injected; n=8 bisazir/saline injection.

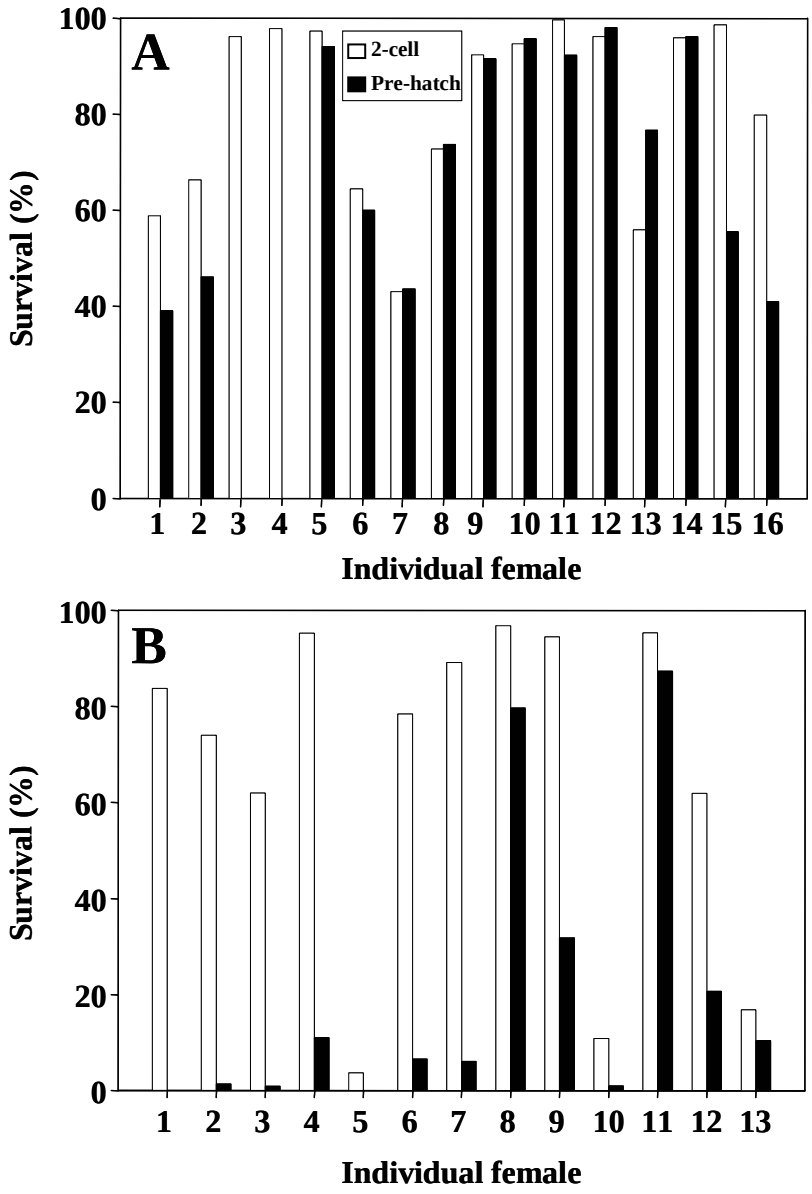

Figure 23: Sea lamprey survival rates at 2-cell embryo and pre-hatching stages for individual control (A) and bisazir treated (B) females. Sperm from 3-4 control males was used in all experiments.

Table 4: Sea lamprey survival rates at 2-cell embryo and pre-hatching stages. Data was combined from three batches of fish obtained from Lake Huron Biological Station, Millersburg, Michigan. The first two batches of fish originated from the Cheboygan River. All others originated from the St. Mary's River.

	Males		Females	
	Control	Bisazir	Control	Bisazir
2-cell embryo				
N	5	7	16	13
Survival (%)	73.4 ± 21.3	74.2 ± 25.8	81.8 ± 18.8	66.3 ± 34.0
Pre-hatching				
N	5	7	14	12
Survival (%)	72.9 ± 21.7	8 ± 18.6	71.7 ± 23.3	21.4 ± 30.5

Objective 7. To optimize 8-hydroxy-deoxyguanosine assay of sea lamprey sperm and ova and measure its concentration in urine.

This protocol was based on Huang et al. (2001).

DNA extraction

Milt from sea lamprey was collected by gentle stripping and placed on ice. Sperm motility of each individual was assessed and sperm concentration estimated microscopically using a Double Neubauer Counting Chamber. The milt was centrifuged and the resulting supernatant (seminal plasma) was measured and removed leaving behind a sperm pellet. The sperm pellets were stored in eppindorf tubes at -70°C until the initiation of DNA extraction. All buffers and reagents used for DNA extraction, DNA digestion, and HPLC / EC analysis were prepared with C18 triple filtered HPLC water and filtered through a 0.2 μm filter.

Sperm pellets were removed from storage, placed on ice, and diluted with 1 ml of room temperature phosphate buffered solution (PBS, 10 mM) to restore previously measured sperm concentrations. The tubes were gently tapped and aspirated with a pipet to facilitate dispersal of all cells. Persistent cell clumps were carefully disrupted with a hand-held, battery-powered mini-homogenizer fitted for 1.5 ml Eppendorf tubes. A volume of sperm + PBS corresponding to a total of 75 million cells was pipeted into a 15 ml tube. Each tube received 6 ml of Cell Lysis Solution (CLS) (Puregene,Gentra Systems, Minneapolis, MN) and 36 ul of diethylenetriamine pentaacetic acid (DTPA). DTPA prevents artificial DNA oxidation by chelating iron that may be released from red blood cells (RBC) or cytoplasm. The contents of each tube were mixed by pipeting up and down. Next, 240 ul of 1M dithiothreitol (DTT) (Sigma, St. Louis, MO) solution and 30 ul of Proteinase K (20 mg/ml)(Puregene,Gentra Systems, Minneapolis, MN) were added

to each sample. The tubes were inverted 25 times and incubated at 55°C for 1 hour in a water bath. Tubes were inverted 25 times after 30 minutes of lysis.

Samples were removed from the water bath, inverted 25 times, and 30 ul of Rnase-A Solution (Puregene,Gentra Systems, Minneapolis, MN) was added to each tube. The samples were mixed by inverting 25 times and incubated at 37°C in a water bath for 30 minutes. After cooling the samples to room temperature, 2 ml of Protein Precipitation Solution (Puregene,Gentra Systems, Minneapolis, MN) was added to the RNAse-A treated cell lysate and vortexed vigorously at high speed for 20 seconds. Samples were cooled on ice for 5 minutes and centrifuged at 2000 x g for 10 minutes to precipitate all proteins. The supernatant containing suspended DNA was carefully poured off the protein pellet into a clean 15ml tube containing 100% isopropanol (HPLC grade, Fisher Scientific, Pennsylvania) and 6 ul of glycogen (Puregene, Gentra Systems, Minneapolis, MN). Glycogen increases DNA yields by acting as a carrier. The tubes were gently mixed on a rocker for 1 minute and centrifuged at 2000 x g for 3 minutes, precipitating the DNA into a small white pellet. The supernatant was poured off and the tubes were drained on a paper towel.

DNA was washed twice with 70% isopropanol, centrifuging after each washing. The isopropanol was poured off the pellet and the DNA was dissolved in HPLC grade water. The absorbance of the DNA was measured at 260 nm to determine the ug of DNA successfully isolated (Beckman 640 DU Spectrophotometer) and the 260 / 280 nm ratio determined.

DNA Digestion (performed at New York University School of Medicine, Department of Environmental Medicine, NY, NY, courtesy of Dr. Krystyna Frenkel's lab)

Preparation of standards:

Nucleosides (2'-deoxycytidine (dC), 2'-deoxyinosine (dI), 2'-deoxyguanosine (dG), thymidine (dT), and 2'-deoxyadenosine (dA)) ("cigta") (Sigma, St. Louis, MO) were weighed and added to triple filtered HPLC grade water at a concentration of 5 mM, except for dA, which was prepared to a concentration of 2.5 mM. The solutions were incubated at 37°C for 5 minutes and vortexed to ensure complete dissipation. A 1 mM concentration of total nucleosides was prepared by mixing 1 ml each of dC, dI, dG, dT and 2ml of dA. Aliquots of 200 ul of the mixture were pipeted into eppindorf tubes, dried under vacuum centrifugation (SpeedVac, Savant, Holbrook, NY) and stored at -70°C. The concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Sigma, St. Louis, MO) was measured by dissolving ~ 1 g in 1 ml of triple filtered HPLC water, measuring the UV absorbance at 248 nm and dividing by the extinction coefficient of 12,300. 8-OHdG was diluted to a final concentration of 1 uM and aliquoted into 100 ul portions, dried and stored at -70°C. Standards were prepared for HPLC analysis by adding 1 ml of HPLC water to the 8-OHdG tube and 200 ul of water to the "cigta" tube. The solutions were allowed to sit for 20 minutes prior to HPLC and then combined in equal amounts for a final concentration of 0.5 mM cigta and 0.05 uM 8-OHdG. During HPLC sampling, 5, 10, 20 and 40

ul of the standard (corresponding to 2.5-20 nmoles nucleosides and 0.25-2 pmoles 8-OHdG) were used to create a standard curve.

Deoxyribonucleic acid from salmon testes (ST DNA) (Sigma, St. Louis, MO) was hydrated in triple filtered HPLC water at a concentration of 5 ug/ul. Thirty ug (6ul) of ST DNA was aliquoted into one set of eppendorf tubes and 100 ug (20ul) of ST DNA was aliquoted into another set of tubes to serve as DNA standards. The portions were stored at -70°C until needed for use as digestion controls.

DNA Digestion and HPLC:

Isolated and hydrated DNA samples from sea lamprey were aliquoted in ~ 40 ug portions into eppendorf tubes for digestion. One tube containing 30 ug of ST DNA and one tube of 100 ug ST DNA were pulled from storage for digestion. HPLC water was added to the 100 ul mark on all sample tubes. Each tube was gently tapped to ensure even dispersal. The following solutions, previously prepared, were added to each tube: NaCl, 2 ul 5M; $MgCl_2$, 10 ul 100mM; Tris, 1 ul 1M (pH=7.4); DTPA, 2 ul 2mM; and Dnase I, 2 ul 20 units/ul (Sigma, St. Louis, MO). Each tube was gently tapped to create a homogenous mixture, quickly spun in a mini-centrifuge and incubated in a 37°C water bath for 30 minutes. Samples were acidified with 1 ul 3M (pH=5.2) acetate buffer and digested with 1 ul 1 unit/ul NP1 (Sigma, St. Louis, MO). After 1 hour of incubation, the PH was raised with Tris, 10 ul 1 M (pH=8.0) and 1 ul 1 unit/ul AP added for digestion. Following 30 minutes of incubation, 1 ul each of PDE I (0.1 unit/ul) and PDE II (0.01 unit/ul) were added to ensure complete digestion of DNA and incubated another 30 minutes.

Samples were transferred to filter tubes with a molecular weight cut-off of 5,000 (Sigma, St. Louis, MO) and spun at ~ 12,000 x g for 20 minutes. The filtered samples were transferred to HPLC sample collection tubes and 60 ul of DNA hydrolysates were analyzed by HPLC (Beckman Coulter Inc., Fullerton, CA) coupled with UV (Model 166) and EC (Coulochem II, ESA Inc., Chelmsford, MA) detectors, via an autosampler (Model 507e). The system utilized 2 pumps with 4-way solvent programming (Model 126). Data from the UV and EC detectors was collected by Gold Nouveau Software (Beckman Coulter Inc., version 1.6) installed on an IBM computer. The mobile phase incorporated 50 mM sodium acetate buffer (pH 5.2) in 6% aqueous methanol with a flow rate of 1 ml/min. Normal nucleosides (dC, dI, dG, dT, dA) were detected by UV absorption at 254 nm and the EC detector using analytical cell 1 at 400 mV and 20nA identified 8-OHdG. The column used on the HPLC system was a Rainin ODS C_{18} protected by an Ultrasphere ODS guard column. A standard curve was created for normal and oxidized nucleosides and was used to quantify the levels of each compound in the samples. The degree of DNA damage was expressed as 8-OHdG per 10^6 total normal nucleosides or as 8-OHdG per 10^6 dG.

Results

We successfully utilized a commercial kit for isolation of sperm DNA and prepared a digest of lamprey sperm for nucleoside chromatography (Fig. 24). More specifically, the degradation product of 8-hydroxy-deoxyguanosine was identified and measured. There were no significant changes in nucleoside concentrations between bisazir-treated and control lamprey sperm (Figure 25). When 8-OH-dG was expressed in terms of total dG or total nucleosides, it has shown numerically higher value for bisazir treated males (Figure 26). These results have to

be viewed in the context of the earlier analysis of bisazir concentrations in testes (Tables 2 and 3) and sperm viability (hatching rate, Table 4). For the purpose of the present analysis, one sample of the combined two fish was excluded as clear "outlier". However, further analysis is required to compare the level of 8-OH-dG with individual fertility. Results of 2003 indicated that bisazir concentrations varied in sperm by a factor of 8-10 suggesting that treatments (doses) were extremely variable among individual fish. Secondly, some bisazir treated males provided sperm with comparably high fertility. Therefore, we can conclude that despite the relatively high complexity of 8-OH-dG analysis, it may offer a very reliable measure of DNA degradation (oxidation) resulting in sperm infertility when physiological inferiority (motility, insemination capacity, i.e., percentage of 2-cell embryo) was not observed.

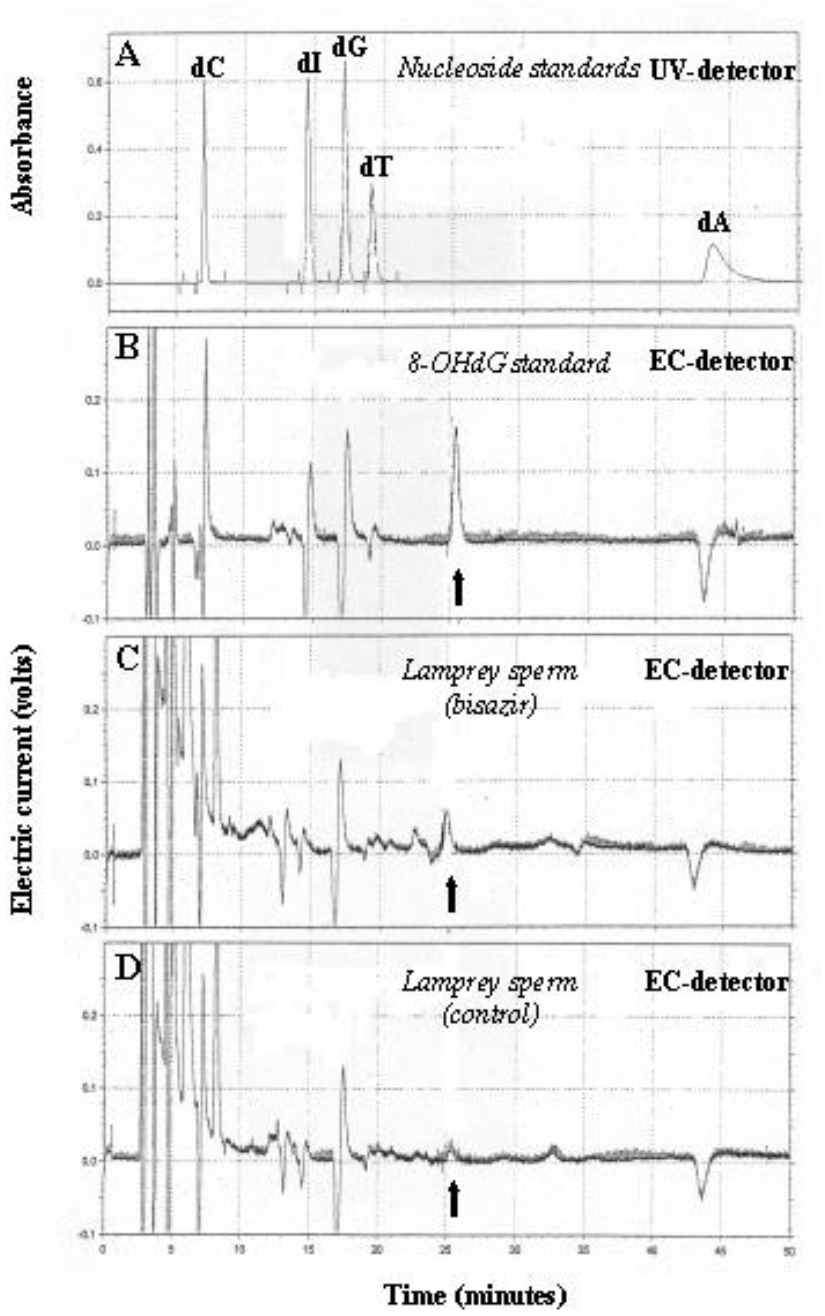

Figure 24. Representative HPLC-UV (A) and EC (electrochemical detector) (B-D) chromatograms showing peaks of total nucleotides in standard solution and 8-hydroxydeoxyguanosine (8-OH-dG) (arrows) in DNA digests from standard (salmon DNA) and lamprey sperm (control, C) and lamprey treated with bisazir.

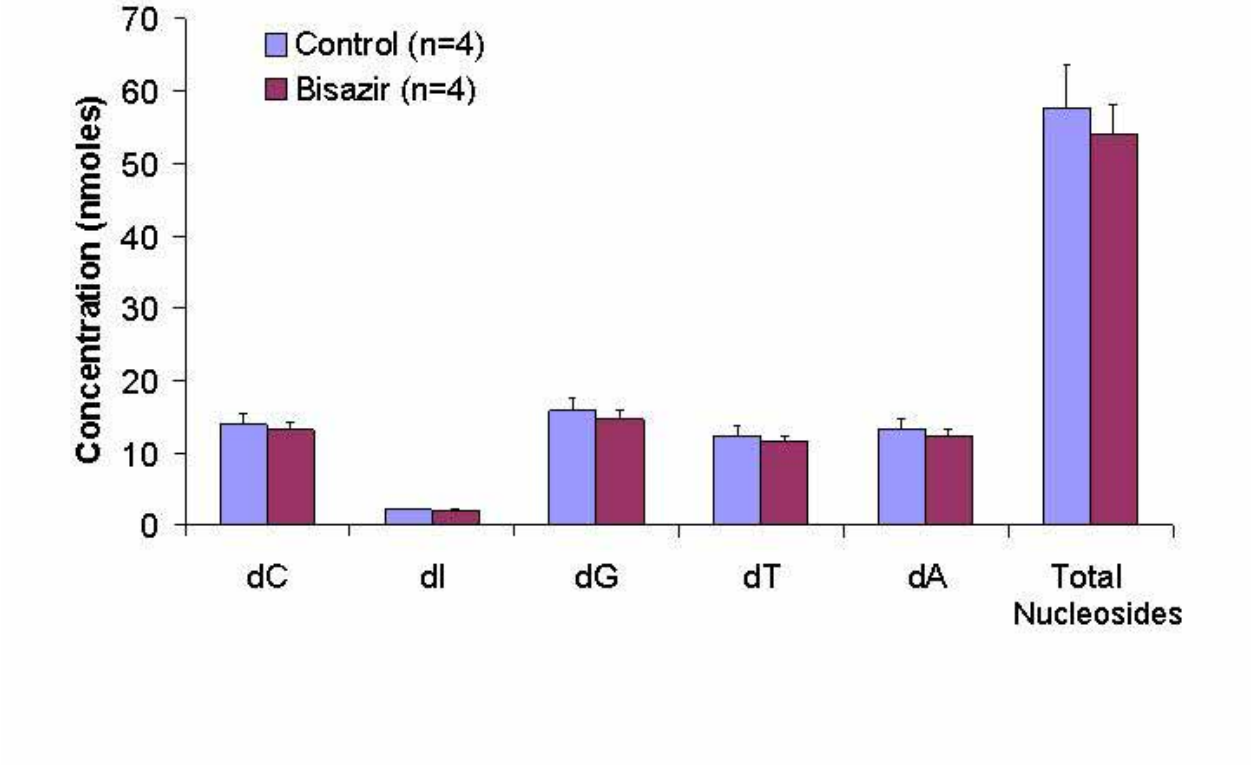

Figure 25. Concentration of individual nucleosides (dC, dI, dG, dT, and dA) and total nucleosides in control and bisazir-treated animals. See page 59 for explanations.

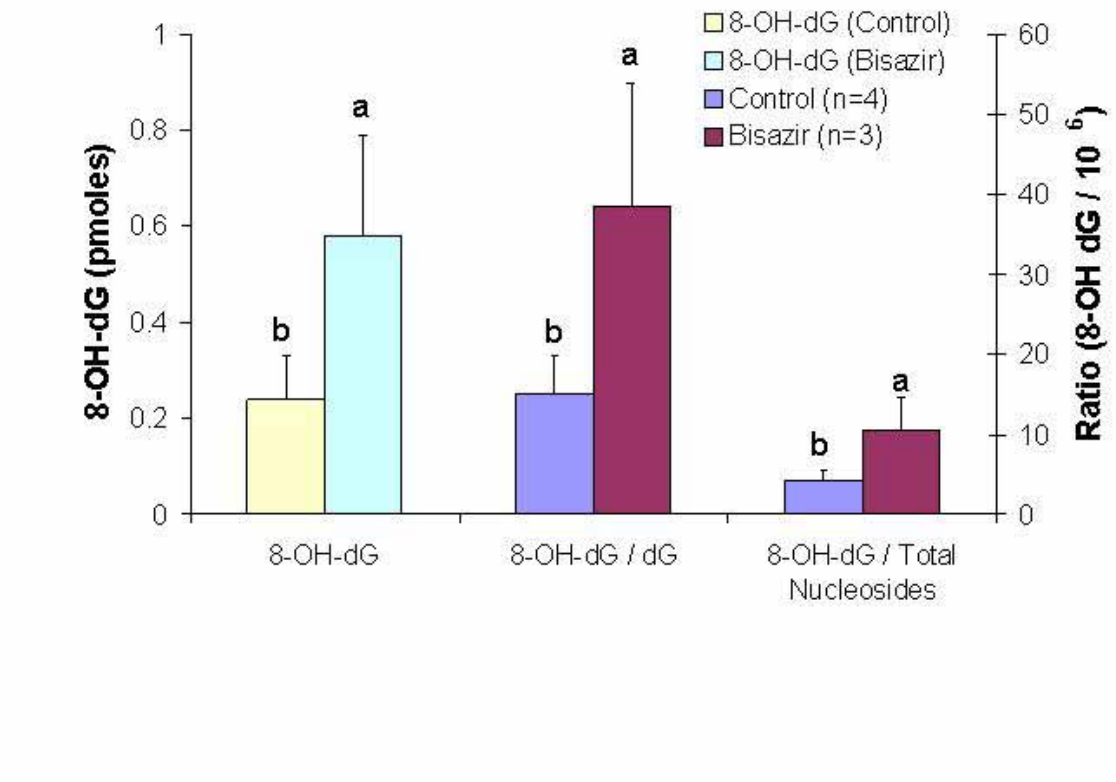

Figure 26. Levels of 8-OHdG in control and bisazir-treated males.

Deliverables/Major Findings/Conclusions

1. Neutral and alkali comet assays were evaluated for their effectiveness in determining DNA lesions in sperm cells and erythrocytes. Both assays revealed more DNA damage to cells obtained from bisazir treated animals than from controls (non-treated). Likewise, microscopic evaluation of bisazir spermatozoa revealed sub-standard concentrations and impaired motility. This data affirms the mutagenic action of bisazir to gonadal cells (spermatozoa) and to cells not directly involved in reproduction (erythrocytes). Further studies on the effects of bisazir on tissue cells from organs such as the liver, kidney, muscle and even heart may prove helpful in fully understanding the mode of action of bisazir. This could prove important in elucidating the effects of bisazir on the health, performance, and mortality of sea lamprey; all critical factors when evaluating the ability of treated animals to compete for spawning with non-treated males.

2. Maximal damage to sea lamprey spermatozoa was induced by exposing control spermatozoa to concentrations of H_2O_2 up to 1000 μM. This data served as a positive control with which to compare control and bisazir treated cells. DNA damage was evaluated by using an unbiased, computer-based scoring program. A dose-dependent increase in damage to DNA of sea lamprey sperm in relation to concentration range (0-1000 μM) was observed in all the major comet measurements, thus validating the usefulness and accuracy of the scoring program.

Spermatozoa from bisazir treated animals exhibited greater DNA damage when compared to controls. The damage displayed itself less dramatically as the maximal damage induced by H_2O_2 illustrated by its long tails. Bisazir damage presented slightly enlarged heads (over control) with small amounts of DNA spilling out into a tail. This, however, make comet assay a perfect validation assay for quality control purposes of bisazir treatments.

Storage of sperm on ice was tested to screen for the possibility of spurious DNA damage due to the effects of storage. An increase in DNA fragmentation was indeed detected in direct relation to storage time. It is therefore imperative that cells targeted for DNA studies are immediately prepared for evaluation following recovery or be stored at --70ºC until further studies can be conducted.

3. Comet assay proved further usefulness for detecting UV-induced damage to DNA. Dose-dependent effects of UV on DNA fragmentation were clearly illustrated by several major comet parameters. These changes parallel the dose-dependent decrease in sperm fertilizing ability as measured at pre-hatching stage.

Furthermore, KMnO4 was investigated *in vitro* as a possible DNA mutagen. KMnO4 affected damage to sea lamprey spermatozoa as detected by comet assay. The damage incurred by the cells more closely paralleled the conservative damage seen in bisazir treated cells. It would be interesting to further explore the fertilizing ability of spermatozoa following exposure to $KMnO_4$.

4. Bisazir concentrations in both liver and water samples (~10 hours after intraperitoneal injection of bisazir) of male lamprey were 26% and 10% higher, respectively, than those found in females. However, the average whole body weights of males exceeded females by only 1%. The females appear to be eliminating bisazir from the liver more rapidly than the males but are not excreting the chemical from their bodies as quickly.

5. In an attempt to synchronize ovulation and spermiation of sea lamprey, animals were injected intraperitonealy with luteinizing hormone releasing hormone a (LHRHa). LHRHa significantly accelerated the rate of maturation and onset of ovulation and spermiation of biszir treated animals. While we were not attempting to study the effects of LHRHa, it should be noted that in light of these results, further studies of the effects of bisazir on gonadal maturation of males and females should be conducted. Current sterilization protocols targeting injection of both males and females with bisazir may be assisting these animals in reaching reproductive maturity sooner than their control counterparts, thus reducing competition between treated and controls. This finding should be considered as a priority research goal in "lamprey control" program.

6. The assay for determination of 8-OHdG in tissues was successfully implemented in the laboratory following DNA isolation and digestion. Spermatozoan DNA from bisazir-treated lamprey contained higher ratios of 8-OHdG per dG (2'-deoxyguanosine) when compared to controls. This is indicative of DNA damage due to oxidative stress. Again, this procedure, although time consuming and more expensive than comet assay, can be used for quality control of bisazir treatment in lamprey sperm.

References

Coble, D.W., Druesewitz, R.E., Fratt, T.W. and Scheirer, J.W., 1990. Lake trout, sea lampreys, and overfishing in the UpperGreat Lakes: a review and reanalysis. *Trans. Am. Fish. Soc.* 119, 985-995.

Ciereszko, A., Glogowski, J., and Dabrowski, K. 2000. Fertilization in landlocked sea lamprey: storage of gametes, optimal sperm:egg ratio, and methods of assessing fertilization success. *J. Fish Biol.* 56, 495-505.

Ciereszko, A., Dabrowski, K., Christ, S.A., Toth, G.P., and Glogowski, J. 2002. Factors affecting motility characteristics and fertilizing ability of sea lamprey spermatozoa. *Trans. Am. Fish. Soc.* 131, 193-202.

DuBois, R.B. and Blust, W.H., 1994. Effects if lampricide treatments, relative to environmental conditions, on abundance and sizes of salmonids in a small stream. *N. Am. J. Fish. Mngt.* 14, 162-169.

Foksinski, M., Bialkowski, K., Skiba, M., Ponikowska, I., Szmurlo, W. and Olinski, R. 1999. Evaluation of 8-oxodeoxyguanosine, typical oxidative DNA damage, in lymphocytes of ozone-treated artriosclerotic patients. *Mutation Res.* 438: 23-27.

Green, M.H., Lowe, J.E., Delaney, C.A. and Green, I.C., 1996. Comet assay to detect nitric oxide-dependent damage in mammalian cells. *Meth. Enzymol.* 269, 243-266.

Huang, X., Powell, J., Mooney, L.A., Li, C., and Frenkel, K. 2001. Importance of complete DNA digestion in minimizing variability of 8-oxo-dG analyses. *Free Rad. Biol. Med.* 31:1341-1351.

Lee, K.J. and Dabrowski, K. 2002. Gossypol and gossypolone enantiomers in tissues of rainbow trout fed low and high levels of dietary cottonseed meal. *J. Agric.Food Chem.* 50: 3056-3061.

Michelmore, C.L. and Chipman, J.K. 1998. Detection of DNA strand breaks in brown trout (*Salmo trutta*) hepatocytes and blood cells using the single cell gel electrophoresis (comet) assay. *Aquat. Toxicol.* 41:161-182.

Saperas, N., Chiva, M., Pfeiffer, D.C., Kasinsky, H.E. and Ausio, J., 1997. Sperm nuclear basic proteins (SNBPs) of Agnathas and Chondrichthyans: variability and evolution of sperm proteins in fish. *J. Mol. Evol.* 44, 422-431.

Scholefield, R.J., Slaght, K.S., and Allen, J.L. 1997. Liquid chromatographic method for determining the concentration of bisazir in water. *J. AOAC Int.* 80, 1111-1116.