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Airgun blasts used in marine seismic surveys have limited effects on mortality, and no sublethal effects on behaviour or gene expression, in the copepod *Calanus finmarchicus*

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Seismic surveys use airguns that emit low frequency high magnitude sound to detect subsea resources and to map seabed geology. The effect of seismic blasts on *Calanus spp*., a key food source for commercially important fish, was assessed in field experiments. Immediate mortality of copepods was significantly different from controls at distances of 5 m or less from the airguns. Mortality 1 week after the airgun blast was significantly higher—by 9% relative to controls—in the copepods placed 10 m from the airgun blast but was not significantly different from the controls at a distance of 20 m from the airgun blast. The increase in mortality—relative to controls—did not exceed 30% at any distance from the airgun blast. Only two genes changed in response to the airgun blast; however, their function is unknown. There were no sublethal effects of the seismic blasts on the escape performance or the sensory threshold needed to initiate an escape response at any of the distances from the airgun blast that were tested. Results from these experiments suggest that seismic blasts have limited effects on the mortality or escape response of *Calanus sp*. within 10 m of the blast and no measurable impact at greater distances.

Keywords: acoustic, *Calanus*, predator escape response, subsea geological surveys

Introduction

Seismic surveys are used to detect petroleum, natural gas, and other subsea resources, and also to map seabed geology. Although underreported, the areal coverage of seismic surveys is significant. For example, according to figures from the Norwegian Petroleum Directorate, the extent of seismic surveys in the Norwegian Exclusive Economic Zone has been relatively stable over the last 25 years—about 133 000 vessel kilometres per year (Kvadsheim *et al*., 2017). In Australian waters, an average of 63 000 km of surveying for subsea petroleum (using seismic airguns) was conducted per year during 2014 and early 2015 (APPEA, 2016).

Airgun arrays have been the most widely used acoustic sources in marine seismic surveys during the past 50 years (Duren, 1988; Watson *et al*., 2016). Such surveys are often conducted in areas that support high-value fisheries and sometimes within or near the spawning grounds of commercially or ecologically important species, creating spatial and resource use conflicts between different stakeholders (e.g. Blanchard *et al*., 2014). A recent review of

70 laboratory and field studies found only a few reports describing physical damage to adult fish (e.g. mortality, barotrauma; Carroll *et al.*, 2017) all of which occurred when the fish were in proximity to the airguns (McCauley *et al.*, 2003; Popper *et al.*, 2005). Field data examining fish behaviour and their distribution in response to seismic blasts suggests that fish detect the airgun blasts and generally move away from the source in response (Dalen and Knutsen, 1987; Pearson *et al.*, 1994; Engås *et al.*, 1996; Løkkeborg *et al.*, 2012; Peña *et al.*, 2013), although that is not always the case (Peña *et al.*, 2013; Bruce *et al.*, 2018).

Investigations into the effects of seismic airgun blasts on ichthyoplankton and zooplankton are scarce, despite their importance in marine ecosystems. Fish larvae and juveniles, unlike larger fish, are unable to move away from a seismic survey transect line to avoid exposure to the airgun blasts. Survival of anchovy (*Engraulis encrasicolus ponticus*) and Mediterranean horse mackerel eggs (*Trachurus mediterraneus ponticus*) was reduced after exposure to airgun blasts (Kostyuchenko, 1973). Similarly, survival of northern anchovy (*Engraulis mordax)* eggs and yolk-sac larvae was reduced, and their swimbladders and otoliths damaged (Holliday *et al.*, 1987), as were the neuromasts in the lateral line (Booman *et al.*, 1996). Most of the damage that has been reported in fish larvae occurred when they were 1.5 m or closer to airguns and little or no effect has been observed at distances of >5 m (although see McCauley *et al.*, 2017 for an exception).

Very few studies have assessed the possible effect of seismic airgun blasts on invertebrates (Carroll *et al.*, 2017) and there are even fewer on planktonic crustaceans (Kosheleva, 1992; Pearson *et al.*, 1994; Christian *et al.*, 2003; Day *et al.*, 2016). Few of these studies have been conducted using realistic exposure levels (Christian *et al.*, 2003; Celi *et al.*, 2013; Day *et al.*, 2016). However, a recent field experiment reports high mortality in zooplankton populations at distances up to 1.2 km from the airguns (McCauley *et al.*, 2017). Modelled scenarios based on that finding suggests a mortality of 14% in zooplankton at a distance of 15 km from the seismic blast (Richardson *et al.*, 2017).

Copepods are the dominant metazoan in most pelagic communities (Schminke, 2006). In the North Atlantic, a region in which there is significant seismic survey activity, the *Calanus* spp. complex is the dominant copepod genus, numerically and by biomass (Melle *et al.*, 2014). Both juveniles and adults of many commercially important fish stocks (Atlantic cod, herring, capelin, mackerel, blue whiting) depend on the production of lipid-rich adult *Calanus* spp. during spring and summer (Melle *et al.*, 2014; Skjoldal, 2004), and interannual variability of *Calanus* spp. production is linked to recruitment in these species (Ellertsen *et al.*, 1989; Runge *et al.*, 1999; Kristiansen *et al.*, 2011). Additionally, *Calanus* spp. are important prey for larger zooplankton such as euphausiids (*Meganyctiphanes norvegica, Thysanoessa inermis*) and amphipods (*Themisto* spp.) and for the North Atlantic Right Whale (*Eubalaena glacialis*; Michaud and Taggart, 2011). There is no published information on the effect of seismic airgun blasts on *Calanus* spp. The objective of this study was to test if exposure to blasts from airguns used in seismic surveys affects mortality, predator escape response, or gene expression of *Calanus finmarchicus.*

Material and methods

The experiments were conducted during 2009 (Experiment 1) and 2010 (Experiment 2) at the Institute of Marine Research, Austevoll Research Station, Norway (60°05′20″N 5°15′57″E).

Source of animals and experimental design

Calanus finmarchicus were cultured at 13°C in 3000 l silos and fed a mixture of the algal species *Isochrysis galbana*, *Rhodomonas lens*, and *Chaetoceros muelleri* at a total cell concentration of 5×10^3–10^4 cells ml^{-1} (a mixture that is known to sustain healthy cultures, Runge *et al.*, 2016). Copepods were collected from the silos using a light trap (BellaMare, San Diego, CA, USA) and were carefully transferred to experimental bags that were positioned at different depths and distances from the airguns (Figure 1 and Table 1, see below). The experimental bags were 90 µm thick, 20 l plastic bags (Baca Plastindustri AS, Norway) filled with 5 l of seawater taken from the silo in which the animals were being cultured. These plastic bags are acoustically transparent to the frequency range emitted by the airguns [as established using Equation (6.3.9) in Kinsler *et al.*, 2000], but could have affected the fluid flow. The latter possibility is discussed below.

During the 2009 experiment, the plastic bags containing *C. finmarchicus* were deployed at a depth of 6 m, the same depth as the airguns, at horizontal distances of 0 m (i.e. between the two airguns), 0.7, 1.5, 3.0, and 5.0 m respectively from the centre of the airguns hanging from a floating raft (Figure 1 and Table 1). Additional bags were positioned at 0.3 m below the surface (5.7 m directly above the airguns). During the 2010 experiment, horizontal distances of 20 and 25 m from the cluster were added and 0.7 and 3.0 m were removed (Table 1).

At each location, two groups of experimental bags were deployed, each in triplicate. One group of bags contained 60 adult *C. finmarchicus* in each bag, which were used to determine changes in gene expression (genomics). The second group of bags contained 400 adult *Calanus* in each bag, which were used for behavioural analysis (∼300 animals) and to determine mortality (immediate and after 1 week; ∼100 animals). Copepods were counted and transferred to the bags between ½ and 1 h before exposure. The bags were sealed with no air space, placed in coolers, and brought directly to the site in a small boat. Deployment of the bags was randomized to ensure that differences in the time that copepods were in bags, or the time that bags were on the

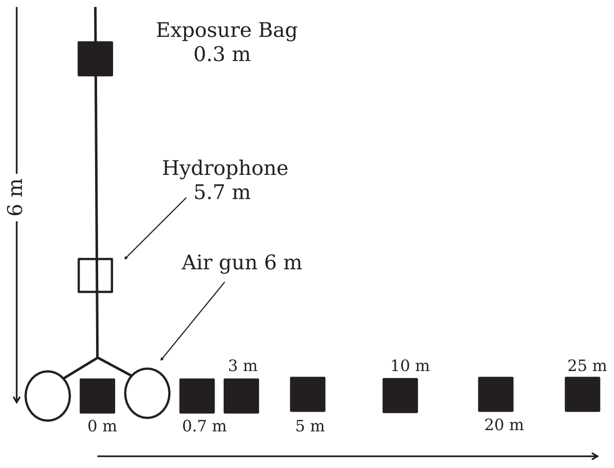

Figure 1. Schematic diagram of the position of both the controls and treatment bags (black squares) relative to the airguns (open circles). The open square denotes the position at which hydrophone measurements were made. The top of the illustration represents the sea surface.

Table 1. Distances from the centre of the airguns to the hydrophone, the acoustic doppler velocimeter (ADV) and the replicate sample bags (controls and those containing the copepods) during the experiments in 2009 and 2010.

	2009			2010		
Distance (m)	**Hydrophone positions**	**ADV measurements**	**No. of bags**	**Hydrophone positions**	**ADV measurements**	**No. of bags**
Control	–	2	6	–	2	6
5.7[a]	2	2	3	1	2	3
1.5[a]	2	–	–	–	–	–
0[b]	3	–	3	–	–	–
0.7	4	2	3	–	–	–
1.5	4	2	3	2	2	3
3.0	2	2	3	–	–	–
5.0	3	2	3	3	2	3
7.0	–	–	–	3	2	3
10.0	–	–	–	1	2	3
20.0	–	–	–	2	–	3
25.0	–	–	–	2	2	3

The distance “0” are measurements made between the two airguns which was positioned 0.5 m apart and 6 m below the surface. An ‘a’ identifies measurements made vertically above the air guns and a ‘b’ identifies measurements made between the airguns.

boat, did not affect the results. The bags were deployed from a floating raft at the prescribed distances from the airguns. Allocation of the bags to different distances was randomized using a random number generator.

At the experimental site a small weight was connected to the bags then the bags were lowered to the desired depth. Bags were held at depth for 15 min prior to the blast. The control groups were treated in exactly the same way, except that they were deployed after the blast then retrieved after the last treatment bags were collected. The temperature at the surface and at 6 m depth ranged from 14.8 to 15.4°C in 2009 and 2010.

The bags were collected and brought back to the laboratory (a 2 min boat ride) immediately after the seismic blast. Some of the copepods in the bags containing 60 animals were preserved in RNAlater (Qiagen) for genetic analysis (as described below).

Setup of the airguns

Two Bolt 1500 LL airguns, each with chamber volume of 4.31 (260 cu.in.), i.e. the total cluster volume equals 8.61 (520 cu.in.), were used in the experiment. The firing of the guns, and the measurements of the sound that they produced, were synchronized using the same standard source controller as is used in commercial seismic operations (TRISOR, WesternGeco). The vessel from which the airguns were deployed was leased from Fjord Instruments (WesternGeco). The airguns were deployed to a depth of 6 m using a crane on the vessel. Water depth at the blasting site was 40 m. The supply pressure to the airguns was kept as close to 13.8 MPa (2000 psi) as possible but varied between 13.0 and 14.4 MPa during the experiments. The number of samples collected at each distance for the 2009 and 2010 experiments is listed in Table 1.

Characterizing the exposures produced by the airguns

Sound pressure produced by the airgun blasts was measured inside the bags during exposures using a HD1-TC WesternGeco hydrophone. One sound measurement was taken at each exposure distance. Additional measurements were made at a depth of 12 and 0.3 m directly below and above the airguns. The nominal sensitivity of the hydrophone was $5.6\,10^{-11}\,\mathrm{V}\,\mu\mathrm{Pa}^{-1}$ with a flat frequency response between 0.7 and 500 Hz. The fixed and variable gains were compensated for before sampling the voltage, and the voltage signal was converted to a 16 bit digital signal at a sample rate of 4 kHz (0.25 ms steps). To avoid aliasing, the signal was low-pass filtered using a finite impulse response filter with a cut-off at 500 Hz (-3 dB) and a slope or roll-off of $18\,\mathrm{dB\,octave}^{-1}$ (third order). The hydrophone data set are available online (c.f. Handegard *et al.*, 2019).

In addition to the sound pressure pulse and associated particle acceleration (the latter was not measured in this study), the airguns also generate fluid flows as a result of the released air mass forming an oscillating cloud of bubbles. Fluid flow was measured using an Acoustic Doppler Velocimeter (ADV, Nortek AS, Norway), which measures three-dimensional fluid flow velocities within a $1\,\mathrm{cm}^3$ sampling volume (i.e. approximately the scale of the copepods being studied). Prior to the blasts, the surrounding water was seeded with a high density of either $MgCO_3$ particles (in the water) or *Rhodomonas* sp. (in the bags; $10^5\,\mathrm{cells\,ml}^{-1}$) as particle tracers for the ADV. The fluid flow velocity was measured at a sampling frequency of 16 Hz. The measurements were made at a depth of 6 m at the same distances from the airguns as the copepods. An additional measurement was made in the surface bag position 0.3 m above the airguns in 2009. Data were collected for a minimum of 30 s before and after the blast. Measurements were made inside and outside the experimental bags.

Characterizing the effect of airgun blasts on *C. finmarchicus*

Immediate post-exposure mortality was measured by counting the number of dead individuals in the treatment bags within 1 h after exposure. Longer-term mortality rates (7 days) were determined by transferring 100 live *C. finmarchicus* from each of the bags that contained 400 animals to 50 l tanks where they were fed as described above. Temperature was maintained at 12°C and flow rate in each tank was $120\,\mathrm{l\,h}^{-1}$. Dead *C. finmarchicus* were counted and removed every day for 7 days to obtain cumulative mortality.

The behaviour of animals after exposure to seismic airgun blasts was observed to assess whether there were any sublethal effects, specifically in reference to predator avoidance. The animals were removed from the experimental bags (those containing 400 animals) and gently transferred to a transparent tank

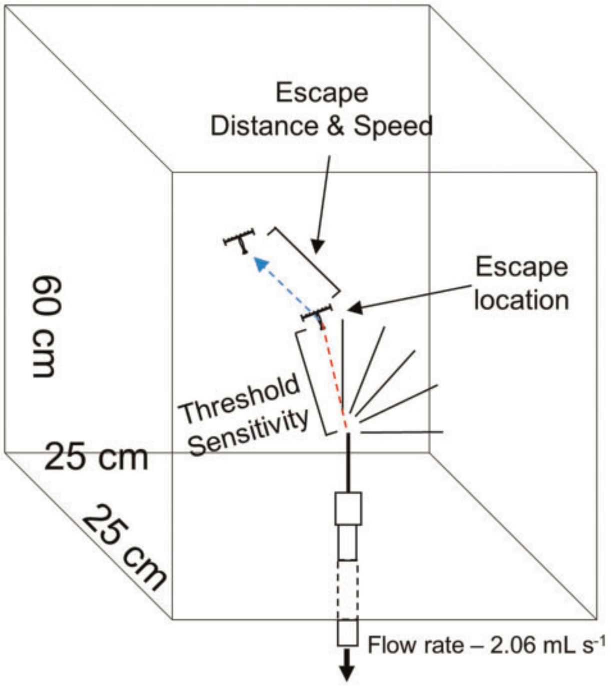

Figure 2. Schematic diagram showing the configuration of the experimental tank and the escape response variables measured on *Calanus finmarchicus*.

(19 cm × 14 cm × 50 cm; 13 l) for observations. Silhouette imaging was used to observe copepod behaviour in three-dimensional space. This optical setup provides fine-scale behavioural observations with an image quality that is unaffected by ambient light levels (described in Browman *et al.*, 2003). In short, the system consists of two orthogonally-oriented cameras with a far-red light emitting diode placed at the focal point of a biconvex collimating lens, the output beam (15 cm diameter) of which passes through an aquarium placed at the intersection of the two lines of sight. Video images were collected at 25 Hz sampling rate.

A siphon flow was used to create a stable fluid mechanical disturbance (Fields and Yen, 1996). The resulting flow fields are well-characterized and have been used to analyse copepod escape behaviour (Fields and Yen, 1997; Fields *et al.*, 2012; Kiørboe and Visser, 1999). The flow rate (2.0 ml s^{-1}) was generated by a gravity-forced drain through a 16-gauge (1.194 mm ID), stainless steel, and flat-tip hypodermic needle mounted 70 mm above the bottom of the tank. A constant head pressure was maintained by simultaneously returning the drained water to the top of the tank using a peristaltic pump. To diminish the disturbance to the calibrated flow field created by the siphon, incoming water was pumped back into the tank through a 105 mm diameter cylinder with a 35 µm mesh screen located just below the water's surface (Figure 2). The total volume filmed around the mouth of the siphon was ≈2.5 l (10 × 10 × 25 cm), to ensure that all of the responses by the copepods were observed. The velocity (V) of the water entrained by the siphon decreased exponentially with distance from the siphon as:

$$V = Q \cdot (4\pi \cdot r^2)^{-1}, \qquad (1)$$

where Q is the volume exiting the siphon (Fields and Yen, 1996). At the edges of the viewing area (5 cm from the siphon) the flow created by the siphon is 60 µm s^{-1}. This is well below the escape threshold for most species (Fields and Yen, 1997) and near the neurophysiological threshold for detection (Fields *et al.*, 2002).

The threshold and magnitude of escape response of *C. finmarchicus* to the flow from the siphon was quantified using three characteristics. The behavioural threshold of the escape response was determined as the distance from the flow source at which the copepod initiated an escape reaction (suction flow: see below). Once initiated, the magnitude of the escape reaction was assessed by the measuring the average speed of the entire escape reaction and the total distance travelled during the escape. The appendages involved in an escape reaction (and their motion) are well described (Fields *et al.*, 2012) and can easily be differentiated from a simple flick response or an attack response (Fields and Yen, 2002) based on the appendages used. Because the threshold for the escape reaction decreases with multiple sequential escapes (Fields, 2000), in cases where the flow re-entrained the same animal after an escape, only the first escape reaction was used for further analysis. Escape reactions that occurred below the mouth of the siphon, or whose location was obstructed by another animal in one of the views, were discarded. The escape distance was calculated as the cumulative distance travelled over the entire escape sequence. The distance was measured between sequential video frames to capture the total length of a complex non-linear path. The average speed of the escape reaction was calculated as the total distance travelled during the escape response divided by the duration of the entire escape reaction. Animal position, speed, and distance travelled were measured using custom designed software (Measure, by JASCO Research; described in Browman *et al.*, 2003).

Behavioural observations were made in a climate-controlled room at 12.5 ± 0.5°C. Observations were made on animals from each exposure distance in triplicate with 100–150 animals per replicate (2.6–4.0 animals l^{-1}). Each replicate was filmed for 30 min. Animals were not used more than once.

Microarray analysis

Microarray analysis was performed on 95 adult female *C. finmarchicus* (single animals) representing the six different groups: two control groups, one without any handling sampled directly from the tank (five parallels) and one with the same handling as the blast group (15 parallels), four groups exposed to the seismic blast at 0, 0.7, 1.5, and 5.0 m (ten animals each). As the experiment extends over 2 days, and time of day could have an impact on gene expression, animals were randomized according to the time of airgun blasts (see Table 2).

Two different EST libraries were constructed. RNA extracted from different stages of *C. finmarchicus* was used for library construction: egg, nauplii, copepodite II to V, adult female and male, unfertilized female. In addition, RNA from starved females and from individuals from all experimental groups was included. Total RNA was extracted using a combination of RNeasy Micro Kit (Qiagen) and Trizol as described in Eichner *et al.* (2014). The libraries were constructed using In-fusion SMARTer cDNA library construction kit (Evrogen) in accordance with supplied protocols, one with and one without application of RiboMinus$^{\mathrm{TM}}$ Eukaryote Kit for RNA-Seq (Ambion) for depletion of ribosomal RNA. Normalization was performed with Trimmer (Evrogen) according to the supplied protocol. Sequencing was done using 454 (Beckman Coulter Genomics).

Table 2. Characteristics of the escape response of *Calanus finmarchicus* adults.

Treatment	Total escapes (*n*)	Distance from siphon (mm)	Escape distance (mm)	Escape velocity (mm s^{-1})
2009	**286**	**9.5 (±0.2)**	**16.5 (±0.6)**	**92.6 (±2.2)**
2010	**244**	**4.7 (±0.1)**	**7.9 (±0.4)**	**50.1 (±1.1)**

Values are means (+/− SE).

Clustering and annotation were conducted using an in-house pipeline. This contains base calling by using Phred to assign quality scores to each base. For trimming and vector masking RBR and SeqClean respectively was used. Masking was done against *Escherichia coli* and univec core. Clustering and assembly was done with the TGI Clustering tool, which uses CAP3 for the assembly phase, using default parameters. The clustered sequences were blasted vs. UniRef 90. A significant hit was defined as one with an exact match of at least four characters and an Expectation value (E-value) of $<10^{-7}$ (using BLAST options "-e = 1e-7 -W4"). The sequences were matched to Gene Ontology with GO associations for UniProt (GOA).

A 44k Agilent oligo microarray was designed using the Agilent eArray program according to the supplier's instructions. For each contig, three different 60mer oligos with 3 prime biases were chosen for each singleton. Besides custom oligos, spikes and controls were included following Agilent recommendations. Additional linkers to attach the 60mer oligos were used.

All animals preserved in RNAlater® were photographed and grouped for analysis on the basis of size measurements (NIH ImageJ). The experimental replicates (three at each treatment distance) and the controls (five in total) were exposed at a different time of the day (three replicates), which could have an impact on gene expression. Therefore, time of day was treated as a potential factor in the variance of the data. For all further steps, animals were randomized to exclude batch effects. RNA was isolated from individual animals using the RNeasy Micro kit (Qiagen) in combination with Trizol extraction as described above. DNAse treatment was done on column according to standard protocol. The RNA samples were frozen at −80°C until usage. One aliquot was used for RNA integrity and quantity analysis using the Agilent 2100 Bioanalyzer and NanoDrop Spectrophotometer (OD 260:280 and 260:230 ratios). Another aliquot was used for cDNA synthesis and labelling. Amplification, labelling, and hybridization were carried out with Low Input Quick Amp Labelling Kit for one-color Microarray-Based Gene Expression analysis (Agilent). One hundred nanograms total RNA was used as input for the reaction. Labelling efficiency and quantity of labelled cDNA was determined using a NanoDrop Spectrophotometer. Scanning was performed using an Agilent scanner (5 µm resolution) with settings according to standard protocol. For data extraction, feature extraction software 9.5.3 was used. Data analysis was done in J-Express (Dysvik and Jonassen, 2001). Correspondence analysis was performed on log2 transformed data. Significance analysis of microarray (SAM) analysis was executed for the different groups.

Data analysis

Mortality was analysed from data collected immediately after the exposure and 7 days after exposure. To test the dose effect of the seismic impact, mortality was modelled separately for the two different years using a generalized linear model (GLM) (binomial) with distance from the source as a factor. Data analysis was conducted using R (R Core Team, 2014).

The three components of the behavioural response were analysed as a function of distance from the seismic blast using a linear regression. In cases where no significant difference in the behavioural variable with distance from the airguns was found, the data from all distances were pooled and tested against the control (*t*-test) to determine if copepods exposed to the seismic blast responded differently from the control. The behavioural data were analysed using Systat Software (Sigmaplot version 11.2, San Jose, CA, USA).

Genetic data analysis was performed using J-Express software (2012). Samples were grouped as follows: (1) distance to airguns, (2) daytime (early: 10:00–13:00, midday: 13:00–16:00, late: after 16:00), (3) size of animal (smaller than median, about median, and bigger than median), and (4) airgun blast number. SAMs (*q*-value 0.05; Tusher *et al.*, 2001; 400 permutations) were used to identify differentially express genes within the different groups.

Results

Characterizing the airgun exposures

Each blast from the airguns produced a peak in sound pressure associated with the acoustic pulse (hydrophone; Figure 3a), followed by a peak in the fluid flow (ADV; Figure 3b). The time-lag between blast and peak values varied by orders of magnitude between the sound pressure pulse and the hydrodynamic fluid flow, with pressure values reaching a maximum in milliseconds after blasts and fluid flow speed achieving a maximum after seconds (Figure 3). The peak pressures measured in the acoustic nearfield and appurtenant estimated sound exposure levels (SEL) integrated over the duration of the pulse, between the two airguns (0 m) and at 25 m, was 1363 kPa, yielding SEL = 221 dB re 1 μPa^2 s, and 25 kPa yielding SEL = 183 dB re 1 μPa^2 s, respectively (Figure 4).

The pressure recordings made 0.3 m above the airguns showed higher pressure amplitudes than that at the corresponding horizontal distance. The recordings close to the surface had lower amplitudes than that at the corresponding horizontal distances. This is as expected because of interference from the surface-reflected pressure wave (ghost wave). The sampling rate was too low to accurately determine the rise times at different ranges, but the approximate range was 1.2 to 1.7 ms.

By fitting the $SEL \sim SEL_0 + b \cdot \log_{10}(r)$ to the observations, we obtained estimates of $\hat{b} = -16$ and $\widehat{SEL_0} = 208\ dB$ re 1 $\mu Pa^2 s$, which indicates a transmission loss between spherical and cylindrical spreading (Figure 4a). A comparison of the pressure recordings inside and outside the bags at the same locations showed no significant difference in magnitude, confirming that the bags were acoustically transparent (Pearson *et al.*, 1994).

Fluid flow speed as measured by the ADV was highest near the airgun cluster and decreased exponentially with distance (Figure 5a). Calculated maximum horizontal and vertical speeds

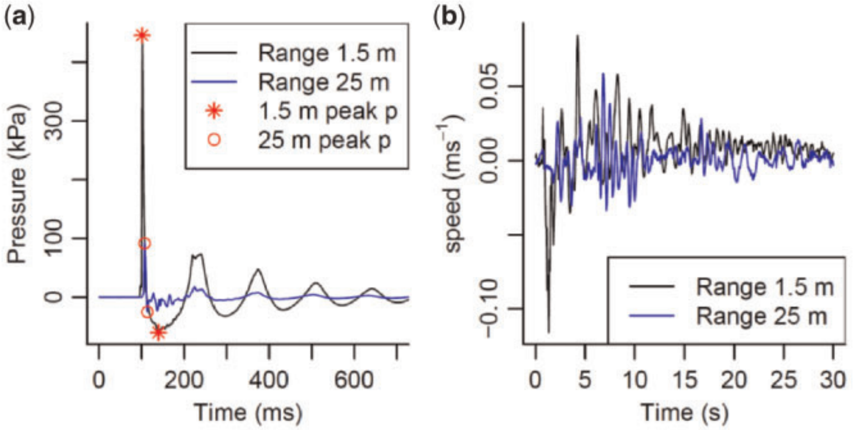

Figure 3. Airgun blast exposures characterized by (a) the acoustic pressure recorded from hydrophones and (b) the vertical fluid flow speed measured from the Acoustic Doppler Velocimeter as a function of time. Blue and black (gray and black in print) lines denote the observations taken at 1.5 and 25 m, respectively. In (a), the upper and lower asterisks and circles denote the positive 0-p$^+$ and negative 0-p$^-$ pressure peaks, for the 1.5 and 25 m range, respectively. Note the different timescales on the two plots.

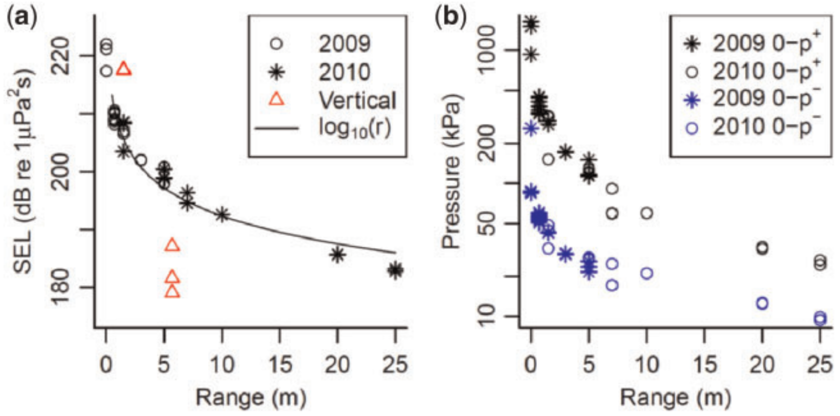

Figure 4. (a) The calculated sound exposure level (SEL) and (b) the zero to positive peak pressure (0-p$^+$) and the absolute value of the zero to negative peak pressure (0-p$^-$) as a function of distance from the airguns. The asterisks and circles denote the 2009 and 2010 data, respectively, and the triangles are observations vertically above the airguns from 2009.

were 0.60 and 0.42 m s^{-1} respectively at the sound source and decreased rapidly to 0.03 m s^{-1} at 1.5 m from the source. Fluid flow speed data were fit to an exponential function:

$$S = S_{max} \cdot \exp(-bd), \qquad (2)$$

where S equals speed at distance, d, from the source in metres. S_{max} (maximum speed) and the coefficient, b, were solved through numerical iteration using Sigmaplot (V11; Systat Software; San Jose, CA USA). Fluid speed was measured inside and outside the experimental bags (Figure 5b). The speed at 1.0 m from the source was 35% higher inside the bag than outside the bag. At 5 m from the source there was no difference in the fluid speed measured inside and outside the bag.

Responses of *C. finmarchicus* to airgun exposures

Mortality

Results of the 2009 and 2010 experiments were generally consistent. Immediate mortality (within 1 h of exposure) was significantly different from controls at distances of 5 m or less from the airgun. Overall, immediate mortality in both the exposed copepods and the controls were significantly higher in 2010 compared with 2009 (GLM, $p < 0.0001$; Figure 6a). In 2009, the immediate mortality was significantly higher than the controls at distances of 0, 0.3, and 5 m from the seismic gun ($p = 0.0058$; $p < 0.0001$; $p < 0.0001$). In 2010, the immediate mortality was significantly higher than the controls only directly in front of the seismic gun (0 and 1.5 m; $p = 0.0111$ and $p = 0.045$). After 1 week, the cumulative mortality was higher in 2009 compared with 2010 (GLM, $p = 0.0203$; Figure 6b). Mortality 1 week after the airgun blast was significantly higher—by 9% relative to controls—in the copepods placed 10 m from the airgun blast but was not significantly different from the controls at a distance of 20 m from the airgun blast. Compared with the controls, significantly greater mortality occurred at 0, 0.3, 0.7, and 5 m in 2009 ($p < 0.0001$; $p = 0.00013$; $p = 0.0126$; $p < 0.0001$) and at 0.3, 1.5, and 10 m in 2010 ($p < 0.0001$; $p = 0.0155$; $p = 0.0087$). The increase in mortality—relative to controls—resulting from the airgun blast did not exceed 30% at any distance from the airgun blast.

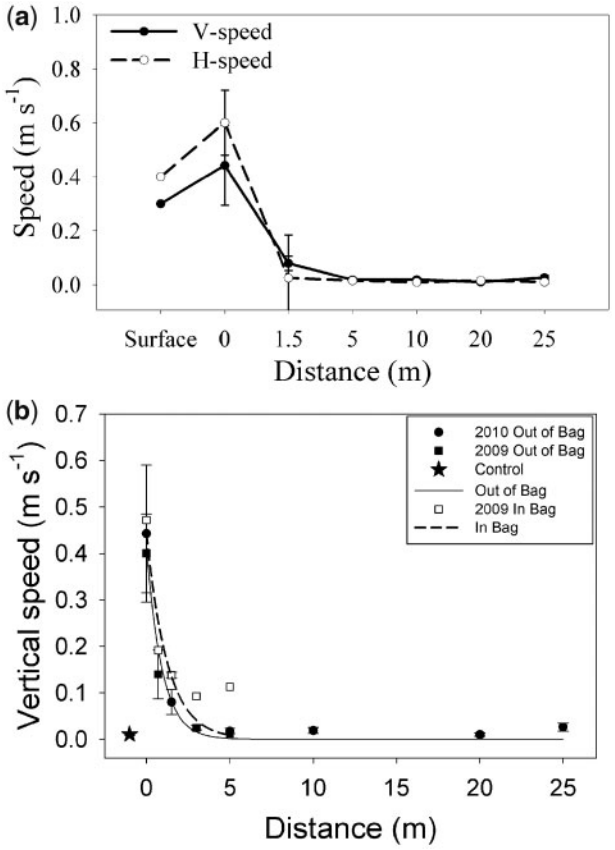

Figure 5. Acoustic Doppler Velocimeter measurements. (a) Maximum fluid speed inside the bags in the horizontal (H) and vertical (V) direction. (b) Fluid speed in the vertical direction measured inside and outside the experimental bag at different distances from the airguns. Measurements within the bag were made only during the 2009 sampling year.

Effects of airgun exposure on the escape behaviour of C. finmarchicus

In all, 530 escape reactions (286 escape reactions in 2009 and 244 in 2010) were analysed to evaluate the effect of the airgun blasts on the behavioural responses of *C. finmarchicus* (Table 2). Each year was analysed individually (Figure 7). There were no significant effects of distance from the seismic blast on any of the behavioural metrics (Supplementary Table S1). Nor were there any differences in behaviour between unexposed (control) and exposed groups (Supplementary Table S2).

Genetic analysis

Neither the time after exposure nor the size of the animal had any discernible effect on gene expression (Figure 8a). Copepods positioned 1.5 and 5 m from the seismic blast exhibited elevated gene expression at two oligos (unannotated genes; Figure 8b). There was no differential gene expression relative to the controls in copepods exposed to airgun blasts at distances of >5 m.

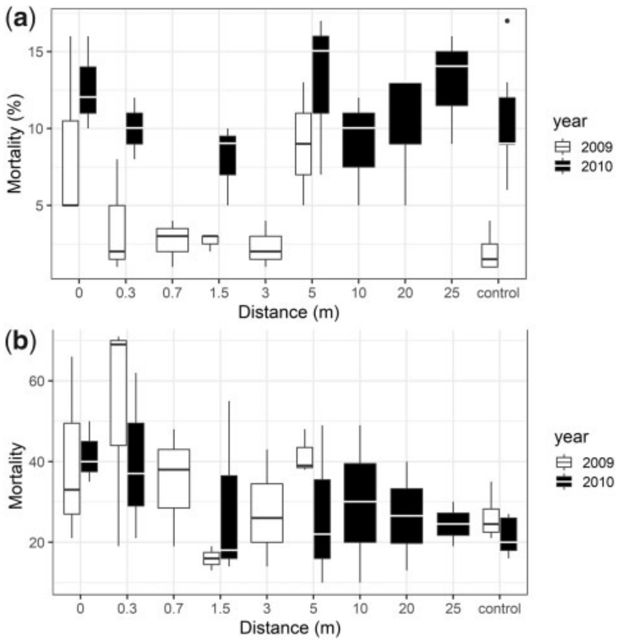

Figure 6. *Calanus finmarchicus* mortality in the experimental bags measured (a) immediately after exposure to the airgun blast and (b) after 1 week. The boundary of the box denotes the 25th and 75th percentiles, and the horizontal line denotes the mean. The whiskers (error bars) above and below the box indicate the 90th and 10th percentiles, respectively. Overall, the airgun blast caused higher mortalities compared with controls but only at close range (<5 m instantaneously and <10 m after 1 week; see text for details).

Discussion

Effects of exposure to seismic airgun blasts on *C. finmarchicus*

Seismic airgun blasts had no effect on the escape response of *C. finmarchicus*. There was, however, a significant change in the expression of two genes of unknown function and a significantly higher immediate mortality at distances of <5 m from the airgun and a higher cumulative mortality (7 days after exposure) at a distance somewhere between 10 and 20 m from the airgun. However, the increase in cumulative mortality after 1 week—relative to the controls—did not exceed 30% at any distance from the airgun blast. This higher mortality may be partly because of bag effects. Flow speed at distances up to 5 m from the source was higher in the bag than outside the bag (Figure 5b) and may have caused higher mortalities close to the airguns. Therefore, mortality of free-swimming animals (outside the bag) might have been lower than what was observed inside the bags.

These results are consistent with previous field studies of acoustic impact on a variety of invertebrates, none of which showed increased mortality because of seismic airgun blasting. For example, in scallops up to 10 months after exposure (Parry *et al.*, 2002; Harrington *et al.*, 2010; Przeslawski *et al.*, 2018b), clams 2 days after exposure (La Bella *et al.*, 1996), or lobsters from weeks (Parry and Gason, 2006) up to 8 months after exposure (Payne *et al.*, 2007; Day *et al.*, 2016). In contrast, Day *et al.* (2017) reported elevated mortality in scallops 120 days after exposure to airgun blasts. Mortality increased (by up to 15%) as the number of blasts to which animals were exposed increased. In

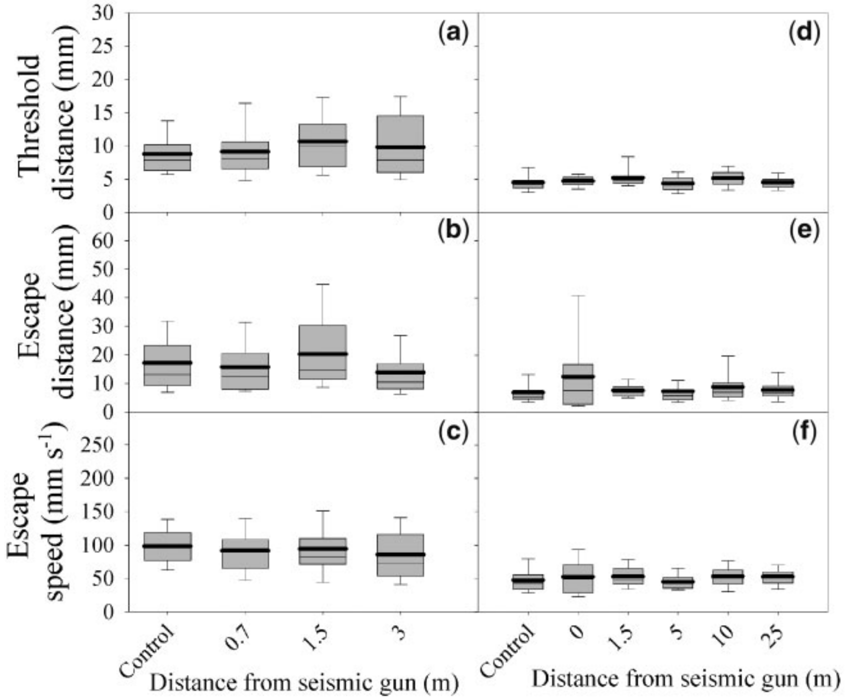

Figure 7. Behavioural responses of *Calanus finmarchicus* in threshold distance (a, d), escape distance (b, e) and escape speeds (c, f), for the 2009 (a–c) and 2010 (d–f) experiments, respectively, as a function of distance to the seismic airguns. The boundary of the box denotes the 25th and 75th percentiles, and the thin and bold line denotes the mean and median, respectively. The whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. No statistically significant difference was found between exposed groups and the control groups in either year.

other studies, there was no evidence of reduced abundance or catch rates in plankton (that would indicate large-scale mortality) a few hours after exposure (Parry *et al.*, 2002) or in benthic organisms such as reef-associated invertebrates 4 days after exposure (Wardle *et al.*, 2001), snow crabs up to 12 days after exposure (Christian *et al.*, 2003), or shrimp 2 days after exposure (Andriguetto-Filho *et al.*, 2005).

There were no sublethal effects of the seismic blasts as assessed by the copepods' escape reaction and gene expression. The exposed copepods showed no change in the threshold stimulus needed to initiate an escape reaction or in the distance or speed of the escape response. These results suggest that the airgun blast did not cause significant morphological damage to the antennae nor to the mechanosensors that measure fluid mechanical disturbances (Fields *et al.*, 2002).

Airgun exposure resulted in a change in the expression of only two genes (unannotated), which were significantly up-regulated, and only at 1.5 and 5 m from the airguns. *Calanus finmarchicus* differentially expresses a range of genes involved in protein folding, transcription, translation, and metabolism when exposed to high stress environments, including heat shock (Lauritano *et al.*, 2012; Semolina *et al.*, 2015). Airgun exposure did not produce differential expression in any of the heat-shock stress genes, supporting the conclusion of the behavioural assay; airgun blasts do not strongly affect these copepods physiologically and the effects are limited to distances of 5 m from the blast.

Our observations, and the results of the studies mentioned above, are inconsistent with a recent report concluding that airgun blasts produced high mortality in zooplankton at distances up to 1200 m (McCauley *et al.*, 2017). McCauley *et al.* reported the equivalent of the immediate mortality that was measured in this study. Unlike McCauley *et al.* exposure to airgun blasts in this study did not result in immediate mortality at distances >5 m from the airgun.

The bags in which the copepods were held in this study were transparent to the acoustic pressure, suggesting that the pressure pulse itself was insufficient to cause high mortality. Importantly, McCauley *et al.* (2017) reported an SEL of 156 dB re 1 μPa^2 s at a range of 509–658 m and reported mortality in the zooplankton, whereas the SEL of our setup was 186 dB re 1 μPa^2 s at a range of 25 m and we observed no mortality at that distance. Therefore, it is difficult to reconcile the high mortality reported by McCauley *et al.* (2017) with the low mortalities reported in the body of earlier research cited above and in the experiment that we report here. It is possible that our results can be attributed to *C. finmarchicus* having a high tolerance to airgun blasts and that other species would be more sensitive. Other possible causes for the high mortality observed by McCauley *et al.* (2017)—unrelated to the airgun blast itself—could be bubbles because of cavitation or small-scale shear produced by the sampling boat (see Bickel *et al.*, 2011). Additional field studies, coupled with more controlled laboratory experiments, are required to resolve these inconsistencies (Przeslawski *et al.*, 2018a).

Realism of the exposures

The airgun setup used in this experiment was operated at similar pressures but was an order of magnitude smaller (8.6 l) than a full airgun array applied for two- and three-dimensional seismic surveys. Typical two- and three-dimensional airgun arrays consist of

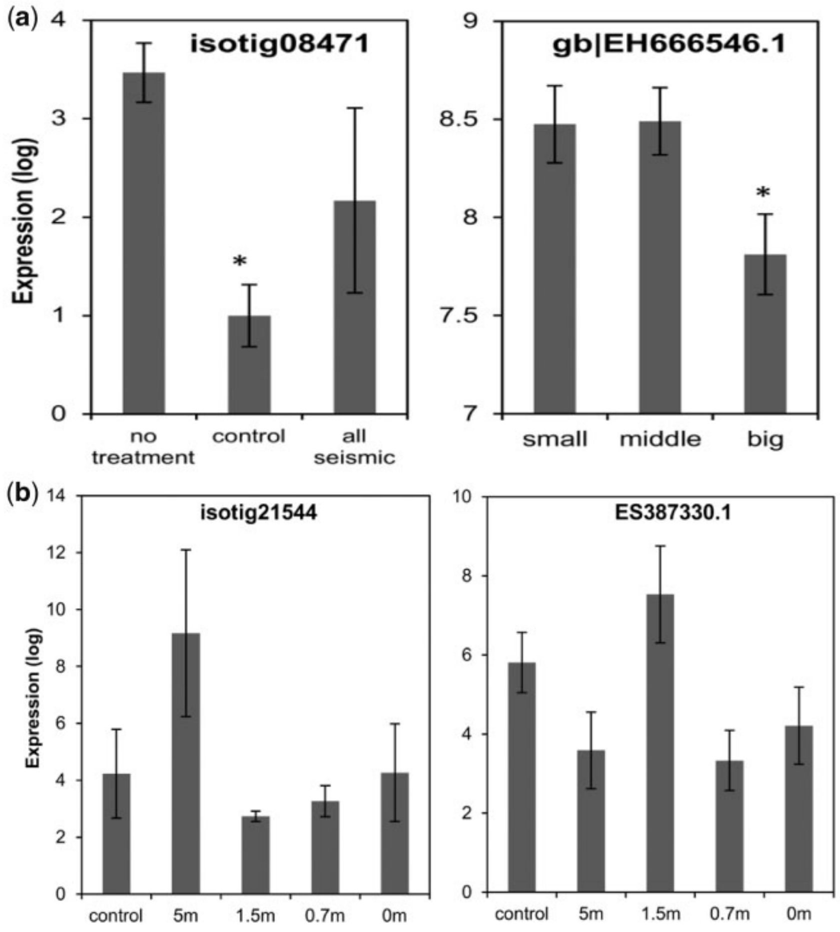

Figure 8. (a) Differentially expressed genes in untreated groups vs. control groups of *Calanus finmarchicus* (isotig08471, left panel) and between different sizes classes (EH666546.1, right panel). Small individuals are those that are 0.05 mm or smaller than the median (2.60 mm) of all analysed animals, big animals are defined as 0.05 mm or larger than the median of all analysed *C. finmarchicus*. Bars indicate confidence interval ($\alpha = 0.05$). Asterisks are those that are significantly different. (b) Genes expressed differently in the different blast groups. Only two genes (isotig21544 and ES387330.1) were differentially expressed in *C. finmarchicus* that were placed at different distances from the airguns. Neither gene has been annotated.

12–48 airguns ranging in total chamber volume from 20 to 87 l (1220–5300 in^3) operating at 13.8 MPa (2000 psi; Slabbekoorn *et al.*, 2019). The applied airgun setup corresponds to parts of ordinary applied airgun arrays both in two- and three-dimensional surveys and is, therefore, representative of what the organisms are exposed to. Similar basic setups have been used by several research groups performing stationary and controlled experiments on the effects of airguns on marine organisms (Dalen and Knutsen, 1987; Holliday *et al.*, 1987; Kosheleva, 1992; Pearson *et al.*, 1992, 1994; Booman *et al.*, 1996; Wardle *et al.*, 2001; Christian *et al.*, 2003; Popper *et al.*, 2005).

Because peak pressure scales with the cube root of volume (Caldwell and Dragoset, 2000), the peak pressure of the industry airgun arrays are 2.2–3.3 times higher than the airgun setup used in our experiment. In the far field, the pressure will be linearly proportional to the number of airguns in the array, but the experiment that we conducted was at distances typically found *within* or close to an array. An estimate of the maximum pressure *within* an array can be approximated by adding the estimated pressures referred to 1 m from the two largest guns (source levels), then applying spherical spreading that corresponds to half of the nominal airgun spacing (Caldwell and Dragoset, 2000). Our setup had a spacing of 0.5 m with a 2.2–3.3 times lower pressure than most industry standard setups. The maximum pressures that we observed would correspond to two airguns with a nominal spacing of 3–3.6 m [this is determined by reversing the calculation outlined in Caldwell and Dragoset (2000)]. Importantly, although the airgun setup used in this study is smaller than the industry airgun array standard, the pressure waves created are very similar at the distances examined in this study.

What is damaging marine organisms?

There are several potential sources of damage (some of which could be mortal) to animals exposed to the airgun blasts: the acoustic pressure pulse and associated particle acceleration and velocity caused by the acoustic pulse itself, and/or fluid motion occurring after the seismic pulse caused by bubble formation of the released air. Most studies examining the impact of seismic survey activity on organisms measure the acoustic signal, i.e. the propagating acoustic waves result in fluctuations in acoustic pressure, and associated particle displacement, particle velocity, and particle acceleration. Changes in pressure are measured directly and may harm animals, e.g. low pressures may damage organisms that have discrete areas of different density in their bodies (e.g. gas filled enclosures or lipid storage vacuoles; Bamber and Seaby, 2004). The time averaged intensity (energy per unit area per unit time) of sound is defined as the integrated product of pressure and particle velocity (Carey, 2006) and provides a measure of its potential damage. The acoustic particle acceleration has been cited as an important measure to characterize the impact of sound (Nedelec *et al.*, 2016) and is particularly relevant to hearing in fish because the otoliths are essentially acceleration sensors. It is possible to relate sound pressure and particle velocity through the conservation of acoustic momentum and assuming perfect surface reflections, but acceleration can also be measured directly (Sigray and Andersson, 2011). This should be done in future studies.

Acoustic pulses from reflections at the surface (ghost echoes) invert the pressures and cause a drop in pressure. The amplitudes of the negative pressure fluctuations are typically lower than the maximum pressures (e.g. Figure 4) but, depending on the airgun configuration, the negative pressure fluctuations may induce cavity clouds in regions close to the airguns (Khodabandeloo and Landrø, 2018). Collapsing cavity bubbles are used to kill or stun animals by snapping shrimps (Versluis *et al.*, 2000) and, because of the stronger pressure drop induced by the airgun arrays, it is expected that the cavity collapses are stronger than those of snapping shrimps (Khodabandeloo and Landrø, 2018). Our setup did not cause pressure drops great enough to induce cavity clouds, but it is important to note that this effect will not extend beyond the range of an airgun array.

The airgun blasts also generate large-scale fluid motion that may have an impact on the copepods (Figure 5). This is not the oscillatory particle acceleration associated with the acoustic pulse, but rather directional water motion that occurs over longer duration than the airgun pulse itself. If the flow generates enough shears along the surface of the organism, it may cause morphological damage. This is consistent with experiments that show elevated mortality in zooplankton caused by vessel propellers (Bickel *et al.*, 2011) and may explain the elevated mortality observed close to the seismic blast in this study.

Whether it is the sound pulse itself (i.e. the energy, peak pressures, or particle acceleration), the (turbulent) fluid flow occurring more slowly (i.e. not related to the sound pulse), or other effects such as the bubble cloud that caused the higher mortality in the copepods placed near the seismic blast, remains unknown and will have to be investigated by independently manipulating these different components (e.g. Schuijf, 1975). Regardless of the mechanism, the effects of seismic airgun blasts on *C. finmarchicus* are much less than on the zooplankton reported by McCauley *et al.* (2017) and, as a result, model assessments of the broader impacts of seismic surveys on zooplankton (such as Richardson *et al.*, 2017) will have to be revisited.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

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