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Chronic Effects of Seismic Energy on Snow Crab *(Chionoecetes opilio)*

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By

LGL LIMITED
environmental research associates

and

Oceans LTD.

For

Environmental Studies Research Fund
444-7th Avenue S.W.
Calgary, AB
T2P 0X8

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Chronic Effects of Seismic Energy on Snow Crab (*Chionoecetes opilio*)

by

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EXECUTIVE SUMMARY

Introduction

LGL Limited of St. John's, Newfoundland and Labrador was contracted by the Environmental Studies Research Fund (ESRF) to conduct a study of the effects of seismic air gun energy on snow crab (*Chionoecetes opilio*) and snow crab catches. Loud noise has the potential for detrimental effects on animals by physical damage to sensitive organs (e.g., ear structures in the case of vertebrates), by causing increased stress, or by causing changes in behaviour. At present, the snow crab fishery in Atlantic Canada is concentrated in Newfoundland and Labrador and it is the most important commercial fishery in that province. The snow crab is also an important commercial species for fishers in the southern Gulf of St. Lawrence and off southern Nova Scotia, particularly for those who fish the waters off Cape Breton's west coast.

The study was one of the recommendations of an ESRF workshop convened in Halifax, Nova Scotia (September 2000) where seismic issues were discussed by local, national and international experts. Until recently, most of the concerns related to seismic exploration have focused on certain species and/or life stages of marine mammals and fish that are known to have sensitive hearing abilities. Little research has been conducted on the effects of seismic energy on invertebrates in general, and crab in particular, because these species do not have hearing structures, although some can detect pressure waves. Furthermore, unlike vertebrates, the bodies of marine invertebrates are generally the same density as the surrounding water and therefore, theoretically, sudden change in pressure, such as that caused by sudden loud noise, would be unlikely to cause physical damage. Nonetheless, commercial fishers in Cape Breton, Nova Scotia, have expressed concern that seismic surveys may affect the health and/or behaviour of snow crab. In addition, there also have been anecdotal suggestions in Newfoundland that seismic surveys may affect crab trap catch efficiency. Given the concerns of the fishers, the high value of the fishery, the general lack of hard evidence for or against effects, and the increasing amount of seismic exploration in waters inhabited by snow crab, the ESRF commissioned an initial study of the topic.

The study, conducted in the fall of 2002, examined a number of health, behavioural, and reproductive variables before, during, and after, seismic shooting in a preliminary attempt to assess potential effects on physical, biochemical, or activity patterns in the subject animals. The majority of the results have been reported in Christian et al. (in press). The present report details the materials and methods, results and discussion of that part of the study that assessed the potential for delayed (chronic) effects of seismic energy on the health of snow crabs.

Methodology

Study Site

The field component of the study was conducted on a commercially-fished snow crab ground off the northeast Avalon Peninsula, Newfoundland, approximately five km north-northeast of Cape

St. Francis in the mouth of Conception Bay (47° 50.7' N, 52° 45.4' W). The field work was conducted between mid-September and mid-December, 2002. Water depth at the study site was approximately 165 to 175-m. The 15-m longliner, the *FV Rough and Wild*, was used to access the study site and to serve as a study platform.

Noise Sources

Seismic air guns were used for noise sources, including two 10 cubic inch (in^3) sleeve air guns, one 20-in^3 gun, and four 40-in^3guns. The air guns were used singly (40-in^3) and combined in an array of seven guns (200-in^3). For each firing session, the guns were set at two metres below the surface of the water and fired 200 times at 10-second intervals. The number of shots was chosen to be representative of the dosage that an individual animal might be exposed to during an operational seismic program.

Exposure of Crabs to Seismic Energy

Seven legal-sized males and three egg-bearing females were exposed to the energy from the 40-in^3 gun. These 10 animals, along with an equal number of unexposed animals (the 'controls'), were transported to Fisheries and Oceans in St. John's and placed into holding tanks. In addition, ten legal-sized male crabs treated with the energy from the 200-in^3 seismic array and nine legal-sized male control crabs were taken to DFO in St. John's and placed into holding tanks.

Analyses of Crab Health

Crabs were held at DFO for approximately seven months and during that time three of thirty-nine crabs died. These mortalities included one control male crab and one treated male crab associated with the single gun exposure, and one control male crab associated with the seven-gun array experiment. The causes of these mortalities are unknown but are not unusual when holding animals in aquaria for long periods (J. Payne, pers. comm.). They may have died from natural causes or from unknown injuries or disease incurred during capture and holding.

At the end of the holding time at DFO, selected analyses of physiological indicators of stress were conducted on the crabs. Samples of haemolymph were collected from both the control and treatment male snow crabs and subsequently analyzed for various parameters including haemolymph solute, serum protein, serum enzymes, serum glucose, serum ions and differential haemocyte counts. Haemolymph was not collected from the female crabs because the volume of a single sample from a female is too low. A sample of sufficient volume would require numerous attempts at bleeding which in itself could alter various haemolymph parameters.

A "stress test" using wet ice was then performed on both the bled male crabs and the unbled female crabs. Crabs were placed on ice in coolers with tops slightly ajar to allow for oxygenation. Changes in crab condition, including time to death, were monitored and recorded at least twice per day. The four degrees of condition noted were (1) very active with legs drawn

up to the underside of the body, (2) active but with some drooping of the legs, (3) alive but movement restricted to eyes and mandible appendages, and (4) dead. As each crab died, the head was excised and fixed in 10% formalin for subsequent histopathological examination of the eye tissue.

Results and Discussion

Mortality during Holding Period

As stated above, three of the thirty-nine crabs died during the seven-month holding period at DFO. These mortalities included one control male crab and one treated male crab associated with the single gun exposure, and one control male crab associated with the seven-gun array experiment. The causes of these mortalities are unknown but are not unusual when holding animals in aquaria for long periods (J. Payne, pers. comm.). They may have died from natural causes or from unknown injuries or disease incurred during capture and holding. None of the female crabs died during the holding period.

Analyses of Male Snow Crab Haemolymph

Haemolymph Solute

Haemolymph refractive indices can provide a measure of change in total dissolved substances or solutes. In the present experiment, there were no significant differences in refractive index between control and exposed crabs.

Serum Protein

Protein concentrations (mg/ml) were also measured in the serum of control and exposed male crabs from both experiments. Changes in serum protein concentrations may indicate osmoregulatory or physiological disturbances. There was a significant difference in serum protein concentration between the control and treated male crabs of the exposure experiment using the single gun. The approximate received sound level was 221 dB re 1 $\mu Pa_{0\ to\ peak}$. The mean protein concentration of the exposed animals was significantly higher than that for the control animals. However, no significant differences between control and treated crabs were indicated for the animals from the 200-in^3 exposure experiment or when the male crabs from both exposures were combined. The approximate received sound level from the 200-in^3 array was 224 dB re 1 $\mu Pa_{0\ to\ peak}$.

Serum Enzymes

Enzymatic activities of the sera of the control and treated male crabs were assessed using the Api-Zym system. Major changes in serum enzyme levels may indicate the occurrence of tissue damage. Of the 19 enzymes tested, five were demonstrable in most of the samples analyzed.

These included two esterases (esterase and esterase lipase), one phosphatase (naphtol-AS-BI-phosphohydrolase) and two glycosidases (α-glucosidase and N-acetyl-β-glucosaminidase). Two other phosphatases (alkaline and acid phosphatases) were also detected in a few of the samples. However, no significant differences in the prevalence of samples showing these two phosphatase reactions were noted between control and exposed groups (Fisher's Exact Test).

There was some indication of increased activity of three enzymes (aspartate aminotransferase, alanine aminotransferase and α-amylase) in the sera of exposed crabs although the differences between control and treated crabs were not statistically significant.

Serum Glucose

There were no significant differences in glucose concentrations between control and exposed crabs of either exposure experiment, or when the crabs from both exposure experiments were combined.

Serum Ions

There were no significant differences in ion concentrations between the control and treated male crab groups.

Differential Haemocyte Counts

There were no significant differences in percentages of granulocytes, semi-granulocytes and hyalinocytes between the control and treated males of either exposure experiment or when the males from both exposures were combined.

Wet Ice Stress Test

Animals subjected to the 'wet ice stress test' were monitored over a period of several days until all individual crabs died. The longest period to mortality was 14 days. No statistically significant differences in mortality time between the control and treated groups, including the females, were noted.

Observations of general morbidity were also carried out during the wet ice stress test. No overt differences in morbidity characteristics were noted between the control and experimental groups throughout the 14-day observation period.

Eye Histopathology

No structural differences between the eyes of exposed crabs and the eyes of control crabs were observed during examination using light microscopy.

Conclusions

Experiments that exposed legal-sized male snow crabs and female snow crabs to the energy of a single air gun and/or seven-gun array did not indicate any chronic or longterm effects on a variety of haematological indices, eye tissue histopathology, and time to mortality during an extreme stress test. All snow crabs analyzed for chronic effects were exposed at either two or four metres from the seismic source, much closer than the several hundred metres or so where a crab might be exposed during a real seismic survey. Although the source sound levels were lower than those typically associated with seismic surveys, the received sound levels were equal to or greater than those to which snow crab would be exposed during normal seismic operations.

Of the 39 crabs held at DFO after completion of exposure to seismic, three animals died. Two of the three mortalities occurred among the control animals.

Mean refractive indices (measure of haemolymph solutes) of the treated and control groups were very similar, regardless of the seismic source.

The mean serum protein concentration of male crabs exposed to the single airgun was significantly higher than that of the control male snow crabs. However, the mean serum protein concentration of the male crabs exposed to the seven-gun array was not significantly different from that of the control animals. Thus, results on serum protein are inconclusive, probably due to the small sample sizes (e.g., n = 6 and n = 6, respectively).

Serum enzyme analyses indicated that mean levels of aspartate aminotransferase, alanine aminotransferase and α-amylase were higher in the haemolymph of exposed male crabs than in the haemolymph of control males, although not significantly higher. These three enzymes are often associated with liver and pancreatic damage in humans. Other haemolymph analyses (alkaline phosphatase, serum glucose, serum ions, differential haemocyte counts) did not indicate any differences between treated and control animals.

Results of the wet ice stress test did not indicate any significant differences in 'time to mortality' between treatment and control animals. The male snow crabs tended to survive longer than the females.

Histopathological analysis of eye soft tissue did not indicate any structural differences between the treated and control animals.

In conclusion, there did not appear to be any differences of chronic effect of seismic energy between the treated and control snow crabs.

RÉSUMÉ

Introduction

LGL Limited, de St. John's (Terre-Neuve-et-Labrador), a entrepris pour le Fonds pour l'étude de l'environnement (FEE) une étude portant sur l'effet de l'utilisation des canons à air sismiques sur le crabe des neiges (*Chionoecetes opilio*) et la pêche de ce crustacé. Les bruits intenses peuvent nuire aux animaux en entraînant des lésions dans les organes sensibles (l'oreille interne dans le cas des vertébrés), du stress ou des changements de comportement. La pêche du crabe des neiges dans les eaux canadiennes de l'Atlantique est présentement centrée sur les côtes de Terre-Neuve-et-Labrador où elle est la plus importante des pêches commerciales. Le crabe des neiges est également une espèce commerciale importante pour les pêcheurs qui travaillent dans le sud du golf du Saint-Laurent et au large du sud de la Nouvelle-Écosse, en particulier pour ceux qui pêchent le long de la côte ouest du cap Breton.

L'étude faisait partie des recommandations formulées à l'issue de l'atelier du FEE tenu à Halifax (Nouvelle-Écosse) en septembre 2000, au cours duquel les enjeux liés à la prospection sismique ont été débattus par des experts locaux, nationaux et internationaux. Jusqu'à récemment, la plupart des préoccupations concernant ce type de prospection visaient certaines espèces et/ou étapes de vie de mammifères marins et de poissons réputés avoir une ouïe sensible. Peu d'études ont été effectuées sur les effets que pourrait avoir l'énergie sismique sur les invertébrés en général et sur les crabes en particulier, parce que ces espèces ne possèdent pas de structures auriculaires même si certaines peuvent détecter les ondes de pression. De plus, contrairement au corps des vertébrés, celui des invertébrés marins a généralement la même densité que l'eau environnante et en théorie, les changements brusques de pression dans l'eau, tels que ceux causés par des bruits intenses, ne sont donc pas susceptibles d'entraîner des dommages physiques. Les pêcheurs commerciaux du cap Breton (Nouvelle-Écosse) ont cependant fait part de leurs préoccupations concernant les relevés sismiques qui, selon eux, pourraient affecter la santé et le comportement des crabes des neiges. De plus, à Terre-Neuve-et-Labrador, on a suggéré, sans preuve scientifique, que les relevés sismiques pourraient nuire à l'efficacité des pièges à crabe utilisés par les pêcheurs. Compte tenu de ces préoccupations, de la grande valeur de cette pêche, du manque général de preuves solides établissant l'existence ou l'absence d'effets et de l'intensification de l'exploration sismique dans l'habitat du crabe des neiges, le FEE a demandé que soit menée cette étude.

Effectuée à l'automne 2002, l'étude a pris en considération des variables sur la santé, le comportement et la reproduction avant, pendant et après le déclenchement de plusieurs tirs sismiques afin de tenter, dans un premier temps, d'évaluer les effets potentiels de tels bruits sur la physiologie, la biochimie et l'activité des animaux étudiés. La plus grande partie des résultats ont été publiés par Christian *et al* (sous presse). Le présent rapport décrit en détails le matériel et les méthodes, les résultats et les discussions du volet de l'étude portant sur la possibilité que l'énergie sismique entraîne des effets tardifs (chroniques) sur la santé du crabe des neiges

Méthodologie

Secteur d'étude

Les activités sur le terrain ont été menées sur un fond marin situé dans un secteur de pêche commerciale du crabe des neiges, au nord-est de la presqu'île d'Avalon (Terre-Neuve-et-Labrador), à environ cinq km au nord-est de Cape St. Francis, dans le passage de la baie de la Conception (47° 50,7' N, 52° 45,4' O). Les travaux sur le terrain ont été effectués de la mi-septembre à la mi-décembre 2002. La profondeur de l'eau du secteur à l'étude était de 165 à 175 m. Un palangrier de 15 m, le *FV Rough and Wild,* a permis d'accéder à la zone d'étude et servi de plate-forme d'étude.

Sources de bruit

Le bruit a été produit par des canons à air sismiques : deux canons de 10 pouces cubes (po^3), un canon de 20 po^3 et quatre canons de 40 po^3. On a tiré les canons un par un (40 po^3) ou en faisceau de sept canons (200 po^3). Lors des séances de tirs, les canons étaient plongés à deux mètres sous la surface et 200 coups étaient tirés à la cadence d'un coup toutes les 10 secondes. Le nombre de coups devait représenter le bruit auquel un animal pourrait être exposé durant un programme de levés sismiques réels.

Exposition des crabes à l'énergie sismique

On a exposé sept mâles de taille légale et trois femelles oeuvées à l'énergie émise par un canon de 40 po^3. Ces dix crabes, ainsi que dix crabes n'ayant pas été exposés au bruit (groupe témoin) ont ensuite été placés dans des aquariums situés dans les laboratoires de Pêches et Océans Canada (POC) à St. John's. Dix autres mâles de taille légale soumis à l'énergie sismique émise par le faisceau de canons de 200 po^3, ainsi que neuf mâles de taille légale (groupe témoin) ont été transportés aux laboratoires de POC à St. John's.

Analyses de la santé du crabe

On a gardé les crabes en captivité à POC pendant environ sept mois au cours desquels on a observé la mort de 3 des 39 crabes, dont un mâle exposé au bruit d'un seul canon, un mâle du groupe témoin associé à cette expérience et un mâle du groupe témoin associé au bruit émis par le faisceau de sept canons. On ne sait pas ce qui a causé leur mort, mais de telles mortalités ne sont pas rares chez les animaux gardés en captivité dans des aquariums pendant de longues périodes (J. Payne, entr. pers.). Leur mort pourrait avoir été naturelle ou fait suite à des blessures ou à des maladies entraînées par leur capture ou leur captivité qui n'auraient pas été observées.

Après la période de captivité, on a analysé certains indicateurs physiologiques du stress ainsi que des échantillons d'hémolymphe prélevée chez les crabes mâles des groupes témoins et les mâles exposés au bruit selon un certain nombre de critères, dont la chimie de l'hémolymphe, les protéines sériques, les enzymes sériques, le glucose sérique, les ions sériques et le taux différentiel d'hémocytes. On n'a pas prélevé l'hémolymphe des femelles en raison de la faible

quantité que représente un échantillon d'hémolymphe prélevé chez elles. Obtenir un échantillon suffisant aurait nécessité tant d'essais qu'on aurait risqué de modifier certains paramètres de l'hémolymphe.

On a ensuite mené des épreuves d'effort sur glace. Des crabes mâles dont on avait prélevé l'hémolymphe et des crabes femelles ont été déposés sur de la glace dans des contenants dont le couvercle n'était pas scellé pour leur permettre de respirer. On a surveillé et noté les changements subis par les crabes, y compris le temps létal, au moins deux fois par jour. On a observé quatre niveaux de l'état des crabes : 1) très actif, pattes repliées sous le corps; 2) actif, pattes relâchées; 3) vivant, mouvements limités aux yeux et aux mandibules; et 4) mort. Après la mort, la tête était détachée et archivée dans une solution de 10 % de formaldéhyde en vue d'un examen histopathologique des tissus oculaires.

Résultats et discussion

Mortalité en captivité

Tel qu'énoncé ci-haut, 3 des 39 crabes sont morts au cours des sept mois de captivité. Parmi ces crabes, on compte un mâle exposé au bruit d'un seul canon, un mâle du groupe témoin associé à cette expérience et un crabe du groupe témoin associé au bruit de sept canons. On ne sait pas ce qui a causé leur mort, mais de telles mortalités ne sont pas rares chez les animaux gardés en captivité dans des aquariums pendant de longues périodes (J. Payne, entr. pers.). Elle pourrait avoir été naturelle ou fait suite à des blessures ou à des maladies entraînées par leur capture ou leur captivité qui n'auraient pas été observées. Aucune mortalité n'a été observée chez les femelles pendant la captivité.

Analyses de l'hémolymphe des crabes des neiges mâles

Chimie de l'hémolymphe

Bien que l'indice de réfraction de l'hémolymphe permette parfois de mesurer les changements dans les substances dissoutes que l'on y retrouve, on n'a observé aucune différence significative de l'indice de réfraction entre l'hémolymphe des crabes du groupe témoin et celle des crabes exposés au bruit.

Protéines sériques

Après chacune des expériences, on a mesuré la concentration (mg/ml) en protéines sériques chez les crabes mâles du groupe témoin et les mâles exposés au bruit. Des changements dans la concentration en protéines sériques peuvent indiquer des perturbations physiologiques ou des troubles de l'osmorégulation. Les résultats de l'exposition au bruit d'un seul canon ont révélé une différence significative dans la concentration en protéines sériques entre les crabes du groupe témoin et les crabes exposés au bruit. Lors de cette expérience, les crabes ont été soumis à des niveaux de bruit d'une pression de crête d'environ 221 dB re 1 µPa. La concentration moyenne en protéines chez les crabes exposés au bruit dépassait considérablement celle des crabes du groupe témoin. Cependant, aucune différence significative n'a été observée entre les

crabes du groupe témoin et les crabes exposés au bruit du faisceau de canons de 200 po^3 ni en combinant les résultats des deux expériences. Les crabes soumis au bruit émis par le faisceau de canons de 200 po^3 ont été exposés à des niveaux de bruit d'une pression de crête d'environ 224 dB re 1 µPa.

Enzymes sériques

On a mesuré l'activité des enzymes sériques chez les mâles du groupe témoin et les mâles exposés au bruit à l'aide du système Api-Zym. Des changements importants des niveaux enzymatiques sériques peuvent indiquer la présence de lésions sur les tissus. Des 19 enzymes analysés, 5 étaient observables dans la plupart des échantillons analysés, dont deux estérases (estérase et estérase lipase), une phosphatase (naphtol-AS-BI-phosphohydrolase) et deux glycosidases (α-glucosidase et N-acetyl-β-glucosaminidase). On a aussi décelé la présence de deux autres phosphatases (phosphatase alcaline et phosphatase acide) dans quelques spécimens. Cependant, aucune différence significative n'a été observée chez les crabes du groupe témoin et les crabes exposés au bruit quant à la prévalence des échantillons présentant ces deux réactions à la phosphatase (méthode exacte de Fisher).

On a observé les signes d'une activité accrue des trois enzymes (sérum glutamo-oxalacétique transaminase, glutamate pyruvate transaminase et α-amylase) dans les sérums des crabes exposés au bruit, bien que les différences n'aient pas été statistiquement importantes entre les crabes exposés et les crabes du groupe témoin.

Glucose sérique

On n'a observé aucune différence importante dans la concentration glucidique des crabes des groupes témoin et des crabes exposés lors de l'une ou l'autre des deux expériences, ni en combinant les résultats des deux expériences.

Ions sériques

On n'a observé aucune différence significative dans la concentration ionique chez les mâles du groupe témoin et les mâles exposés au bruit.

Taux différentiel d'hémocytes

On n'a observé aucune différence significative dans le pourcentage de granulocytes, de semigranulocytes et d'hyalinocytes présents chez les mâles du groupe témoin et les mâles exposés au bruit lors de l'une ou l'autre des deux expériences, ni en combinant les résultats pour tous les mâles des deux expériences.

Épreuve d'effort sur glace

Les crabes soumis à l'épreuve d'effort sur glace ont été surveillés pendant plusieurs jours jusqu'à la mort de tous les crabes, c'est-à-dire pendant 14 jours. On n'a enregistré aucune différence

significative d'un point de vue statistique entre le temps létal chez les crabes du groupe témoin et chez les crabes exposés au bruit, mâles ou femelles.

On a aussi observé la morbidité générale lors de l'essai sur glace. On n'a relevé aucune différence flagrante entre les caractéristiques de morbidité des crabes du groupe témoin et celles des crabes exposés au bruit pendant les 14 jours de l'observation.

Histopathologie oculaire

Un examen microscopique photonique n'a révélé aucune différence dans la structure oculaire des crabes exposés au bruit et celle des crabes du groupe témoin.

Conclusions

Les expériences visant l'exposition de crabes des neiges mâles de taille légale et de crabes des neiges femelles à l'énergie dégagée par un seul canon à air ou par un faisceau de 7 canons n'ont permis de déceler aucun effet chronique ou à long terme sur une gamme d'indices hématologiques, dans l'histopathologie des tissus oculaires ni dans le temps létal lors d'un essai d'effort intense. Tous les crabes des neiges qui ont fait l'objet d'essais pour les effets chroniques étaient situés à deux ou quatre mètres de la source d'énergie sismique, beaucoup plus près que les centaines de mètres qui séparent les crabes de la source lors d'un véritable levé sismique. Bien que le niveau de bruit à la source était plus bas que celui normalement associé aux levés sismiques, les crabes ont été exposés à un niveau de bruit égal ou supérieur à celui auquel ils sont exposés lors d'activités sismiques normales.

Chez les 39 crabes examinés dans les laboratoires de POC après l'exposition au bruit, on a enregistré trois mortalités, dont deux chez les crabes du groupe témoin.

L'indice de réfraction moyen (composition chimique de l'hémolymphe) des crabes du groupe témoin et des crabes ayant été exposés au bruit était très comparable, peu importe la source d'énergie sismique.

La concentration moyenne en protéines sériques était beaucoup plus haute chez les crabes des neiges mâles exposés à l'énergie d'un seul canon à air que chez les crabes du groupe témoin. Cependant, cette concentration n'était pas très différente entre les crabes exposés à l'énergie sismique des sept canons et ceux du groupe témoin. Ainsi, les résultats de l'analyse du taux de protéines sériques sont inconcluants, probablement en raison de la petite taille de l'échantillon (p. ex. : n = 6 et n = 6, respectivement).

L'analyse des enzymes sériques a révélé que les niveaux moyens de sérum glutamo-oxalécetique, de glutamate pyruvate transaminase et d'α-amylase étaient plus élevés dans l'hémolymphe des mâles exposés au bruit que dans celle des mâles du groupe témoin, bien que cette différence ne soit pas significative. Chez l'humain, ces trois enzymes sont souvent associés aux lésions hépatiques et pancréatiques. D'autres analyses de l'hémolymphe (phosphatase

alcaline, glucose sérique, ions sériques, taux différentiel d'hémocytes) n'ont révélé aucune différence entre les crabes exposés au bruit et les crabes du groupe témoin.

Les résultats de l'épreuve d'effort sur glace n'ont révélé aucune différence significative entre le temps létal des crabes exposés au bruit et celui des crabes du groupe témoin. En général, les mâles ont survécu plus longtemps que les femelles.

L'analyse histopathologique des tissus oculaires n'a révélé aucune différence dans la structure oculaire des crabes exposés au bruit et celle des crabes du groupe témoin.

En conclusion, il semble n'y avoir aucune différence dans les effets chroniques de l'énergie sismique subis par les crabes exposés au bruit et les crabes du groupe témoin.

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- J. Nielsen, seismic equipment operator
- D. Hannay, Jasco acoustics expert
- R. Soper, J. Slade, P. Chafe, and B. French, Oceans technicians
- Afonso Diving Contractors

INTRODUCTION

LGL Limited of St. John's, Newfoundland and Labrador, conducted a study during the fall of 2002 to assess the effects of seismic energy on the behaviour and physiological health of snow crabs (*Chionoecetes opilio*) (Christian et al. in press). The study was funded by Environmental Studies Research Fund (ESRF). It was conducted on a natural snow crab ground off the Newfoundland coast near St. John's. A study of this nature had been suggested during a September 2000 Halifax workshop held to develop methodologies for conducting research on the effects of seismic exploration on the Canadian East Coast Fishery (Thomson et al. 2001).

One major aspect of the Christian et al. (in press) study involved the treatment of snow crabs with various seismic exposure levels to determine any acute and/or chronic physiological and/or pathological effects. The present report further details the materials and methods, results and discussion of an additional laboratory part of the study that assessed the potential for delayed (chronic) effects of seismic energy on the health of snow crabs.

MATERIALS AND METHODS

Study Site

The field component of the study was conducted on a commercially-fished snow crab ground off the northeast Avalon Peninsula, Newfoundland, approximately five kilometres north-northeast of Cape St. Francis in the mouth of Conception Bay (47° 50.7' N, 52° 45.4' W) (Figure 1). The field work was conducted between mid-September and mid-December, 2002. Water depth at the study site was approximately 165 to 175-m. The 15-m longliner, the *FV Rough and Wild*, was used to access the study site and to serve as a study platform.

Seismic Array

The seismic system used in this study consisted of a Price Compressor Co. A-300 4-Stage 300 SCFM compressor, four 40-in^3 Texas Instrument (TI) sleeve air guns, one 20-in^3 TI sleeve air gun, two 10-in^3 TI sleeve air guns, a 5,000 psi air tank with pressure regulator set @ 2,000 psi, a 50-m multihose and firing line umbilical via distribution panel, and a Macha International Inc. gun firing box, Model TGS-8.

Measurement of Seismic Acoustic Signals

A RESON TC4014 hydrophone was attached to the topsides electronics via a 200-m cable. The hydrophone output was fed to a Wavetek System 816 multi-channel filter that was configured as a 48 dB/octave, 8-pole Butterworth low pass filter with a 500 Hz cutoff frequency. This band-limited the analog hydrophone signals prior to sampling and the analog-to-digital conversion to prevent 'aliasing' of out-of-band components into the frequency band of interest.

The filtered hydrophone signal was brought to a Datel PCI-416 data acquisition board housed in an Advantech industrial computer. The Datel C++ source code was modified for this project to provide the necessary data collection, graphics and storage routines for the hydrophone data.

The data were collected in 16-second file blocks at a sampling rate of 2,048 Hz resulting in at least one seismic shot (1 shot every 10 seconds) being included in each data file. The data files were scanned for the start of the sleeve gun burst and this offset in the file was recorded with the filename for the subsequent data processing. Many of the files contained data from two shots.

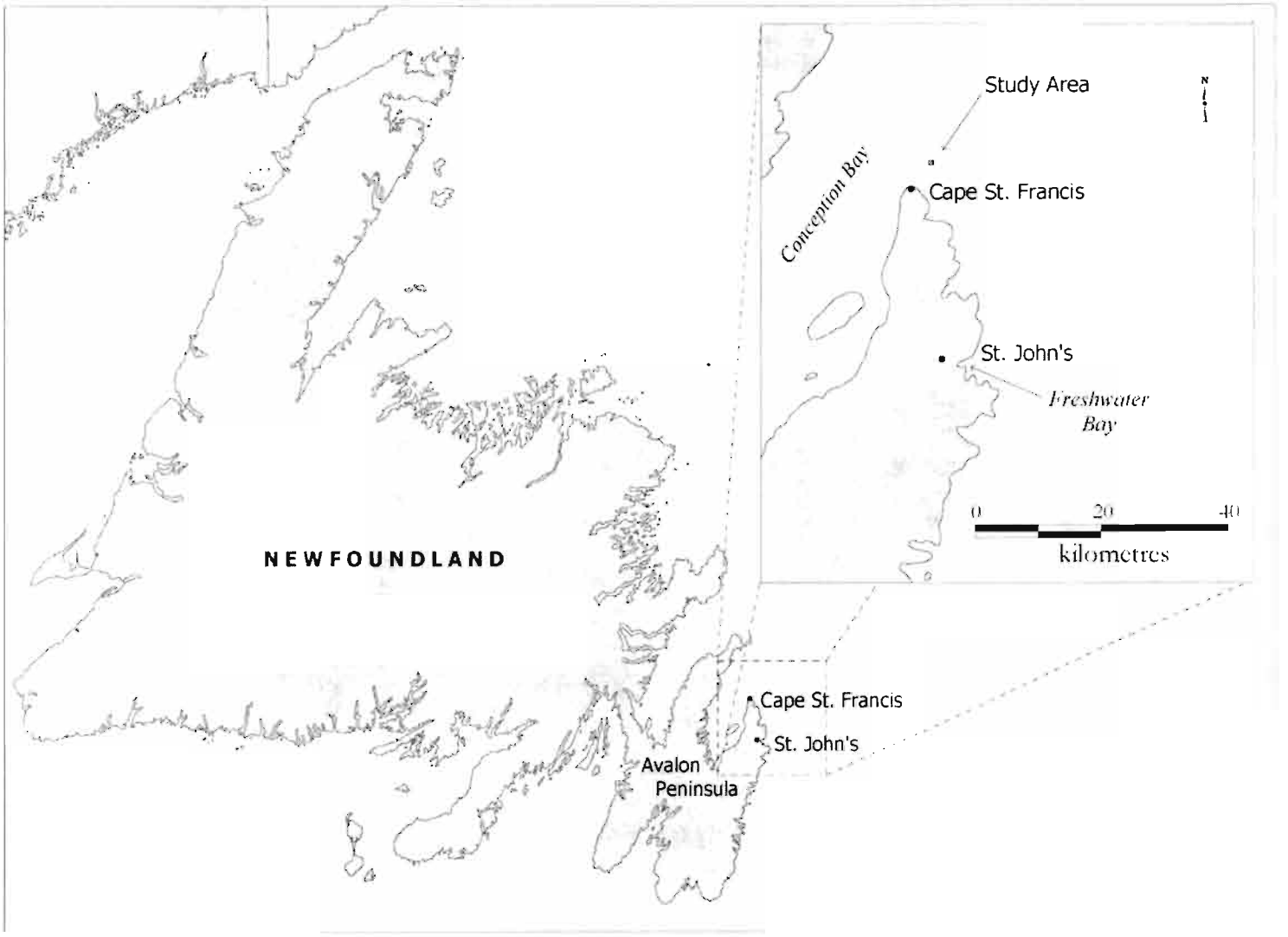

Figure 1. Location of study site where snow crab were collected and treated with seismic energy.

Exposure of Crabs to Seismic Energy

Two separate seismic sources were used during the study: (1) a single 40-in^3 airgun, and (2) a 200-in^3 seven-airgun array.

1. Hard-shelled, legal-sized male crabs and egg-bearing female crabs were exposed to the seismic energy from the single 40-in^3 sleeve air gun. An experimental trap holding these animals was suspended directly below the gun at a distance of 2-m. The approximate received sound level at this distance was 221 dB re 1 $\mu Pa_{0\ to\ peak}$.

2. Hard-shelled, legal-sized male crabs were also exposed to seismic energy from the 200-in^3 seven-air gun array. The array was suspended within a rectangular steel frame (1.25-m width x 1.25-m height x 2.5-m length). Four large inflatable buoys were attached to the top corners of the frame to provide floatation. The experimental trap holding these animals was suspended directly below this array at a distance of 4-m. The approximate received sound level at this distance was 224 dB re 1 $\mu Pa_{0\ to\ peak}$.

Each exposure session consisted of 200 shots at a firing rate of one every 10 seconds (i.e., approximately 33 minutes per session). The seismic source was set at 2-m depth during all shooting sessions. The number of shots was intended to be representative of the dosage that an individual animal might be exposed to during an operational seismic program.

Seven legal-sized males and three egg-bearing females were exposed to the energy from the 40-in^3 gun. These 10 animals, along with an equal number of control animals, were taken to Fisheries and Oceans in St. John's and placed into holding tanks. Treatments using the 200-in^3 array were conducted on legal-sized male crabs only. Ten crabs treated with the energy from the seismic array and nine control crabs were taken to DFO in St. John's and placed into holding tanks (Table 1).

Table 1. Specifics of exposure of 'delayed effects' crabs to seismic energy from both the single 40-in^3 gun and the 200-in^3 seven-gun array.

Distance between seismic source and crabs (m)	Gun/array volume (in^3)	Crab Gender	Control	Treated
2	40	Male	7	7
2	40	Female	3	3
4	200	Male	9	10

The control and treatment male and female crabs at DFO were observed for manifestation of any delayed lethal and/or sub-lethal effects. Four weeks after the exposure to seismic energy, samples of haemolymph were taken from both the treatment and control crabs. Samples from all

of the crabs were fixed for haemocyte counts while only the samples from treatment and control animals associated with exposure to the 200-in^3 array were subjected to protein and enzyme analysis. The remaining serum samples were archived at –80°C.

Crabs were held at DFO for approximately seven months and during that time three of thirty-nine crabs died. These mortalities included one control male crab and one treated male crab associated with the single gun exposure, and one control male crab associated with the seven-gun array experiment. The causes of these mortalities are unknown but are not unusual when holding animals in aquaria for long periods (J. Payne, pers. comm.). They may have died from natural causes or from unknown injuries or disease incurred during capture and holding.

At the end of the holding time at DFO, samples of haemolymph were collected from both the control and treatment male snow crabs and subsequently analyzed for various parameters including haemolymph solute, serum protein, serum enzymes, serum glucose, serum ions and differential haemocyte counts. Haemolymph was not collected from the female crabs because the volume of a single sample from a female is too low. A sample of sufficient volume would require numerous attempts at bleeding which in itself could alter various haemolymph parameters.

A "stress test" using wet ice was then performed on both the bled male crabs and the unbled female crabs. Changes in crab condition, including time to death, were noted during the stress test. As each crab died, the head was excised and fixed for subsequent eye histopathology.

Haemolymph Sampling

Haemolymph was withdrawn at the body-leg joint of the 2nd or 3rd thoracic legs of live male snow crab with 3 ml sterile syringes (23-ga). A drop of each sample was placed on a hand held refractometer (Westover Model RHS-10ATC) capable of automatic temperature compensation and measurement of specific gravity readings between 1.000 and 1.070. Half a milliliter of each sample was carefully expelled into a tube containing 10% buffered formalin and subsequently used for differential haemocyte counts. Two and a half milliliters of each sample were centrifuged for two minutes at 2,000 rpm and the resultant supernatant or serum was divided between three tubes that were frozen immediately at –60°C. These frozen samples were used for biochemical analyses for serum proteins, serum enzymes, serum glucose and serum ions.

Haemolymph Analysis

The following haemolymph analysis procedures were performed on the haemolymph sample of each control and treatment male snow crab.

Haemolymph Solute

Relative concentrations of solute or total dissolved substances in serum were determined by refractometry. Comparisons between the control and treatment groups were conducted using the Unpaired t-Test or the Mann-Whitney Rank Sum Test, depending on the normality of the data distributions. Differences were considered significant at $p<0.05$.

Serum Proteins

Protein concentration of each serum sample was determined using the Lowry protein method (Lowry et al., 1951) with bovine albumin standards. Comparisons between the control and treatment groups were conducted using the Unpaired t-Test. Differences were considered significant at $p<0.05$.

Serum Enzymes

Two hundred and sixty microlitres (µl) of serum from each crab exposed to either the 40-in^3 gun or the 200-in^3 array were diluted five times with distilled water and examined with the API ZYM^R system. This is a commercial kit that provides a semi-quantitative method for rapidly screening 19 enzymatic reactions (Monget 1978). The system consists of a strip with 20 microwells containing enzyme substrates and buffer for assaying various hydrolases including phosphatases, esterases, aminopeptidases and glycosidases. Seventy-five µl of diluted serum were placed in each well. Metabolic byproducts produced during the 45-minute incubation period at room temperature were indicated by coloured reactions as a result of the addition of reagents. Based on the intensity of colour, the reactions were visually graded from 0 to 5 (0 = no enzymatic activity, 5 = maximum activity), with reference to the Api Zym colour reaction chart.

Alkaline phosphatase levels of the same samples were quantitatively measured using the method of Bessey et al. (1946). Pilot studies were first carried out to determine the optimal conditions (protein concentration, incubation time, and substrate concentration) for assays with snow crab serum. Based on the pilot studies, the following protocol was adopted for each sample. Fifty microlitres of pure serum were incubated at room temperature for 30 minutes in one millilitre of 100 mM TRIS buffer (pH 8.5) containing 2.5 mM p-nitrophenylphosphate and 400 mM NaCl. The reaction was stopped with 100 µl of 10N NaOH. The mixture was centrifuged at 4,000 rpm for five minutes and the optical density read at 410 nm. Comparisons between the control and treatment groups were conducted using the Unpaired t-Test or the Mann-Whitney Rank Sum Test, depending on the normality of the data distributions. Differences were considered significant at $p<0.05$.

The serum of each male crab exposed to the 40-in^3 gun and each associated control animal was also analysed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and α-amylase (AMY). These pilot studies were performed outside the framework of this study in collaboration with Dr. Anver Rahimtula, a biochemist at Memorial University of Newfoundland, St. John's. Assays were carried out using a Sequential Multiple Analyser designed for clinical

tests on mammalian blood. Aminotransferases were assayed in reactions coupled to the formation of NAD while amylase was measured through a series of reactions coupled to the formation of NADH (Suber 1994). Comparisons between the control and treatment groups were conducted using the Unpaired t-Test or the Mann-Whitney Rank Sum Test, depending on the normality of the data distributions. Differences were considered significant at $p<0.05$.

Serum Glucose

Glucose concentrations in sera of control crabs and crabs exposed to both the single gun and the seven-gun array were measured by the Hexokinase/glucose-6-phosphatase dehydrogenase method (Farrance 1987) with the supplied Infinity™ Glucose Hexokinase Liquid Stable Reagent (ThermoDMA USA). This reagent is intended for in vitro diagnostic use in the quantitative determination of glucose in human serum or urine. Pilot studies were first carried out to determine if this reagent could also be used with snow crab serum. Based on the pilot studies, the following protocol was adopted for each sample. Nine microlitres of serum was incubated at room temperature for 5 minutes with 1350 µl of reagent and then the absorbance at 334 nm was read on a Cary 300 spectrophotometer. The concentration of glucose in mmol/L was calculated from the regression of absorbance against standard concentrations of the sugar. Comparisons between the control and treatment groups were conducted using the Unpaired t-Test. Differences were considered significant at $p<0.05$.

Serum Ions

Serum chloride was measured with an automated colorimetric analyser (Roche Cobas Fara/BMC Hitashi) according to USEPA method no. 325.1. The other major ions including sodium, potassium, calcium, and magnesium were analysed by ICP-OES (de optima 3000) according to USEPA Method No. 200.7.

Differential Haemocyte Counts

Haemolymph fixed in 10% buffered formalin was centrifuged and the resultant pellet was then washed three times with distilled water. The haemocytes in the pellets were then resuspended in a methanol/acetic acid (4/1) solution. A drop of cell suspension was placed on a microscope slide, smeared, air dried, stained with GIEMSA and examined with a Wild Leitz Aristoplan bright field microscope in order to identify three types of haemocytes: (1) hyalinocytes, (2) semi-granulocytes, and, (3) granulocytes). These haemocyte types have been defined in previous studies on crustaceans (Bauchau 1981; Soderhall and Cerenius 1992), including one with snow crab (Christian et al. in press). A differential count was made and each of the three types of cells was then expressed as a percentage of all counted haemocytes. Percentages were transformed using arcsin square root before analysis using the Unpaired t-Test or Mann-Whitney Rank Sum Test, depending on the normality of data distributions (Sokal and Rohlf 1981). Results of the comparisons between the control and treated groups having a $p<0.05$ were considered to be statistically significant.

Wet Ice Stress Test

Immediately after haemolymph samples were collected from the male crabs, control and exposed crabs were subjected to a 'wet ice stress test'. The crabs, in groups of three, were placed on plastic-covered ice in coolers with slightly opened lids to allow for oxygenation. Water resulting from ice melt was continuously drained from each cooler by attached hoses. The ice was changed every 24 hours. Crabs were initially assessed every 6 hours but it soon became apparent that 12-hour internals between assessments were appropriate. Four condition categories were used during the assessments: (1) very active with walking legs fully drawn upwards, (2) walking legs drooping but still responsive to stimuli, (3) movement restricted to eyes and mandible appendages, and (4) mortality. Once a crab died, its carapace width was measured and its head with eyes was excised and fixed in 10% formalin.

Light microscopy was opportunistically conducted on the eye tissue of some of the crabs. Although it may have been as long as 12 hours between time of crab death and eye tissue fixation, the eye tissue probably did not start to degrade as quickly as would be expected for tissues rich in degradative enzymes (e.g., hepatopancreas, digestive tract).

Eye Histopathology

Eyes fixed in 10% buffered formalin were decalcified in Cal-Ex™ for one hour and then stored in 70% methanol. Regular processing and paraffin embedding did not provide suitable sections for microscopic examination. These preparation attempts included embedding under vacuum conditions. Instead, samples were prepared using the methacrylate resin embedding method. Eyes were dehydrated in 70% and 95% ethanol and then infiltrated with methacrylate. The eyes were then placed longitudinally in molds and embedded in historesin. Samples were then trimmed about halfway through and four 3 µm serial sections were taken. These sections were stained with Mayers haemotoxylin and then counterstained with 1% aqueous phloxine solution. Coverslips were applied using Entellan® and the slides were left to air dry and harden overnight.

Once hardened, the slides were examined under different magnifications using transmission light microscopy (Wild Leitz Aristoplan bright field microscope) to determine any structural differences between exposed and control animals. Representative photographs were taken with a Nikon digital camera.

RESULTS AND DISCUSSION

Mortality During Holding Period

As stated above, three of the thirty-nine crabs died during the seven-month holding period at DFO. These mortalities included one control male crab and one treated male crab associated with the single gun exposure, and one control male crab associated with the seven-gun array experiment. The causes of these mortalities are unknown but are not unusual when holding animals in aquaria for long periods (J. Payne, pers. comm.). They may have died from natural causes or from unknown injuries or disease incurred during capture and holding. None of the female crabs died during the holding period.

Crab Carapace Width

There were no significant differences in carapace width (mm) between control and treated crabs of the three groups (Table 2). Raw data for individual crabs are contained in Appendix 1.

Table 2. Carapace width of control and treated snow crabs held for seven months after exposure to seismic.

	Males @ 2-m from 40-in^3 gun	Males @ 4-m from 200-in^3 array	Males (combined)	Females @ 2-m from 40-in^3 gun
Exposed (n)	104.3 ± 12.0 (6)	104.9 ± 7.2 (10)	104.7 ± 6.3 (16)	47.0 ± 8.6 (3)
Control (n)	112.5 ± 12.0 (6)	101.7 ± 6.3 (8)	106.4 ± 10.4 (14)	46.8 ± 3.3 (3)
p Value a	0.160	0.341	0.597	0.943

All data are expressed as mean ± standard deviation (mm)
[a] p Value obtained with Unpaired t-test

Haemolymph Parameters

The various haemolymph parameters were measured only in male crabs.

Haemolymph Solute

Haemolymph refractive indices can provide a measure of change in total dissolved substances or solutes. Results of refractive index measurements of control and treated male crabs are summarised in Table 3. There were no significant differences in refractive index between control and exposed crabs. The raw data are contained in Appendix 1.

Table 3. Comparison of haemolymph refractive index of control and treated male crabs, seven months after exposure to seismic.

	Males @ 2-m from the 40-in^3 gun	Males @ 4-m from the 200-in^3 array	Males (combined)
Exposed (n)	1.047 ± 0.012 (6)	1.044 ± 0.008 (10)	1.045 ± 0.009 (16)
Control (n)	1.042 ± 0.005 (6)	1.047 ± 0.009 (8)	1.045 ± 0.008 (14)
p Value a	0.367	0.347	0.930

All data are expressed as mean ± standard deviation
[a] p Value obtained with Unpaired t-test

Serum Protein

Protein concentrations (mg/ml) were also measured in the serum of control and exposed male crabs from both experiments. Changes in serum protein concentrations may indicate osmoregulatory or physiological disturbances. Results of protein concentration measurements of control and treated male snow crabs are summarised in Table 4. There was a significant difference in serum protein concentration between the control and treated male crabs of the exposure experiment using the single gun. The mean protein concentration of the exposed animals was significantly higher than that for the control animals. No significant differences between control and treated crabs were indicated for the animals from the 200-in^3 exposure experiment or when the male crabs from both exposures were combined (Table 4). The raw data are contained in Appendix 1.

Table 4. Comparison of serum protein concentration of control and treated male crabs, seven months after exposure to seismic.

	Males @ 2-m from the 40-in^3 gun	Males @ 4-m from the 200-in^3 array	Males (combined)
Exposed (n)	23.27 ± 3.28 (6)	16.16 ± 6.72 (10)	18.83 ± 6.58 (16)
Control (n)	17.29 ± 5.24 (6)	20.47 ± 5.89 (8)	19.11 ± 5.65 (14)
p Value [a]	0.039	0.173	0.902

All data are expressed as mean ± standard deviation (mg/ml)
[a] p Value obtained with Unpaired t-test

Serum Enzymes

Api-Zym Enzymatic Activities

Enzymatic activities of the sera of the control and treated male crabs were assessed using the Api-Zym system. Major changes in serum enzyme levels may indicate the occurrence of tissue damage.

Of the 19 enzymes tested, five were demonstrable in most of the samples analyzed. These included two esterases (esterase and esterase lipase), one phosphatase (naphtol-AS-BI-phosphohydrolase) and two glycosidases (α-glucosidase and N-acetyl-β-glucosaminidase). Two other phosphatases (alkaline and acid phosphatases) were also detected in a few of the samples (see Appendix 2). However, no significant differences in the prevalence of samples showing these two phosphatase reactions were noted between control and exposed groups (Fisher's Exact Test).

Alkaline Phosphatase

Alkaline phosphatase (ALP), which was detected in the sera of some crabs using the Api Zym system, was also measured quantitatively. Although little studied in crustaceans, this enzyme is common in a variety of animal tissues. In the sera of the single gun exposure male crabs, the activity of ALP was either not detected or detected at very low levels in both the control and exposed animals. In the sera of the seven-gun array exposure male crabs, results were somewhat similar to those of the single gun exposure crabs except for one treated and three control crabs. The sera of these four individuals exhibited high alkaline phosphatase activity (Table 5).

Table 5. Alkaline phosphatase activity in the sera of control and treated male crabs, seven months after exposure to seismic.

Exposure Experiment	Group	Individual Male Crabs									
Males @ 2-m from the 40-in^3 gun	**Exposed**	0.0	0.0	0.0	0.0	0.0	0.2	-	-	-	-
	Control	0.0	0.0	0.0	1.9	1.3	1.1	-	-	-	-
Males @ 4-m from the 200-in^3 array	**Exposed**	0.0	1.8	0.1	194	0.0	0.0	0.0	0.0	2.2	1.0
	Control	0.0	0.0	92	0.3	0.0	94	280	0.0	-	-

Activity expressed as OD units per ml

Overall, the above quantitative results "correlated" with those obtained with the semi-quantitative Api Zym method. However statistical analysis was not meaningful on such a small set of numbers with high variability and numerous zero values.

Aminotransferase and Amylase Enzymes

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and α-amylase (AMY) were also measured in the sera of male crabs exposed to the 40-in^3 airgun at 2-m. These particular enzymes are often associated with liver and pancreatic damage in humans (Adolph and Lorenz 1982; Suber 1994) and were investigated in a pilot study here. Results expressed as U/L are summarised in Table 6. The raw data are contained in Appendix 3.

Table 6. Aminotransferase and amylase enzymes in the sera of control and treated male crabs, seven months after exposure to the single 40-in^3 gun.

PARAMETER	AST [a]	ALT [b]	AMY [c]
Exposed (n = 6)	6.50 ± 3.21	20.67 ± 12.97	32.17 ± 21.20
Control (n = 5)	3.60 ± 1.95	9.40 ± 4.62	14.00 ± 12.73
p Value [d]	0.112	0.100	0.129

All data are expressed as mean ± standard deviation (U/L)
[a] AST = aspartate aminotransferase,
[b] ALT = alanine aminotransferase,
[c] AMY = α-amylase
[d] p Value obtained with Unpaired t-test or Mann-Whitney Rank Sum test

There was some indication of increased activity of the three enzymes (aspartate aminotransferase, alanine aminotransferase and α-amylase) in the sera of exposed crabs. Although the differences between control and treated crabs were not statistically significant, these differences are still worthy of note since enzyme level changes are sometimes indicative of tissue damage.

Serum Glucose

Concentrations of glucose (expressed in mmol/L) in the sera of control and treated male crabs of both exposure experiments are summarised in Table 7. The raw data are contained in Appendix 1.

Table 7. Glucose concentrations in the sera of control and treated male crabs, seven months after exposure to seismic.

Group	Males @ 2-m from the 40-in^3 gun	Males @ 4-m from the 200-in^3 array	Males (combined)
Exposed (n)	1.223 ± 0.448 (6)	0.941 ± 0.385 (10)	1.047 ± 0.419 (16)
Control (n)	1.282 ± 0.304 (5)	1.224 ± 0.376 (8)	1.246 ± 0.338 (13)
p Value [a]	0.808	0.138	0.177

All data are expressed as mean ± standard deviation (mmol/L)
[a] p Value obtained with Unpaired t-test

There were no significant differences in glucose concentrations between control and exposed crabs of either exposure experiment, or when the crabs from both exposure experiments were combined.

Serum Ions

The concentrations of various major ions in the sera of control and treated male crabs of both exposure experiments are summarised in Table 8. The raw data are contained in Appendix 4.

There were no significant differences in ion concentrations between the control and treated male crab groups.

Table 8. Ion concentrations in the sera of control and treated male crabs, seven months after exposure to seismic.

Ion	Group	Males @ 2-m from the 40-in^3 gun	Males @ 4-m from the 200-in^3 array	Males (combined)
Sodium	**Exposed (n)**	10283 ± 117 (6)	10150 ± 97 (10)	10200 ± 121 (16)
	Control (n)	12000 ± 3971 (6)	10125 ± 71 (8)	10929 ± 2645 (14)
	p Value [a]	0.240	0.721	0.677
Potassium	**Exposed (n)**	426 ± 16 (6)	408 ± 19 (10)	415 ± 20 (16)
	Control (n)	414 ± 28 (6)	397 ± 12 (8)	404 ± 21 (14)
	p Value [a]	0.377	0.170	0.170
Calcium	**Exposed (n)**	426 ± 23 (6)	413 ± 22 (10)	418 ± 22 (16)
	Control (n)	439 ± 36 (6)	403 ± 10 (8)	419 ± 30 (14)
	p Value [a]	0.465	0.241	0.959
Magnesium	**Exposed (n)**	999 ± 61 (6)	967 ± 89 (10)	979 ± 79 (16)
	Control (n)	980 ± 61 (6)	948 ± 42 (8)	962 ± 51 (14)
	p Value [a]	0.603	1.000	0.647
Chloride	**Exposed (n)**	16000 ± 632 (6)	16200 ± 632 (10)	16125 ± 619 (16)
	Control (n)	15933 ± 753 (6)	16000 ± 534 (8)	15929 ± 616 (14)
	p Value [a]	0.699	0.560	0.465

Data are expressed as mean ± standard deviation (mg/L)
[a] p Value obtained with Unpaired t-test or Mann-Whitney Rank Sum test

Differential Haemocyte Counts

Differential haemocyte counts were performed on control and treated male crabs of both exposure experiments. Results of these counts are summarised in Table 9. The raw data are contained in Appendix 4.

Table 9. Differential haemocyte counts in control and treated male crabs, seven months after exposure to seismic.

Exposure Experiment	Group	Granulocytes	Semi-granulocytes	Hyalinocytes
Males @ 2-m from the 40-in^3 gun	Control (n = 6)	22.0 ± 4.0	39.5 ± 4.9	38.5 ± 8.7
	Exposed (n = 6)	23.8 ± 3.4	42.7 ± 2.3	33.5 ± 4.8
	p Value [a]	0.414	0.180	0.246
Males @ 4-m from the 200-in^3 array	Control (n = 8)	22.9 ± 3.6	42.8 ± 4.6	34.4 ± 6.1
	Exposed (n = 10)	23.5 ± 4.0	41.6 ± 5.1	34.9 ± 7.7
	p Value [a]	0.736	0.626	0.877
Males (combined)	Control (n = 14)	22.5 ± 3.6	41.4 ± 4.8	36.1 ± 7.3
	Exposed (n = 16)	23.6 ± 3.7	42.0 ± 4.2	34.4 ± 6.6
	p Value [a]	0.409	0.430	0.493

All data are expressed as mean percentage ± standard deviation of each cell type
[a] p Value obtained with Unpaired t-test or Mann-Whitney Rank Sum test

There were no significant differences in percentages of granulocytes, semi-granulocytes and hyalinocytes between the control and treated males of either exposure experiment or when the males from both exposures were combined.

Wet Ice Stress Test

Animals subjected to the 'wet ice stress test' were monitored over a period of several days until all individual crabs died. The longest period to mortality was 14 days. No statistically significant differences in mortality time between the control and treated groups, including the females, were noted (Table 10).

Table 10. Time to mortality for control and treated male and female crabs, seven months after exposure to seismic.

Group	Males @ 2-m from the 40-in^3 gun	Males @ 4-m from the 200-in^3 array	Females @ 2-m from the 40-in^3 gun
Treated (n)	7.83 ± 5.08 (6)	7.13 ± 5.49 (8)	4.33 ± 1.16 (3)
Control (n)	4.67 ± 2.66 (6)	8.63 ± 4.53 (8)	4.00 ± 1.00 (3)
p Value [a]	0.206	0.561	0.725

All data are expressed as mean ± standard deviation (days)
[a] p Value obtained with Unpaired t-test

Observations of general morbidity were also carried out during the wet ice stress test. No overt differences in morbidity characteristics were noted between the control and experimental groups throughout the 14-day observation period.

Eye Histopathology

The eyes of snow crabs exposed to seismic energy were examined using light microscopy. Sections were examined for overt damage to corneal lens tissue, cone cells and retinula cells. The general anatomy of crustacean eyes has been reviewed (Waterman 1961; Cronin 1986; Fincham 1988). The corneal lens tissue had a characteristic hexagonal array (Figure 2, Photo 1), the cone cells were quadripartite in cross section (Figure 2, Photo 2), and the retinula cells appeared in groups of seven in cross section (Figure 2, Photo 3). The approach of the examination was to inspect for breakage or distortion of the borders of the hexagonal arrays of lens tissue, and major breakage of the "rosettes" associated with the cone and retinula cells. Approximately 800 control and 700 treated cone cell rosettes and 400 control and 500 treated retinula cell rosettes were examined. No differences in the hexagonal arrays of the lens tissue, cone cell rosette pattern and retinula cell rosette pattern were observed between control and treated snow crabs.

Photo 1: Cross section of cornea lens showing hexagonal array (x 250).

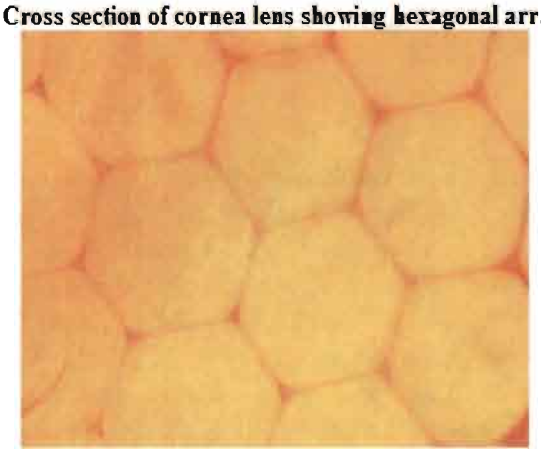

Photo 2: Cross section of cone cells showing quadripartite pattern (x 250).

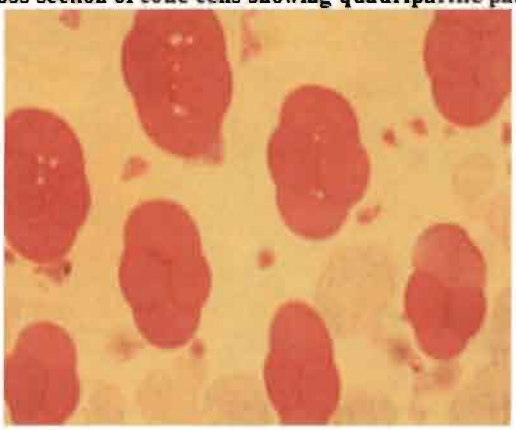

Photo 3: Cross section of retinula cells showing "rosettes" of 7 cells (x 250).

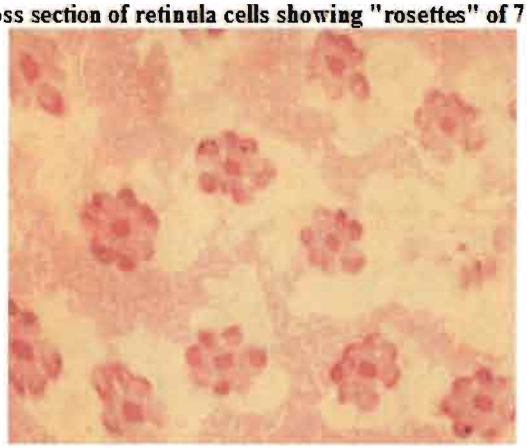

Figure 2. Histological sections of various snow crab eye tissue cell types.

CONCLUSIONS

Experiments that exposed legal-sized male snow crabs and female snow crabs to the energy of a single air gun and/or seven-gun array did not indicate any chronic or longterm effects on a variety of haematological indices, eye tissue histopathology, and 'time to mortality' during an extreme stress test. All snow crabs analyzed for chronic effects were exposed at either two or four metres from the seismic source.

Of the 39 crabs held at DFO after completion of exposure to seismic, three males died. Two of the mortalities occurred among the control animals. In other words, there was not any evidence of delayed mortality for either the control or treated crabs held in the laboratory for seven months after seismic treatments. The causes of these mortalities are unknown but are not unusual when holding animals in aquaria for long periods (J. Payne, pers. comm.). They may have died from natural causes or from unknown injuries or disease incurred during capture and holding.

Mean refractive indices (measure of haemolymph solutes) of the treated and control groups were very similar, regardless of the seismic source.

Although the mean serum protein concentration of male crabs exposed to the single airgun was significantly higher than that of the control male snow crabs, sample sizes were very small (n=6). In contrast, the mean serum protein concentration of the male crabs exposed to the larger seven-gun array was not significantly different than that of the control animals. Changes in serum protein concentrations may indicate osmoregulatory or physiological disturbances.

Serum enzyme analyses indicated that mean levels of aspartate aminotransferase, alanine aminotransferase and α-amylase were higher in the haemolymph of exposed male crabs than in the haemolymph of control males, although not significantly higher. These three enzymes are often associated with liver and pancreatic damage in humans. Other haemolymph analyses (alkaline phosphatase, serum glucose, serum ions, differential haemocyte counts) did not indicate any differences of note between treated and control animals. Major changes in serum enzyme levels may indicate the occurrence of tissue damage.

Results of the wet ice stress test did not indicate any significant differences in 'time to mortality' between treatment and control animals. The male snow crabs tended to survive longer than the females.

Histopathological analysis of eye soft tissue did not indicate any structural differences between the treated and control animals.

Based on these results, there did not appear to be any differences of chronic effect of seismic energy between the treated and control snow crabs.

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APPENDICES

Appendix 1. Data collected from treated and control male and female snow crabs held under laboratory conditions for seven months.

Exposure Experiment	Group	Crab ID	Carapace Width (mm)	Refractive Index	Serum Protein (mg/l)	Serum Glucose (mmol/L)	Alkaline Phosphatase (OD units/ml)
Males @ 2-m from 40 in^3 gun	Treated	M1	112	1.030	21.97	0.497	0.0
	Treated	M2	109	1.049	19.26	1.061	0.0
	Treated	M3	109	1.060	27.76	1.722	0.0
	Treated	M4	105	1.042	26.31	1.204	0.0
	Treated	M5	103	1.044	20.74	1.184	0.0
	Treated	M6	98	1.060	23.60	1.671	0.2
	Control	M7	130	1.036	9.83	1.261	0.0
	Control	M8	110	1.045	22.60	1.388	0.0
	Control	M9	127	1.050	21.00	-	0.0
	Control	M10	116	1.043	15.25	1.051	1.9
	Control	M11	104	1.039	21.74	1.739	1.3
	Control	M12	99	1.040	13.32	0.973	1.1
Males @ 4-m from 200 in^3 array	Treated	M13	101	1.050	21.85	1.077	0.0
	Treated	M14	104	1.050	19.00	1.077	1.8
	Treated	M15	118	1.044	17.38	0.887	0.1
	Treated	M16	109	1.055	26.82	1.867	193.5
	Treated	M17	107	1.036	9.13	1.017	0.0
	Treated	M18	97	1.035	19.27	0.946	0.0
	Treated	M19	117	1.046	19.04	0.811	0.0
	Treated	M20	98	1.035	6.59	0.485	0.0
	Treated	M21	104	1.051	6.55	0.565	2.2
	Treated	M22	109	1.035	15.95	0.683	1.0
	Control	M23	105	1.033	12.52	0.741	0.0
	Control	M24	99	1.045	19.04	1.209	0.0
	Control	M25	100	1.062	28.36	1.743	92.9
	Control	M26	118	1.042	15.30	1.220	0.3
	Control	M27	105	1.047	15.22	0.639	0.0
	Control	M28	100	1.044	21.46	1.269	93.5
	Control	M29	97	1.053	26.67	1.525	280.4
	Control	M30	102	1.054	25.16	1.446	0.0
Females @ 2-m from 40 in^3 gun	Treated	F1	45	-	-	-	-
	Treated	F2	53	-	-	-	-
	Treated	F3	44	-	-	-	-
	Control	F4	51	-	-	-	-
	Control	F5	44	-	-	-	-
	Control	F6	47	-	-	-	-

Appendix 2. Enzymatic reactions (API ZYM) in sera of treated and control male snow crabs held under laboratory conditions for seven months.

Exposure Specifics	Group	Crab ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Crabs @ 2-m from 40-in^3 gun	Treated	M1	0	0	1.0	0.5	0	0	0	0	0	0	0	1.0	0	0	0	0	0	0.5	0	0
	Treated	M2	0	0	1.0	3.0	0	0	0	0	0	0	0	1.0	0	0	0	0.5	0	0.5	0	0
	Treated	M3	0	0	1.0	3.0	0	0	0	0	0	0	0	1.0	0	0	0	1.0	0	0.5	0	0
	Treated	M4	0	0	2.0	3.0	0	0	0	0	0	0	0.5	1.0	0	0	0	0.5	0	1.0	0	0
	Treated	M5	0	0	2.0	2.0	0	0	0	0	0	0	0	1.0	0	0	0	0.5	0	0.5	0	0
	Treated	M6	0	0	2.0	3.0	0	0	0	0	0	0	0.5	1.0	0	0	0	0	0	0.5	0	0
	Control	M7	0	0	1.0	1.0	0	0	0	0	0	0	0	1.0	0	0	0	0.5	0	0.5	0	0
	Control	M8	0	0	1.0	2.0	0	0	0	0	0	0	0	1.0	0	0	0	0	0	0.5	0	0
	Control	M9	0	0	1.0	2.5	0	0	0	0	0	0	0	1.0	0	0	0	0.5	0	0.5	0	0
	Control	M10	0	0	2.0	2.0	0	0	0	0	0	0	0	1.0	0	0	0	1.0	0	1	0	0
	Control	M11	0	0	2.0	2.0	0	0	0	0	0	0	0	1.0	0	0	0	0.5	0	0.5	0	0
	Control	M12	0	0	1.0	1.5	0	0	0	0	0	0	0	1.0	0	0	0	0	0	0.5	0	0
Crabs @ 4-m from 200-in^3 array	Treated	M13	0	0	2.0	1.5	0	0	0	0	0	0	0	0.5	0	0	0	1.0	0	0.5	0	0
	Treated	M14	0	0	1.0	1.5	0	0	0	0	0	0	0	0.5	0	0	0	0.5	0	0.5	0	0
	Treated	M15	0	0	0.5	1.0	0	0	0	0	0	0	0	0.5	0	0	0	0.2	0	0.5	0	0
	Treated	M16	0	4.0	1.0	2.0	0	0	0	0	0	0	2.0	1.0	0	0	0	0.5	0	0.5	0	0
	Treated	M17	0	0	0	0.5	0	0	0	0	0	0	0	0.5	0	0	0	0.2	0	0.5	0	0
	Treated	M18	0	0	1.0	1.5	0	0	0	0	0	0	0	0.5	0	0	0	0.2	0	1.0	0	0
	Treated	M19	0	0	1.0	1.2	0	0	0	0	0	0	0	0.5	0	0	0	0.2	0	0.2	0	0
	Treated	M20	0	0	1.0	1.0	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0.2	0	0
	Treated	M21	0	0	2.0	1.0	0	0	0	0	0	0	0	0.5	0	0	0	0.5	0	0	0	0
	Treated	M22	0	0	0.5	2.0	0	0	0	0	0	0	0	0.2	0	0	0	0.2	0	0.2	0	0
	Control	M23	0	0	1.0	1.5	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0
	Control	M24	0	0	1.0	2.0	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0.5	0	0
	Control	M25	0	3.0	2.0	1.5	0	0	0	0	0	0	1.5	1.0	0	0	0	0.5	0	0	0	0
	Control	M26	0	0	1.0	1.0	0	0	0	0	0	0	0	0.5	0	0	0	0.2	0	0.2	0	0
	Control	M27	0	0	0	1.5	0	0	0	0	0	0	0	0.5	0	0	0	0.2	0	0.2	0	0
	Control	M28	0	3.0	2.0	1.5	0	0	0	0	0	0	1.0	1.5	0	0	0	0	0	1.0	0	0
	Control	M29	0	5.0	0	2.0	0	0	0	0	0	0	3.0	3.0	0	0	0	0.5	0	0.2	0	0
	Control	M30	0	0	1.0	2.0	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0.5	0	0

Appendix 2. Continued.

1=Control
2=Alkaline phosphatase
3=Esterase
4=Esterase lipase
5=Lipase
6=Leucine arylamidase
7=Valine arylamidase
8=Cystine arylamidase
9=Trypsin
10=α-Chymotropsin
11=Acid phosphatase
12=Naphtol-AS-BI-phosphohydrolase
13= α-Galactosidase
14=β-Galactosidase
15= β-Glucoronidase
16= α–Glucosidase
17= β–Glucosidase
18=N-Acetyl- β-glucosaminidase
19= α-Mannosidase
20= α-Fucosidase

The enzymatic reactions were visually graded from 0 to 5, based on the intensity of the colour (0=no enzymatic activity detected; 5=maximum intensity). Reference was made to the API ZYM colour reaction chart.

Appendix 3. Aminotransferase and amylase enzymes in the sera of control and treated male crabs, seven months after exposure to the 40-in^3 seismic airgun.

CRAB ID	ENZYME		
	Aspartate aminotransferase	Alanine aminotransferase	α-amylase
M1	2	4	21
M2	6	35	68
M3	11	17	39
M4	8	37	19
M5	4	12	8
M6	8	19	38
M7	3	10	22
M8	3	11	10
M9	No sample	No sample	No sample
M10	7	16	32
M11	3	5	4
M12	2	5	2

Activity in U/L

Appendix 4. Ion concentrations in the sera of control and treated male crabs, seven months after exposure to seismic.

Exposure Experiment	Group	Crab ID	Sodium (mg/l)	Potassium (mg/l)	Calcium (mg/l)	Magnesium (mg/l)	Chloride (mg/l)
Males @ 2-m from 40-in^3 gun	Treated	M1	10,400	448	390	909	16,000
	Treated	M2	10,100	434	431	958	15,000
	Treated	M3	10,300	425	441	979	16,000
	Treated	M4	10,400	431	452	1,030	17,000
	Treated	M5	10,300	400	408	1,070	16,000
	Treated	M6	10,200	420	434	1,050	16,000
	Control	M7	10,600	468	438	1,010	16,000
	Control	M8	10,500	408	473	978	15,000
	Control	M9	10,300	416	487	952	15,000
	Control	M10	21,100	406	409	898	16,000
	Control	M11	10,300	388	434	964	16,000
	Control	M12	10,200	399	394	1,030	17,000
Males @ 4-m from 200-in^3 array	Treated	M13	10,200	407	450	897	16,000
	Treated	M14	10,300	395	411	895	17,000
	Treated	M15	10,300	384	387	883	16,000
	Treated	M16	10,000	419	446	872	16,000
	Treated	M17	10,100	387	421	1,020	17,000
	Treated	M18	10,100	425	396	970	16,000
	Treated	M19	10,100	446	424	912	16,000
	Treated	M20	10,200	412	392	1,070	16,000
	Treated	M21	10,100	410	398	1,130	17,000
	Treated	M22	10,100	395	407	1,020	15,000
	Control	M23	10,100	394	406	1,010	16,000
	Control	M24	10,100	406	414	926	16,000
	Control	M25	10,000	405	392	963	16,000
	Control	M26	10,200	373	405	972	17,000
	Control	M27	10,100	405	397	990	16,000
	Control	M28	10,100	404	393	895	16,000
	Control	M29	10,200	401	398	920	16,000
	Control	M30	10,200	388	419	907	15,000

Appendix 5. Haemocyte counts in control and treated male crabs, seven months after exposure to seismic (100 cells counted per crab serum sample).

Exposure Experiment	Group	Crab ID		Granulocytes	Semi-granulocytes	Hyalinocytes
Males @ 2-m from 40-in^3 gun	Treated	M1		21	39	40
	Treated	M2		26	43	31
	Treated	M3		29	45	26
	Treated	M4		20	43	37
	Treated	M5		22	45	33
	Treated	M6		25	41	34
	Control	M7		26	41	33
	Control	M8		20	40	40
	Control	M9		23	42	35
	Control	M10		25	44	31
	Control	M11		23	40	37
	Control	M12		15	30	55
Males @ 4-m from 200-in^3 array	Treated	M13		23	42	35
	Treated	M14		26	44	30
	Treated	M15		20	47	33
	Treated	M16		22	30	48
	Treated	M17		29	47	24
	Treated	M18		18	39	43
	Treated	M19		27	40	33
	Treated	M20		22	42	36
	Treated	M21		29	46	25
	Treated	M22		19	39	42
	Control	M23		24	42	34
	Control	M24		30	39	31
	Control	M25		25	53	22
	Control	M26		20	40	40
	Control	M27		22	40	38
	Control	M28		19	43	38
	Control	M29		20	40	40
	Control	M30		23	45	32