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197

# Effects of Seismic Air-Gun Sounds on Lobster (*Homarus americanus*): Pilot Laboratory Studies with (i) a Recorded Track from a Seismic Survey and (ii) Air-Gun Pulse Exposures over 5 Days

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# Effects of Seismic Air-Gun Sounds on Lobster (*Homarus americanus*): Pilot Laboratory Studies with (i) a Recorded Track from a Seismic Survey and (ii) Air-Gun Pulse Exposures over 5 Days

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## Preface

This report presents the findings of two complementary studies commissioned by the Environmental Studies Research Fund. The studies included gross pathology and selective histopathological and serum parameters. The first study which also examined effects on feeding, involved exposure of lobster for eight hours to a recorded sound track from an actual seismic survey. The second study investigated the potential for prolonged or delayed effects in lobster retained for six months after “chronic” exposure to twenty shots per day for five days from an airgun source.

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## Préface

Ce rapport présente les constatations de deux études complémentaires commandées par le Fonds pour l’étude de l’environnement. Les études portaient sur la pathologie clinique et des paramètres choisis d’histopathologie et de sérum. La première étude, qui portait également sur les effets sur l’alimentation, consistait à exposer des homards pendant huit heures à la trame sonore enregistrée d’un levé sismique réel. La seconde étude portait sur le potentiel d’effets prolongés ou retardés sur des homards gardés pendant six mois après une exposition « chronique » à vingt coups de canon à air par jour pendant cinq jours.

# Pilot Study (i) on Lobster Exposed in the Laboratory to a Recorded Sound Track from a Seismic Survey: Mortality, Gross Pathology, Histopathology, Serum Biochemistry and Feeding

## TABLE OF CONTENTS

# 1.0 ABSTRACT

Gross pathology and histopathology were assessed in lobster processed within a few hours after exposure for 8 hours in the laboratory to a recorded sound track from a seismic survey. No mortality was observed and there was no evidence for overt gross pathology on the carapace, appendages or internal organs. There was also no effect on feeding. With respect to histopathology, no effects were noted in tissues of the ovary, an organ to which attention is often drawn. Regarding histopathology of the hepatopancreas, the degree of "necrosis" and periodic acid Schiff (PAS) staining intensity, (a marker for glycogen), of the digestive tubules was similar between the control and exposed groups. However, a higher degree of epithelial vacuolation and tubular dilation was observed in the exposed group compared to the control group.

Some serum biochemical parameters, including protein, calcium, the enzymes aspartate aminotransferase (AST), creatine phosphokinase (CPK), and triglycerides were also examined 3 days post exposure in a separate experiment. No statistical differences ($p<0.05$) were observed but there was a trend for decreased levels of protein, and triglyceride in the exposed animals.

Histopathology is commonly recognized as a higher order effect and could be a useful endpoint to assess for occurrence in association with seismic surveys in lobster (and possibly snow crab) habitat. It is noted in this regard that unlike haemolymph collection and processing, tissues can be fixed in the field without effort.

# 1.0 ABRÉGÉ

On a évalué la pathologie clinique et l'histopathologie de homards traités quelques heures après avoir été exposés pendant 8 heures en laboratoire à la trame sonore enregistrée d'un levé sismique. On n'a observé aucun décès et aucun indice de pathologie clinique manifeste sur la carapace, les appendices ou les organes internes. On n'a constaté aucun effet sur l'alimentation non plus. En ce qui concerne l'histopathologie, on n'a constaté aucun effet dans les tissus de l'ovaire, un organe sur lequel on attire souvent l'attention. En ce qui concerne l'histopathologie de l'hépatopancréas, le degré de « nécrose » et d'intensité de la réaction à l'acide périodique Schiff (PAS) (un indicateur de glycogène) des tubules digestifs étaient semblables chez les groupes témoin et exposé. On a toutefois constaté un plus grand degré de vacuolisation épithéliale et de dilatation tubulaire chez le groupe exposé que chez le groupe témoin.

Certains paramètres biochimiques du sérum, y compris les protéines, le calcium, le sérum glutamo-oxalacétique transaminase (SGOT), la créatine phosphokinase (CPK) et les triglycérides ont également été examinés 3 jours après l'exposition dans le cadre d'une expérience distincte. Aucune différence statistique ($p<0,05$) n'a été constatée, mais on a observé une tendance à la baisse des niveaux de protéines et de triglycérides chez les animaux exposés.

L'histopathologie est généralement reconnue comme un effet secondaire et pourrait être un effet utile à évaluer du point de vue de son occurrence en association avec les levés sismiques dans les habitats de homard (et possiblement de crabe des neiges). On remarque à cet égard que contrairement à la cueillette et au traitement d'hémolymphes, les tissus peuvent être réparés sur le terrain sans effort.

# 2.0 MATERIALS AND METHODS

## 2.1 Experimental Conditions

Female lobsters were held at DFO in aerated 2000L aquaria supplied with flow-through seawater at ambient temperature.

The histopathology experiment was carried out on 24 lobsters, as follows:

- Twelve animals were placed in an aquarium (inside dimensions: 3.63m length x 2.39m width x 1.27m depth) in a crab pot and exposed for 8 hours via an underwater speaker to a recorded sound track from a seismic survey. The speaker was set-up ~ 1m in front of the pot. Sound was recorded by one hydrophone placed ~ 0.5m from the back of the pot. Lobsters were autopsied a few hours after exposure.

- Twelve control animals were handled in the same manner as the experimentals except for the sound exposures.

The experiment to investigate for change in various haematological parameters (serum biochemistry) was carried out on 24 other lobsters under the same conditions described above. However, observations made on lobsters exposed to air-gun pressure previously (Payne et al, 2007) suggested that the appearance of parameters in the haemolymph could be delayed. Therefore, haemolymph was sampled 3 days post-exposure. Three days was an arbitrary period.

## 2.2 Autopsy and Tissue Sample Collection

Lobsters were weighed and killed by severing the “nerve chord” behind the eyes. Each lobster was assessed visually for any external abnormalities. The softness of the shell (carapace) was rated on a 1 to 5 scale. The carapace was then removed to access the dorsal part of the internal organs. Any abnormalities observed on internal organs including hepatopancreas, heart and gonads were to be recorded.

A portion of hepatopancreas and gonad from the right dorsal posterior region was placed in Gendre’s fixative while another portion was placed in Davidson's fixative, for histopathological analysis.

A sample of haemolymph (blood equivalent) was withdrawn using a 10ml syringe (18G 1/2 needle) from underside the tail (section closest to body), placed in a 15ml centrifuge tube, centrifuged at 2,000 rpm for 10 min (4ºC). The supernatant was then transferred to 2ml tubes and frozen at -60ºC.

## 2.3 Tissue Histopathology

Hepatopancreas and gonad samples fixed in Gendre's and Davidson's fixatives were processed for histological analysis (Lynch et al., 1969) using a Tissue-Tek® VIP Processor. A graded ethyl alcohol series of 70%, 80%, 95%, and two changes of 100%, was used for dehydration of the samples. The samples were then cleared in three changes of xylene. Finally, the tissues were impregnated with three changes of molten embedding media, Tissue Prep 2 ™. The processed tissues were embedded in steel molds using molten embedding media, and topped with labelled embedding rings. After cooling, the hardened blocks of embedded tissues were removed from their base molds. The blocks were then trimmed of excess wax. Two to 4 sections per sample were cut at 6$\mu$ on a Leitz microtome, floated on a $47^0$C water bath containing gelatine, and then

picked up on labelled microscope slides. After air drying, the slides were fixed at $60^0$C for approximately 2 hours to remove most of the embedding media and allow the sections to adhere properly to the slide. Hepatopancreas and gonad sections were stained with Mayers Haematoxylin and Eosin (H&E) method (Luna, 1968). Hepatopancreas sections were also stained with Periodic Acid-Schiff (PAS). Coverslips were applied using Permount Mounting Media, and the slides were left to air dry and harden overnight.

One slide with 3-4 sections was examined per lobster under different magnifications by transmission light microscopy (Wild Leitz Aristoplan bright field microscope).

### 2.3.1 Hepatopancreas Histopathology

Sections stained with H&E were assessed microscopically for the presence of any overt differences in general structure or staining characteristics of tissues between control and exposed animals. Special attention was given to the presence in the digestive tubules (tubular structures where digestion occurs) of 1) vacuolation, 2) “necrosis” and 3) dilation. The degree of various conditions was recorded on a 1-3 relative scale (1- no or slight; 2- moderate; 3- high). Any other observation was recorded.

Sections stained with PAS were examined for the presence of PAS positive material in the digestive tubules. The degree of PAS staining was recorded on a 1-3 relative scale.

### 2.3.2 Ovary Histopathology

Sections stained with H&E were assessed for the presence of any overt differences in general structure or staining characteristics between control and exposed animals. Special attention was given to the presence of haemocytes (blood cell equivalents) and/or the occurrence of haemocyte infiltration among the oocytes. The degree of the condition was recorded on a 1-3 relative scale (1- no or a few haematocytes; 2- small number of haemocyte clusters (up to 5); 3- larger clusters or more than 5 small clusters.

## 2.4 Haemolymph

### 2.4.1 Protein Measurement

Protein concentration was determined in serum using the Pierce™ BCA Protein Assay Kit for the colorimetric detection and quantitation of total protein, following the manufacturer’s instructions. Hemolymph samples were diluted 40 times with ultrapure water. Bovine serum albumin is commonly used as a standard in measuring protein and was also used here.

Protein concentration in haemolymph has been recognized as an important health indicator for crustaceans (Mercaldo-Allen 1991; Paterson et al. 1999; Huang & Chen 2001; Perazzolo 2002). Generally, a decrease in total protein has been associated with disease (Floreto et al., 2000), but also has been linked to increased temperature (Lorenzon et al., 2007), low clotting times (Dove *et al.*, 2005) and acidosis (Fotedar *et al.*, 2006).

### 2.4.2 Calcium Measurement

Calcium concentration was determined in serum using the Calcium Arsenazo Reagent from Jas Diagnostics, Inc, following the manufacturer’s instructions. Serum samples were diluted 9 times with ultrapure water. Jas Chemistry Calibrator (Jas Diagnostics Inc.) was used calculate the level of calcium in serum.

Calcium levels in the serum of crustaceans are tightly regulated (Dove et al., 2005). However, changes in serum calcium can provide information on moulting status or could indicate disturbance to osmoregulation. Additionally, calcium changes in crustaceans have been shown to be involved in compensation in dealing with hypoxia (McMahon, 2001), as well as temperature and handling stress (Lorenzon et al., 2007).

### 2.4.3 AST Measurement

AST (aspartate aminotransferase) was measured in serum using the SGOT/AST Reagent Set from Sterling Diagnostics Inc., following the manufacturer's instruction with a slight modification. The recommended sample volume of 100 µl was increased to 500 µl to bring the AST levels that are present in lobster serum within the working range of the kit. An AST calibrator provided with the reagent set was diluted to 500 µl using normal saline (Jas Diagnostics Inc.), and was used to calculate the level of AST.

In vertebrate systems AST is used to indicate hepatocellular damage. A reduction in AST in American lobster has been observed during temperature stress (Dove et al., 2005) and following exposure to a seismic airgun (Payne et al., 2007).

### 2.4.4 CPK Measurement

CPK (creatine phosphokinase) was measured in serum using the Creatine Phosphokinase Colorimetric Assay Kit from Sterling Diagnostics, Inc., following the manufacturer's instructions with a slight modification. The recommended sample volume of 20 µl was increased to 100 µl to bring the CPK levels that are present in lobster serum within the working range of the kit. A CPK calibrator provided with the assay kit was diluted to 100 µl using normal saline (Jas Diagnostics Inc.), and was used to calculate the level of CPK in serum.

Although information is lacking for crustaceans, CPK is commonly found at relatively high levels in muscle tissue of vertebrates (Suber, 1994). The enzyme has been specifically associated with muscle and heart inflammation in salmon (Yousaf and Powell, 2012).

### 2.4.5 Triglyceride Measurement

Triglycerides were measured in serum using the Triglyceride (GPO) Colorimetric Assay Kit from Sterling Diagnostics, Inc., following the manufacturer's instructions with a slight modification. The recommended sample volume of 5 µl was increased to 50 µl to bring TGO levels that are present in lobster serum within the working range of the kit. A TGO calibrator provided with the kit was diluted to 50 µl using normal saline (Jas Diagnostics Inc.), and was used to calculate the level of triglycerides in serum.

A depression in triglycerides has been observed during temperature and handling stress in American lobster (Lorenzon et al., 2007).

## 2.5 Statistical Analysis

Comparisons between control and exposed groups were conducted using Sigma-Stat 3.5:

- Animal weight, carapace length and condition scoring were analysed by the Unpaired t-test or Mann-Whitney Rank Sum test, when the normality test failed.
- Prevalence of various conditions was analysed by the Fisher exact test.

- Feeding was analysed by the Students T test.

In line with common practice, comparisons having a $P < 0.05$ were considered to be statistically significant (with $P < 0.1$ accepted as marginal significance).

## 2.6 Feeding Trials

Animals were fed a weighed amount of shrimp, with the amount remaining after 24 hrs being reweighed. Consumption calculations were carried out in relation to g/Kg lobster.

# 3.0 RESULTS AND DISCUSSION

## 3.1 Received sound levels

The speaker was placed ~ 1m in front of the pot and sound pressure levels were recorded with an hydrophone placed ~ 0.5m back of the pot. Peak-to-peak (P-P), peak (P) and root-mean-squared (RMS) values were approximately 180, 174 and 171 respectively. Peak-to-peak, peak and RMS pressure levels are commonly modelled for a given seismic array and included in environmental assessment statements for many seismic programs. Although little or no empirical information is available from the environment, such modelling can provide a rough approximation of sound penetration at varying distances in the water column (eg. Lawson, 2009). Likewise sound measurements in large tank systems can provide a rough guide of exposure levels.

## 3.2 Necropsy Data and Gross Pathology

Lobsters were weighed and examined visually for gross pathology. There were no abnormalities on internal organs including the gonads, hepatopancreas and heart in any lobsters. The carapace softness was ranked on a 1 (soft) to 5 (hard) scale and missing appendages were recorded. Results are summarised in Tables 1 and 2.

**Table 1 Morphometrics and Prevalence of Missing Appendages**

| | Number of lobsters | Weight [a] (g) | Carapace [a] length (cm) | Missing [b] Appendages |
|---|---|---|---|---|
| **Control** | 12 | 659 ± 99 | 94.1 ± 4.5 | 8.3% |
| **Exposed** | 12 | 655 ± 103 | 93.9 ± 3.3 | 25.0% |
| **p-Value [c]** | | 0.926 | 0.904 | 0.590 |

[a] Mean ± standard deviation

[b] Prevalence expressed as percentage of fish having missing appendages

[c] P-Value obtained after Unpaired t-test on mean of parameters and Fisher Exact test on the prevalence of missing appendages

There were no significant differences in body weight and carapace length (t-test; p = 0.926 and 0.904, respectively) between control and exposed groups (Table 1). The prevalence of lobsters with missing appendages (Fisher's exact test; p = 0.590) was similar between the 2 groups. There was no loss of appendages (namely legs) upon exposure. Previous studies with snow crab (Christian et al., 2003 and 2004; Courtenay et al., 2009) and lobsters (Payne et al., 2007; Oceans Ltd., 2010) also found no difference in loss of legs or other appendages between control and groups exposed to airgun discharges.

There were also no significant differences in carapace softness (Table 2; Fisher Exact test), indicating no apparent differences in moulting status between the 2 groups.

**Table 2 Prevalence of Softness Rank (1-soft to 5-hard)**

| Variable | Rank 1 | Rank 2 | Rank 3 | Rank 4 | Rank 5 |
|---|---|---|---|---|---|
| **Exposed** | 16.70% | 16.70% | 8.30% | 16.70% | 41.70% |
| **Control** | 16.70% | 25.00% | 0% | 16.70% | 41.70% |
| **p Value [a]** | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

[a] p Value obtained after Fisher Exact test

## 3.3 Histopathology

Histopathology forms the basis for diagnosing various health conditions in human and veterinary medicine and is increasingly being used to assess the health of aquatic animals (e.g Hinton et al., 1992; Myers and Fournie, 2002; Payne et al., 2003; Mathieu et al., 2011). Histopathological studies were carried out on the hepatopancreas and ovary.

In the present study, hepatopancreas and gonads were placed in 2 different fixatives (Gendre's and Davidson's), processed, cut and stained by standard techniques. In order to identify the optimal fixative, a general examination of tissues from both fixatives was carried out and the best results were obtained with Gendre's fixative. Detailed histopathological analysis was thus carried out on tissues fixed in Gendre's fluid.

### 3.3.1 Hepatopancreas Histopathology

The hepatopancreas of lobster consists of a multi-branched tubular structure embedded in an extensive network of loose connective tissue. The connective tissue is composed of blood vessels (hepatic arterioles), hemal sinuses and muscular elements. Each digestive tubule is composed of an epithelium, showing 3 principal types of cells (B-, F- and R-cells), surrounding a central lumen. B-cells are the largest cells characterised by a large vacuole. R-cells are the most abundant cells characterised by a columnar shape with nuclei in the basal region of the cell. F-cells are less numerous and have a filamentous appearance (Factor, 1995).

In the present study, based on the histological observations of the sections stained with H&E, the general structure of hepatopancreas in all samples corresponded with the description provided above. However, a number of observations were noted, as follows:

**1 - the presence of eosinophilic staining material** was observed between tubules (Figure 1) in a large number of samples from both control and exposed groups.

**Figure 1. Eosinophilic Staining Material between Tubules (H&E x63)**

**2 - a wide variation in the tubular morphology** was found within and between samples in relation to the degree of epithelial vacuolation (Figure 2), tubular dilation (Figure 3) and epithelial "necrosis" (Figure 4).

**Figure 2 Various degree of Epithelial Vacuolation (H&E x25)**

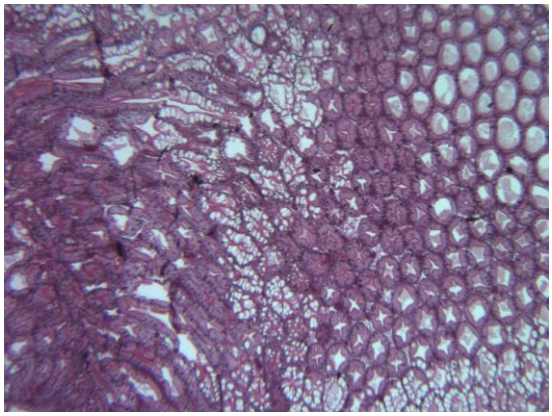


**Figure 3 Focus of Dilated Tubules (H&E x63)**

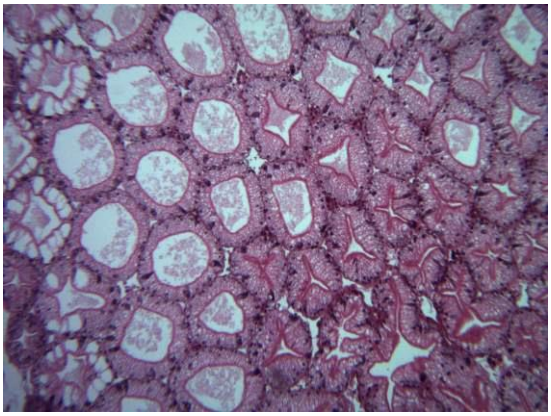
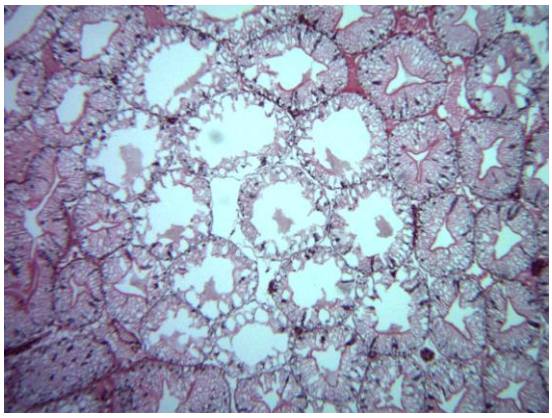


**Figure 4 Focal Necrosis of Tubular Epithelium (H&E x63)**



Due to the wide variation, a scoring system was applied for the degree of various conditions with ranking on a relative scale of 1 to 3. Results, expressed as degree means, for control and exposed groups are summarised in Table 3.

**Table 3 Degree Means of Various Conditions of Hepatopancreas Digestive Tubules in the Control and Exposed Groups**

| | Vacuolation | Dilation | Necrosis |
|---|---|---|---|
| **Control** | 1.167 ± 0.389 | 1.000 ± 0.000 | 1.333 ± 0.492 |
| **Exposed** | 2.000 ± 0.953 | 1.333 ± 0.651 | 1.667 ± 0.651 |
| **P-value** [a] | **0.021** | 0.079 | 0.199 |

Mean ± standard deviations
Scoring on a relative scale of 1 to 3, with 1 being the least and 3 being the most
[a] P-value obtained after Mann-Whitney Rank Sum test – significance in bold.

There was no significant difference in the degree of "necrosis" of hepatopancreatic tubules between the Control and Exposed groups (p=0.199; Mann-Whitney Rank Sum test) (Table 3). However, a significant difference was observed for the degree of vacuolation (p=0.021; Mann-Whitney Rank Sum test) while the degree of dilation of the tubules was also marginally significant (p=0.079; Mann-Whitney Rank Sum test), with higher ranking being obtained for the exposed animals.

The degree of vacuolation and dilation of hepatopancreatic tubules in crustaceans is highly dependent on the stage of reproductive cycle, as well as molting and nutrition status (Al-Mohanna and Not, 1989; Sousa and petriella, 2001), but can also vary with physical and environmental factors such as salinity or pollution (Icely and Nott, 1992; Johnston et al., 1998; Masson, 2001; Cuartas et al., 2003) (in Diaz et al. 2010). In this context, given the apparent similarity of molting status of animals in the present study (see softness ranking in Section 3.2 above) and assumptions of similar reproductive and feeding status (animals were from the same batch and held in the same tank until exposure), it is of interest that the group of lobsters exposed to the sound track exhibited a higher degree of vacuolation and dilation.

When results were expressed as prevalence of scoring (Table 4), significant differences were observed for rating 3 of vacuolation. No cases were noted in control animals, whereas 41% of exposed animals exhibited a high vacuolation.

**Table 4 Prevalence of Scoring of Various Conditions of Hepatopancreas Digestive Tubules in the Control and Exposed Groups**

| | | Rating 1 | Rating 2 | Rating 3 |
|---|---|---|---|---|
| **Vacuolation** | **Control** | 83.3 | 16.7 | 0.0 |
| | **Exposed** | 41.7 | 16.7 | 41.7 |
| | ***P-value*[a]** | *0.089* | *1.000* | ***0.037*** |
| **Dilation** | **Control** | 41.7 | 58.3 | 0.0 |
| | **Exposed** | 8.3 | 66.7 | 25.0 |
| | ***P-value*[a]** | *0.155* | *1.000* | *0.217* |
| **Necrosis** | **Control** | 66.7 | 33.3 | 0.0 |
| | **Exposed** | 41.7 | 50.0 | 8.3 |
| | ***P-value*[a]** | *0.414* | *0.680* | *1.000* |

Prevalence expressed as percentage of animal exhibiting the rating of condition
[a] P-value obtained after Fisher Exact test

**3 - vascular congestion** (engorgement of blood cells or haemocytes) was also observed in intertubular spaces in 2 samples from the exposed group (Figures 5 and 6).

**Figure 5 Vascular Congestion between Tubules (lower magnification, H&E x63)**

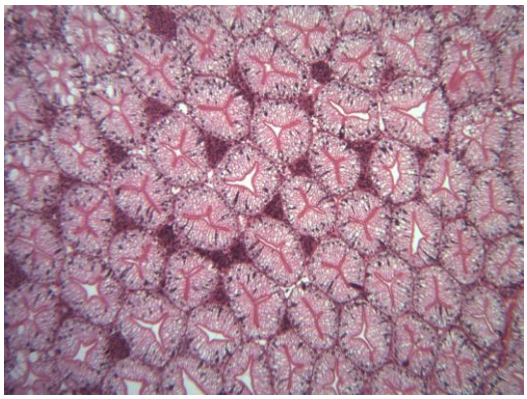
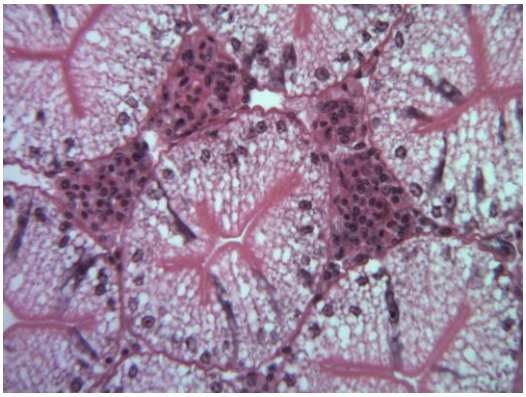


**Figure 6 Vascular Congestion between Tubules (higher magnification, H&E x250)**



Regarding sections stained with PAS for carbohydrate deposits (e.g. glycogen), since staining intensity varied somewhat from one sample to another in both control and exposed groups, sections were ranked for red staining intensity on a relative scale from 1 to 3, with 1 being the least (Figure 7) and 3 being the most (Figure 8).

**Figure 7. Hepatopancreatic Tubules Ranked 1 (PAS x63)**

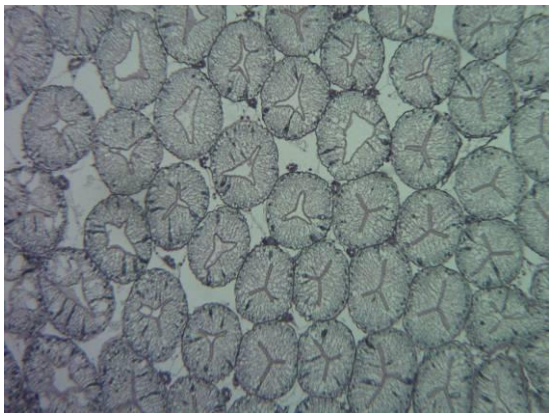
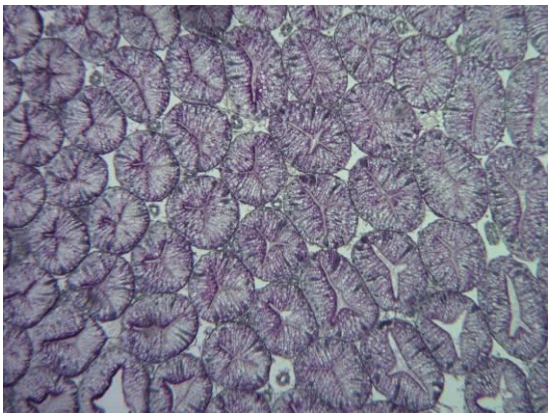


**Figure 8. Hepatopancreatic Tubules Ranked 3 (PAS x63)**



Results were expressed as mean rating (Table 5) as well as prevalence (Table 6) of PAS intensity.

**Table 5 Mean Rating of PAS Intensity in Hepatopancreatic Sections of Control and Exposed Lobsters**

| | Rating |
|---|---|
| **Control** | 1.75 ± 0.75 |
| **Exposed** | 1.83 ± 0.94 |
| **P-value** [a] | 0.926 |

Mean ± standard deviations of rating on a relative scale of 1 to 3, with 1 being the least and 3 being the most

[a] P-value obtained after Mann-Whitney Rank Sum test

**Table 6 Prevalence of PAS Intensity in Hepatopancreatic Sections of Control and Exposed Lobsters**

| | | Rank 1 | Rank 2 | Rank 3 |
|---|---|---|---|---|
| **Control** | Number | 5 | 5 | 2 |
| **N = 12** | % | 41.7 | 41.7 | 16.6 |
| **Exposed** | Number | 6 | 2 | 4 |
| **N = 12** | % | 50.0 | 16.7 | 33.3 |
| **P-Value*** | | 1.000 | 0.371 | 0.640 |

* P-value obtained after Fisher Exact test

There were no significant differences in PAS intensity between control and exposed groups, either for the mean rating (Mann-Whitney Rank Sum test) or the prevalence (Fisher exact test) of the condition. Similar PAS staining characteristics were also noted in the hepatopancreas of lobster examined several months post exposure to airgun discharges (Oceans Ltd., 2010). Payne et al. (2007;2008) reported an increase in staining in lobster but this was upon exposure to a large number of air gun shots (30) at relatively high sound pressure levels.

### 3.3.2 Gonad Histopathology

An effect on reproduction is one of the main concerns when evaluating potential impacts on animal populations. An important reproductive effect could be structural damages to gonads. In the present study, gonadal sections were investigated for infiltration of large numbers of haemocytes, which could be indicative of “hemorrhage” or tissue necrosis whereby haemocytes are involved in the “removal” of effete (damaged or degraded) material.

The general structure of ovaries appeared similar in all samples from both groups. There was no evidence of overt tissue "necrosis" or oedema between the control and exposed groups. A few samples from both groups had one or two very small pockets of haemocytes (Figure 9).

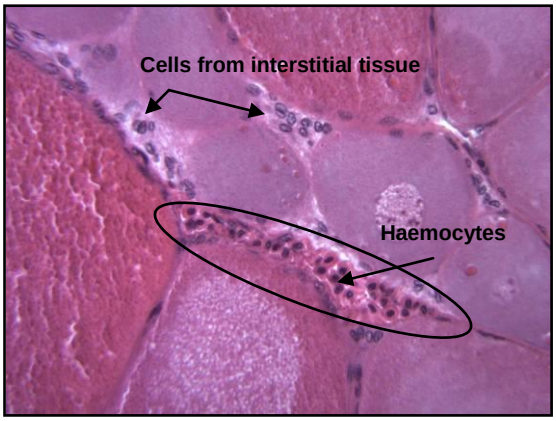



**Figure 9. Gonad Section with a small pocket of Haemocytes (H&E x63)**

Overall, there was no evidence for overt histological differences in the ovaries of lobsters exposed to the sound track in comparison with controls. Likewise, there was no evidence for any major infiltration of haemocytes in the ovary of exposed lobsters versus controls. Similar results were reported with snow crabs exposed to seismic energy in the field (Boudreau et al., 2009) or in lobster examined for delayed effects a number of months after airgun exposure in the laboratory (Oceans Ltd., 2010).

### 3.4 Haemolymph

Serum biochemistry was studied on 12 control and 12 experimental animals 3 days post exposure. The parameters analysed and their respective p values are as follows: protein (0.13); calcium (0.88); AST (0.22); CPK (0.49) and triglyceride (0.07). There was no statistical difference between the groups but there was a trend towards decreased levels of protein, and triglyceride in the exposed animals.

### 3.5 Feeding

Some feeding trials were carried out under "winter" temperature conditions but feeding was practically nil, precluding analysis. Further studies were carried out under "summer" temperature conditions and no difference was found between control and exposed animals in two separate trials (Table 7). An earlier study noted effects on feeding in lobster but this was upon exposure to a relatively high sound level (Payne et al., 2007).

**Table 7 Feeding Trials**

| Trial | Number | Food Consumption | Pvalue (T-test) |
|---|---|---|---|
| Trial #1 | 16C 16E | g/lobster | 0.397244 |
| Trial #1 | | g/kg lobster | 0.481515 |
| Trial #2 | 18C 18E | g/lobster | 0.874044 |
| Trial #2 | | g/kg lobster | 0.867342 |

# 4.0 COMMENT ON USE OF BIOMARKERS

In addition to mortality, gross pathology and feeding, selected serum and histopathological biomarkers were also studied. In a nut-shell, biomarkers encompass sub-lethal effects of a biochemical, physiological and histopathological nature which can be considered to be intermediates between a source of stress and higher order effects or "clinical" effects in human medicine. (Payne et al., 1987; Adams, 1990; Hinton et al., 1992; Peakall, 1992)

Following recommendations by ICES (eg. ICES, 1999; ICES, 2004) and other agencies (as well as the broader scientific community), biomarkers are presently being used extensively in ecotoxicology as early warning indicators of chemical stress. In the case of EEM, they can provide important information on the geographical reach of potential effects, such that if effects are found distant from a pollution source, greater management concern could ensue (eg. Mathieu et al., 2011).

It is important to note that "all" biomarkers are not of equal significance. And although prescriptive statements cannot be made for individual biomarkers (understanding that change in a suite of biomarkers could be more important), tissue change or histopathology is often referred to as the "gold standard" for effects recognized to be adverse or potentially adverse. This study noted change in digestive tubules of lobster exposed to a seismic soundtrack. Different sound exposures and response relationships have not been studied to date in any species of fish or shellfish. However, this study indicates that histopathology of the hepatopancreas could be a practical indicator to assess in lobster in the field in association with seismic surveys. Should any effect be found not only within the immediate area of a sound source but also distant from a seismic array, a case could be made for concern on the health of lobster in the area.

## 5.0 SUMMARY OBSERVATIONS

| Parameter | Observation |
|---|---|
| Mortality | No mortality |
| Loss of appendages (legs) | No loss |
| Other gross pathology (internal and external) | No sign |
| Haemolymph (blood equivalent) | No statistically significant differences for the enzymes AST and CPK; a trend toward slight differences for protein (P=0.13) and triglyceride (P= 0.07) |
| Feeding | No difference in two feeding trials. Grams of food consumed per lobster (P=0.39 and P=0.87). Grams of food consumed per/Kg lobster (P=0.48 and P=0.86). |
| Ovary Histopathology | No difference |
| Glycogen accumulation in Hepatopancreas; (PAS) staining | No difference; PAS staining intensity (P=0.92) |
| Hepatopancreas Histopathology | Higher degree of tubular vacuolation (P=0.02) and tubular dilation (marginal significance, P=0.07)in the exposed group. The highest degree of vacuolation more prevalent in the exposed group (p 0.03)<br>Histopathology is an higher order effect and could be a useful endpoint for environmental monitoring as well as key endpoint to further assess in dose-response and recovery studies. Post exposure recovery studies would be valuable for determining if differences in histopathology are transient or not. |

## 6.0 ACKNOWLEDGMENTS

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# Pilot Study (ii) for Prolonged or Delayed Effects in Lobster Exposed in the Laboratory to Seismic Airgun Pulses over 5 days: Mortality, Gross Pathology, Histopathology and Serum Biochemistry

## TABLE OF CONTENTS

# 1.0 ABSTRACT

The question of potential for prolonged or delayed effects often arises in relation to risks that may be associated with seismic surveys. This is a major knowledge gap albeit a difficult question to address, given the requirement for a series of dose-response and post exposure studies.

General pathology, serum biochemistry and select histopathological studies were carried out on lobsters maintained for approximately 6 months in the laboratory after being exposed during 5 successive days to 20 shots per day of an airgun at relatively high sound pressure levels.

No differences in mortality were noted between the control and experimental groups. Also, no inter-group differences were noted with respect to general pathology, serum concentration of protein, glucose and triglycerides, or overt histological changes in the hepatopancreas and ovary. A slight difference in concentration of serum calcium was observed when standardized to serum protein. However this difference may be due to the slightly higher (but statistically insignificant) level of serum protein in the exposed animals. Although of no apparent “internal” affect, a higher level of shell disease was noted in the control group.

Overall, this pilot study suggests that any changes that may have occurred in selected histological and serum parameters upon airgun exposure were absent several months later, indicating animal recovery.

# 2.0 MATERIALS AND METHODS

## 2.1 Experimental Conditions

Female lobsters were held at DFO in aerated 2000L aquaria supplied with flow-through seawater at ambient temperature. They were acclimated in the laboratory for approximately 4 weeks before commencing the experiment.

Thirty lobsters were exposed during 5 successive days to 20 shots per day from a 10 $in^3$ sleeve air gun with pressure set from 500 to 100 psi as follows: 500 psi on first day, 400 psi on second day, 300 psi on third day, 200 psi on fourth day and 100 psi on fifth day. This approach was adopted because a series of studies would be required to conduct a comprehensive study of exposure response relationships. The objective of this study approach was to "bracket" a high sound pressure amplitude level (ca. 200 dB) and a lower amplitude consistent with that often suggested for cetacean safety of 180 db a level which based on modelling studies is achieved within the 500-2500 range of a source (Lawson 2009).

Exposures were carried out in a large aquarium (inside dimensions: 3.63 m length x 2.39 m width x 1.27 m depth with water depth 1.13 m) with the air gun placed at 1.6 m from the center of the cage containing lobsters. Thirty control animals were handled in the same manner as the experimental animals except for the sound exposures.

Two Reson TC 4014 hydrophones were placed in the middle of the cage on the bottom of the aquarium to monitor received sound levels. One hydrophone (referred to as back hydrophone) was placed approximately 30 cm to the rear of the other hydrophone (referred to as front hydrophone).

After exposures, animals were returned to 2000 L aquaria for approximately 6 months when they were processed and examined for various health effects. They were fed ad libitum every week or so.

Observations were carried out for mortality over the experimental period.

## 2.2 Autopsy and Tissue Sample Collection

Lobsters were weighed and killed by severing the nerve chord located behind the eyes. Each lobster was assessed visually for any abnormalities on the antennas, claws, eyes, pereiopods (legs), abdomen, tail fan and swimmerets. The shell (carapace) of the cephalothorax was removed to reach the dorsal part of the internal organs. The colour of the flesh and the presence of a dark membrane (cuticle) between the shell and the rest of the body were recorded as well as any abnormalities observed on internal organs including hepatopancreas, heart and gonads.

A portion of hepatopancreas from the right dorsal posterior region was placed in Gendre's fixative while a portion of the gonads from the right dorsal posterior region and the last gill from the right dorsal region were placed in Dietrich's fixative.

A sample of hemolymph (blood equivalent) was taken during autopsy, centrifuged at 10,300 rpm (4ºC) and frozen at -60ºC for calcium, protein, glucose and triglyceride analyses.

The first 20 samples were collected using a 1 ml syringe, placed in a 5ml tube, vortexed and centrifuged for 5 minutes. Since samples were gelling before or during the centrifugation, the procedure was changed as follows: blood samples were directly taken from a small incision in tail musculature with blood flowing into a 5ml tube. Tubes were gently rocked back and forth by hand and the time of centrifugation was increased to 10 minutes.

## 2.3 Serum Biochemistry

### 2.3.1 Protein Measurement

Protein concentration was determined in lobster serum using the colorimetric method of Lowry (Lowry et al., 1951) with bovine serum albumin as standard.

### 2.3.2 Calcium Measurement

Calcium was measured in serum by colorimetry with the in-vitro quantitative determination of calcium (Arsenazo) kit (Pointe Scientific Inc.). Hemolymph samples were thawed on ice. A reaction mixture, containing 8 µl of hemolymph, 12 µl of distilled water and 1 ml of Arsenazo reagent, was prepared, mixed, incubated for 1 minute and read at a wavelength of 650 nm using a Milton Roy Spectronic 20 D spectrophotometer. The calcium concentration (mg/dL) was calculating using a calcium standard curve.

### 2.3.3 Glucose Measurement

Glucose was measured in serum by colorimetry with the in-vitro quantitative determination of glucose (enzymatic) kit (Sterling Diagnostics Inc.). Hemolymph samples were thawed on ice. A reaction mixture, containing 20 µl of hemolymph and 2.5 ml of glucose buffer was prepared, mixed and incubated for 3 minutes at 37ºC, followed by the addition of 100 µl of glucose enzyme and 10 minutes of incubation at 37ºC. The final color was read at a wavelength of 510 nm using a Milton Roy Spectronic 20 D spectrophotometer. The glucose concentration (mg/dL) was calculated using a glucose standard curve.

### 2.3.4 Triglyceride Measurement

Triglycerides were measured in serum by colorimetry with the in-vitro quantitative determination of Triglycerides (GPO) kit (Sterling Diagnostics Inc.). Hemolymph samples were thawed on ice. A reaction mixture, containing 20 µl of hemolymph and 1,000 µl of working TRIG-GPO Reagent was prepared, mixed, incubated at 3ºC for 10 minutes and read at a wavelength of 544 nm using Milton Roy Spectronic 20 D spectrophotometer. The triglyceride concentration (mg/dL) was calculated using a triglyceride standard curve.

## 2.4 Tissue Histopathology

Fixed hepatopancreas and gonad samples were processed for histological analysis (Lynch et al., 1969) using a Tissue-Tek® VIP Processor. A graded ethyl alcohol series of 70%, 80%, 95%, and two changes of 100%, was used for dehydration of the samples. The samples were then cleared in three changes of xylene. Finally, the tissues were impregnated with three changes of molten embedding media, Tissue Prep 2. The processed tissues were embedded in steel molds using molten embedding media, and topped with labelled embedding rings. After cooling, the hardened blocks of embedded tissues were removed from their base molds. The blocks were then trimmed of excess wax. Two to 4 sections per sample were cut at 6 microns on a Leitz microtome, floated on a $47^0$C water bath containing gelatine, and then picked up on labelled microscope slides. After air drying, the slides were fixed at $60^0$C for approximately 2 hours to remove most of the embedding media and allow the sections to adhere properly to the slide. Sections were stained with Mayers Haematoxylin and Eosin (H&E) and Periodic Acid-Schiff (PAS) methods (Luna, 1968). Coverslips were applied using Permount Mounting Media, and the slides were left to air dry and harden overnight.

One slide with 3-4 sections was examined per lobster under different magnifications by transmission light microscopy (Wild Leitz Aristoplan bright field microscope). To minimize interpretative bias, a blind system was used in which the examiner is not aware of the group being examined.

### 2.4.1 Hepatopancreas Histopathology

Sections stained with H&E were assessed microscopically for the presence of any overt differences in general structure or staining characteristics of tissues between control and exposed animals. Sections stained with PAS were examined for the presence of PAS positive material in the tubules.

### 2.4.2 Ovary Histopathology

Sections stained with H&E were assessed for the presence of any overt differences in general structure or staining characteristics of tissues between control and exposed animals. Special attention was given to the presence of haemocytes (blood cell equivalents) and/or the occurrence of haemocyte infiltration among the oocytes. The degree of the condition was recorded on a 1-3 relative scale (1- no or a few haematocytes; 2- small number of clusters (up to 5) of haemocytes; 3- larger clusters or more than 5 small clusters.

## 2.5 Statistical Analyses

Comparisons between control and exposed groups were conducted using Sigma-Stat 3.5:

- Animal weight, calcium, protein, glucose and triglyceride concentrations were analysed by the Unpaired t-test or the Mann-Whitney Rank Sum test, when the normality test failed.
- Prevalence of condition was analysed by the Fisher's exact test.

Comparisons having a $p<0.05$ were considered to be statistically significant.

# 3.0 RESULTS AND DISCUSSION

## 3.1 Received sound levels

Received sound levels are provided in Table 1. Note that 2 hydrophones were placed in the cage containing the lobsters in the aquarium; one (referred to as back hydrophone) was placed approximately 30 cm to the rear of the other hydrophone closest to the air gun (referred to as front hydrophone).

**Table 1. Received Sound Levels in the Cage**

| | Pounds per square inch (psi) | Front hydrophone | | | Back hydrophone | | |
|---|---|---|---|---|---|---|---|
| | | Peak to Peak *(dB re 1uPa)* | Peak *(dB re 1uPa)* | Root Mean Square *(dB re 1uPa)* | Peak to Peak *(dB re 1uPa)* | Peak *(dB re 1uPa)* | Root Mean Square *(dB re 1uPa)* |
| **Day 1** | **500** | 200.2 | 194.9 | 171.7 | 196.9 | 191.7 | 165.2 |
| **Day 2** | **400** | 197.6 | 193.5 | 169.7 | 193.9 | 188.7 | 162.7 |
| **Day 3** | **300** | 195.0 | 191.8 | 165.9 | 191.5 | 185.8 | 159.2 |
| **Day 4** | **200** | 190.3 | 187.1 | 161.6 | 185.2 | 179.4 | 153.3 |
| **Day 5** | **100** | 181.1 | 176.8 | 154.1 | 176.1 | 170.5 | 147.9 |

Received peak to peak levels varied from ~ 181 to 200 dB (front) to ~ 176 to 197 dB (back). Peak pressure varied from ~ 177 to 195 dB (front) to ~ 170 to 192 dB (back). Root mean square varied from ~ 154 to 172 dB (front) to ~ 148 to 165 dB (back). Peak-to-peak, peak and RMS pressure levels are commonly modelled for a given seismic array and included in environmental assessment statements for many seismic programs. Although little or no empirical information is available from the environment, such modelling can provide a rough approximation of sound penetration at varying distances in the water column (eg. Lawson, 2009). Likewise sound measurements in large tank systems can provide a rough guide of exposure levels.

## 3.2 Mortality

No mortalities were observed either during or immediately after exposure. Observations were also carried out for delayed mortality in control and exposed groups over the 6 month holding period and no significant differences were observed between the 2 groups (Table 2).

**Table 2. Mortality of Control and Exposed Lobsters**

| Time | Group | | P-Value [a] |
|---|---|---|---|
| | Control N = 30 | Exposed N = 30 | |
| **During or immediately after exposure** | 0 | 0 | 1.000 |
| **Over the 6 months holding period** | 5 | 3 | 0.726 |

(N) = number of lobsters at the beginning of experiment

[a] P-Value obtained after Fisher's exact test

Similar results, with no evidence of either immediate or delayed mortality, were obtained in laboratory and/or field studies with snow crab (Christian et al., 2003; Christian et al., 2004; Boudreau et al., 2009; Courtenay et al., 2009) and lobster (Payne et al., 2007).

## 3.3 Necropsy Data and Gross Pathology

A total of 52 female lobsters were weighed and examined visually for gross pathology. There were no abnormalities on internal organs including the gonads, hepatopancreas and heart in any lobsters. With respect to external organs, missing appendages, observations of flesh colour, presence of brown cuticle between the shell and internal organs and soft carapace as well as shell disease were recorded. Results are summarised in Table 3.

**Table 3. Lobster Weight and Prevalence of some Necropsy Parameters**

| | Number of Animals | Weight (g) | Missing Appendages | Pink Flesh | Brown Cuticle | Soft Carapace | Shell Disease |
|---|---|---|---|---|---|---|---|
| **Control** | 25 | 526 ± 30 | 3 | 4 | 4 | 10 | 16 |
| **Exposed** | 27 | 534 ± 20 | 2 | 4 | 2 | 5 | 9 |
| **p-Value** | | 0.230 | 0.662 | 1.000 | 0.411 | 0.127 | 0.051 |

[a] Mean ± standard deviation

[b] P-Value obtained after Unpaired t-test on mean of weight and Fisher Exact test on the prevalence of the other parameters

There were no significant differences in body weight between control and exposed lobsters (Unpaired t-test; $p = 0.230$).

There were no significant differences in the prevalence of lobsters with missing appendages (Fisher's exact test; $p = 0.662$). Previous studies with snow crab (Courtenay et al., 2009) and lobsters (Christian et al., 2003; Christian et al., 2004; Payne et al., 2007) also found no difference in loss of legs or other appendages between control and groups exposed to seismic energy.

There were no significant differences in the prevalence of lobsters with pink flesh (Fisher's exact test; $p = 1.000$), brown cuticle (Fisher's exact test; $p = 0.411$), and soft carapace (Fisher's exact test; $p = 0.127$). Pink flesh and browning of the cuticle, which may be associated with the growth of a new shell under the old, have been described as changes appearing in premoult lobsters (Waddy et al., 1995). Soft carapace is also a sign of pre-moulting. The results obtained in the present study with these 3 features indicate no apparent differences in moulting status between the control and exposed groups.

It is also noted that the colour of flesh and organs of one lobster from the exposed group was green. Green tint has been reported to occur in natural populations of lobsters if the moulting and reproductive cycles conflict (Ennis, 1984) and is due to the presence of lipovitellin in the hemolymph (Talbot and Helluy, 1995).

Significant differences were observed for shell disease (Figure 1) with a higher prevalence observed in the control group (Fisher's exact test; $p = 0.051$).

**Figure 1. Lobster Demonstrating Shell Disease**

## 3.4 Serum Biochemistry

### 3.4.1 Serum Protein

Protein concentration was measured in the serum of lobsters and results are summarised in Figure 2.

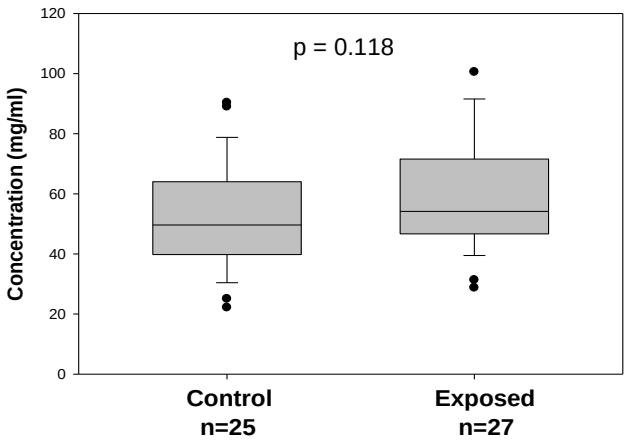


**Figure 2. Protein Concentration in the Serum of Control and Exposed Lobsters**

There were no significant differences in protein concentration between control and exposed lobsters (Unpaired t-test; p = 0.118).

### 3.4.2 Serum Calcium

Calcium levels in the serum of crustaceans can provide information on moulting status with calcium increasing just in advance of moulting. However, change in serum ions such as calcium could also indicate disturbance to osmoregulation. Calcium was measured in the serum of lobsters and expressed in mg/dl as well as standardized to mg of serum protein. Results are summarised in Table 4.

**Table 4. Levels of Calcium in the Serum of Control and Exposed Lobsters**

| Concentration | mg/dl | Standardised to mg of protein |
|---|---|---|
| **Control (N = 25)** | 17.6 ± 4.3 | 0.368 ± 0.150 |
| **Exposed (N = 27)** | 16.0 ± 3.3 | 0.293 ± 0.109 |
| **P-Value** | 0.203 | **0.032** |

Calcium levels in serum were similar in control and exposed lobsters (Mann-Whitney Rank Sum Test; p = 0.203), when expressed as mg/dl, the unit which has been used for assessing if lobsters are in a near moult condition. However, a slight but significant difference was found when calcium levels were standardized to the amount of protein in the serum, with a lower concentration found in exposed lobsters (Mann-Whitney Rank Sum Test; p = 0.032). However this difference may be due to the slightly higher (but statistically insignificant) level of serum protein in the exposed animals.

The calcium results (based on level of calcium in relation to volume of serum (or mg/dl) indicate that both groups were in a similar moulting condition. The results also suggest that seismic energy did not have long term delayed effects on osmoregulation. It is of note that Payne et al. (2007) reported a small decrease in serum calcium in lobsters exposed to air gun noise. Their measurements were made a few days to weeks post exposure whereas the measurements here were carried out approximately 6 months post exposure.

### 3.4.3 Serum Glucose

Glucose was measured in the serum of 10 specimens from each group and expressed in mg/dl or standardised to mg of serum protein. Results are summarised in Table 5.

**Table 5. Glucose Concentration in the Serum of Control and Exposed Lobsters**

| Concentration | mg/dl | Standardized to mg of protein |
|---|---|---|
| **Control (N = 10)** | 10.7 ± 2.3 | 0.201 ± 0.058 |
| **Exposed (N = 10)** | 11.2 ± 5.8 | 0.228 ± 0.098 |
| **P-Value** | 0.798 | 0.475 |

There were no significant differences in glucose concentration between control and exposed lobsters regardless of the unit used (Unpaired t-test; p>0.475).

### 3.4.4 Serum Triglycerides

Triglycerides were measured in serum and expressed in mg/dl or standardised to mg of serum protein. Results are summarised in Table 5.

**Table 6. Levels of Triglycerides in the Serum of Control and Exposed Lobsters**

| Concentration | mg/dl | Standardized to mg of protein |
|---|---|---|
| **Control (N = 25)** | 14.6 ± 9.2 | 0.285 ± 0.181 |
| **Exposed (N = 27 or 26*)** | 25.2 ± 56.4 or 14.5 ± 8.3* | 0.447 ± 0.898 or 0.279 ± 0.222* |
| **P-Value** | 0.978 or 0.851* | 0.840 or 0.658 * |

* After removal of one outliner

There were no significant differences in triglyceride concentration between control and exposed lobsters regardless of the unit used (Mann-Whitney Rank Sum Test; p>0.600).

## 3.5 Histopathology

Histopathology forms the basis for diagnosing various health conditions in human and veterinary medicine and is increasingly being used to assess the health of aquatic animals (e.g Hinton et al., 1992; Myers and Fournie, 2002; Payne et al., 2003; Mathieu et al., 2011). Histopathological studies were carried out on the hepatopancreas and ovary.

### 3.5.1 Hepatopancreas Histopathology

The general structure of hepatopancreatic sections stained with H&E appeared similar in both control lobsters and those exposed to seismic energy. There were no apparent differences of an overt nature. A representative photomicrograph of tubules stained with H&E is provided in Figure 3.

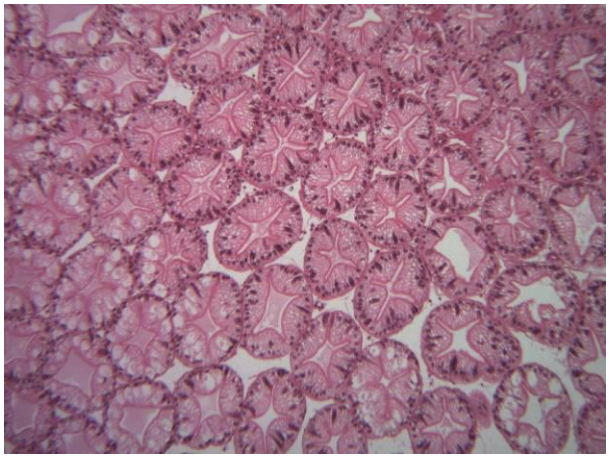

**Figure 3. Hepatopancreatic Tubules (H&E x63)**

With respect to sections stained with PAS for carbohydrate deposits (e.g. glycogen), since staining intensity varied from one sample to another in both control and exposed groups, sections were ranked for red staining intensity on a relative scale from 1 to 3, with 1 being the least (Figure 4) and 3 being the most (Figure 5).

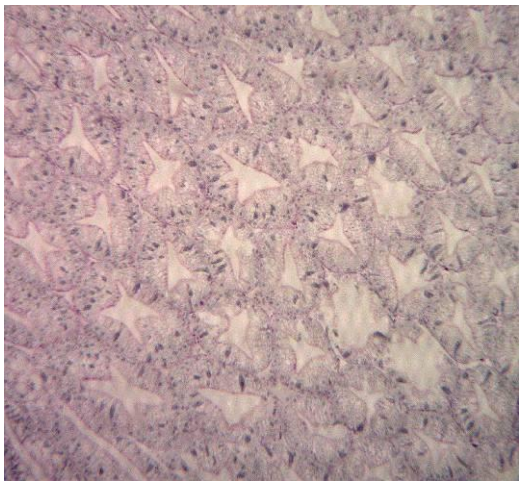

**Figure 4. Hepatopancreatic Tubules Ranked 1 (PAS x63)**

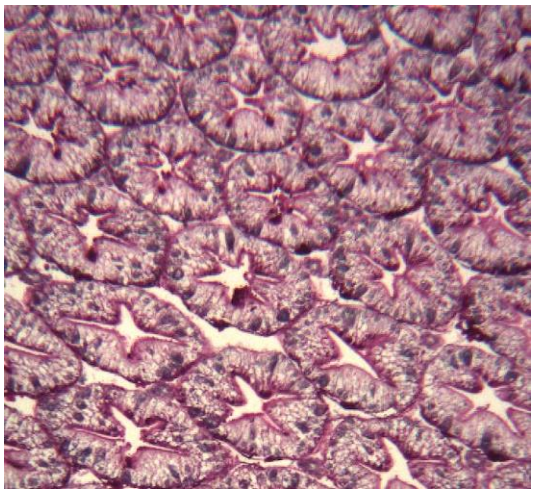

**Figure 5. Hepatopancreatic Tubules Ranked 3 (PAS x63)**

The number of lobsters presenting a score and the prevalence of PAS intensity is provided in Table 6.

**Table 6 Prevalence of PAS Intensity in Hepatopancreatic Sections of Control and Exposed Lobsters**

| | | Rank 1 | Rank 2 | Rank 3 |
|---|---|---|---|---|
| **Control** | Number | 5 | 17 | 3 |
| **N = 25** | % | 20.0 | 68.0 | 12.0 |
| **Exposed** | Number | 10 | 15 | 1 |
| **N = 27** | % | 37.0 | 55.5 | 3.7 |
| **P-Value** | | 0.227 | 0.404 | 0.609 |

There were no significant differences in the prevalence of PAS intensity between control and exposed groups (Fisher exact test) in either ranking.

### 3.5.2 Gonad Histopathology

An effect on reproduction is one of the main concerns when evaluating potential impacts on animal populations. An important reproductive effect could be structural damages to gonads. In the present study, gonadal sections were investigated for infiltration by large number of haemocytes, which could be indicative of haemorrhage or tissue necrosis whereby haemocytes are involved in the removal of effete material.

The general structure of ovaries appeared similar in all samples stained with H&E and PAS. There was no evidence of overt tissue necrosis, difference in staining, oedema or

homogenization of cellular components between the 2 groups. Most of the samples exhibited a few sparse haemocytes between oocytes (Figure 6) while a few samples from both group also had some clusters of haemocytes (Figure 7).

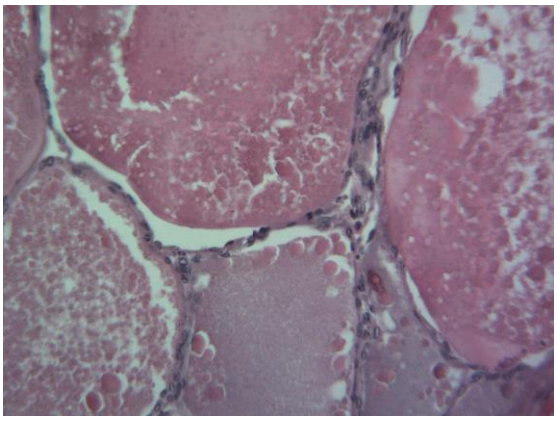

**Figure 6. Gonad Section without Haemocytes (H&E x63)**

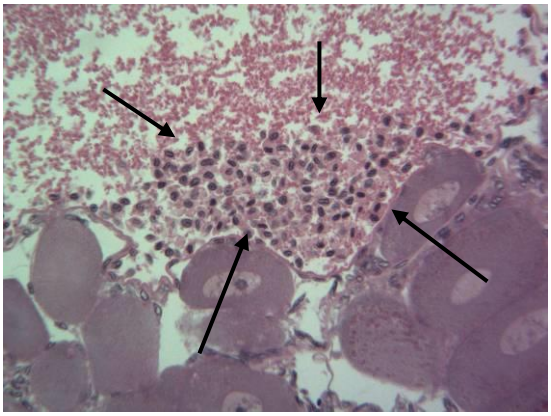

**Figure 7. Gonad Section with a Cluster of Haemocytes (H&E x63)**

Overall, there was no evidence for overt histological differences in the ovaries of lobsters exposed to seismic energy in comparison with controls. Likewise, there was no evidence for any major infiltration of haemocytes in the ovary of exposed lobsters versus controls. Similar results were reported with snow crabs (Boudreau et al., 2009), as well as red lobsters and various shrimp species exposed to seismic energy in the field (GIA, 2002; Andriguetto-Fihlo et al., 2005).

## 4.0 SUMMARY OBSERVATIONS

| Parameter | Observation |
|---|---|
| Mortality | No difference |
| Gross Pathology (external and internal) | No sign other than evidence of shell disease in some controls |
| Loss of legs | No difference |
| Haemolymph (blood equivalent) | No difference for protein, glucose and triglycerides; slight but statistically significant difference for calcium standardized to protein concentration ($P < 0.03$). However this difference may be due to the slightly higher (but statistically insignificant) level of serum protein in the exposed animals. |
| Hepatopancreas Histopathology | No difference |
| Glycogen accumulation in Hepatopancreas (PAS staining) | No difference |
| Ovary Histopathology | No difference |

The question of potential for prolonged or delayed effects often arises in relation to risks that may be associated with seismic surveys. Overall this pilot study indicates that any changes which may have occurred in selected histological and serum parameters upon airgun exposure were absent several months later, indicating animal recovery.

## 5.0 ACKNOWLEDGEMENTS

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