Exposure to seismic air gun signals causes physiological harm and alters behavior in the scallop *Pecten fumatus*

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Seismic surveys map the seabed using intense, low-frequency sound signals that penetrate kilometers into the Earth's crust. Little is known regarding how invertebrates, including economically and ecologically important bivalves, are affected by exposure to seismic signals. In a series of field-based experiments, we investigate the impact of exposure to seismic surveys on scallops, using measurements of physiological and behavioral parameters to determine whether exposure may cause mass mortality or result in other sublethal effects. Exposure to seismic signals was found to significantly increase mortality, particularly over a chronic (months postexposure) time scale, though not beyond naturally occurring rates of mortality. Exposure did not elicit energetically expensive behaviors, but scallops showed significant changes in behavioral patterns during exposure, through a reduction in classic behaviors and demonstration of a nonclassic "flinch" response to air gun signals. Furthermore, scallops showed persistent alterations in recessing reflex behavior following exposure, with the rate of recessing increasing with repeated exposure. Hemolymph (blood analog) physiology showed a compromised capacity for homeostasis and potential immunodeficiency, as a range of hemolymph biochemistry parameters were altered and the density of circulating hemocytes (blood cell analog) was significantly reduced, with effects observed over acute (hours to days) and chronic (months) scales. The size of the air gun had no effect, but repeated exposure intensified responses. We postulate that the observed impacts resulted from high seabed ground accelerations driven by the air gun signal. Given the scope of physiological disruption, we conclude that seismic exposure can harm scallops.

aquatic noise | acoustic stress | hemolymph | reflex behavior | bivalve

Seismic surveys are used to explore the geological structure of the seafloor, using an array of air guns that are slowly (*ca.* 3 km·h^{-1} to 4 km·h^{-1}) towed up and down parallel track lines, generating intense, low-frequency acoustic signals every 8 s to 15 s (every 20 m to 30 m) through the instantaneous release of highly compressed air (1). Surveys operate continuously 24 h a day and last from weeks to months, covering an area hundreds to thousands of square kilometers, with a nearly ubiquitous global distribution. Seismic surveys are commonly used to explore for subseafloor oil and gas deposits, but are also necessary for identifying sites for carbon sequestration, a developing means to cope with anthropogenic production of CO_2 (2).

Seismic signals have a potentially important, yet poorly understood, anthropogenic impact on life in the marine environment. To date, the effects of exposure on whales have received considerable attention (3), and fishes have received somewhat less attention (4). Even less is understood regarding the effect of exposure on invertebrates (5). Field-based experiments have rarely been conducted on invertebrates, and the limited available evidence shows little effect on crab and lobster larvae, while zooplankton show a high level of taxa-specific mortality (6–8). Tank-based experiments simulating exposure have resulted in high levels of damage in several molluscs, including scallop veliger larvae (9) and several species of squid and octopus (10–12), although it is unclear how laboratory experiments conducted in tanks may translate into the field (13, 14).

Among marine invertebrates, bivalves would seem to be particularly vulnerable, as their benthic and largely sessile habit leaves little capacity to avoid the waterborne and groundborne energy of seismic signals. Even the relatively mobile scallop, which swims using jet propulsion, has little chance of escaping exposure, as their energetically demanding form of locomotion leaves even the most competent swimming species depleted after about 4 min of swimming, during which they can cover, at most, a modest 30 m (15, 16).

Bivalves perform a diversity of roles within an ecosystem, including improving water quality through reduction of turbidity, thus increasing light availability for underwater plants; exerting both top-down and bottom-up control on phytoplankton, ameliorating the anthropogenic nutrient inputs that drive eutrophication in coastal waters; and the bio-deposition of pelagic primary production nutrients into benthic systems (17). Bivalves also have substantial socioeconomic value, making potential harm a considerable issue. Global production of bivalves has been constantly increasing over the past 6 decades, as the total annual production (capture + aquaculture) of mussel, oyster, scallop, and clam fisheries has increased from 1 million tons in 1950 to over 14 million tons in 2014, with an annual value of nearly US$17 billion (18). Not only are bivalves increasingly

Significance

Seismic surveys are used around the world as the primary means to explore for oil and gas deposits. Almost nothing is known regarding the impact of these sound signals on marine invertebrates. In this study, the physiological and behavioral effects of exposure on a commercially important bivalve, the scallop, were quantified. Following a field-based air gun exposure regime, exposed scallops were found to have significantly increased mortality rates; disrupted behavioral patterns and reflex responses, both during and following exposure; and altered hemolymph biochemistry, physiology, and osmoregulation capacity. These results indicate that air gun exposure has a harmful impact on scallops and raises concern over the impact on bivalves, due to their global ecological and economic importance.

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relied upon for food security, but a diverse range of value-added industries, including pharmaceuticals, agriculture, building materials, cosmetics, clothing, and jewelry, have developed to take advantage of fishery by-products (19). This increase in global demand has driven advancements of the bivalve mariculture industry, recognized as one the most environmentally sound and sustainable forms of aquaculture, since bivalve aquaculture does not require the addition of food nutrients into a system which may drive eutrophication, as in other forms of aquaculture (19, 20). Rather, bivalves actually improve water quality through biofiltration and biodeposition of organic matter from the water column, assisting in the feeding of other benthic organisms (20). Bivalves have also been incorporated into finfish aquaculture in an effort to reduce the nutrient load, a practice called integrated multitrophic aquaculture (21).

There is considerable overlap between areas supporting bivalve aquaculture and capture fisheries and areas of interest for seismic exploration (Fig. 1). This overlap can result in conflict, as was the case when a scallop mass mortality in Australia's Bass Strait, in which the fishing industry estimated a loss of 24,000 tons of scallops, was anecdotally attributed to a seismic survey. Conflicts between users of marine resources are not new, with oil and fishery industries frequently at odds worldwide and a variety of regulatory and resolution approaches adopted to mitigate the tension (22). Despite the rise in prominence of various forms of renewable energy, oil and gas consumption continues to grow. Exploration using marine seismic surveys will be necessary to meet demand and to ameliorate the resulting carbon emissions, requiring the expansion of surveys into areas of high biodiversity (23) and high-latitude polar seas, particularly the Arctic (24, 25), where bivalves live long, slow lives (i.e., slow to reach reproductive age) with narrow physiological constraints (26).

Here, we use a field-based approach to assess the impact of exposure of a single air gun on the scallop *Pecten fumatus*. Scallops were exposed to air gun signals in a field setting designed to emulate a survey in real-world conditions, and were assessed for mortality, physiology, and behavioral responses over acute (i.e., hours to days postexposure) and chronic (i.e., months postexposure) scales to provide detailed and systematic time series sampling. To evaluate whether exposure causes mortality, mortality rates were assessed through observation at time points ranging from immediately after exposure to 4 mo postexposure. Video recordings of scallops were used to analyze behavior before, during, and following exposure to determine whether air gun signals result in the behavioral alterations posited by the mortality hypothesis. Following exposure, the scallops' recessing reflexes were evaluated by measuring the time taken to recess into the substrate, a state considered the "natural" position of *P. fumatus* (27) and other scallops with a convex lower valve and a flattened upper valve (28, 29). Sublethal physiological effects were quantified through assays of the cellular and humoral components of the hemolymph, the invertebrate analog to vertebrate blood, with measurement of the pH, number of circulating hemocytes, and hemolymph biochemistry compared between treatments. Although there are few investigations into the various parameters of pectinid hemolymph, scallops are osmoconformers and would not be expected to show considerable variation in hemolymph chemistry (i.e., pH, ion concentration) when maintained in stable seawater conditions. The general bivalve hemocyte response, based on a review of studies of mussels, oysters, and clams, is much more dynamic, responding to a range of biotic (reproductive state, nutritional condition, size/age) and abiotic (water temperature, salinity, exposure to pathogens) factors (30). The typical bivalve response to acute environmental stressors described in ref. 30 is an increase in hemocyte numbers, either through cell proliferation or mobilization of cells from tissues to circulation, although some stressors elicit a decrease in hemocyte numbers, driven by cell death or immobilization within tissues. Scallops show a similar response following exposure to environmental pollution (31, 32). We hypothesize that *P. fumatus* will show a response to seismic air gun exposure similar to the response observed in scallops and other bivalves in response to other acute stressors: a stable pH and biochemistry and a transient increase in circulating hemocytes following exposure, with a return to baseline or control levels shortly (i.e., with days) after exposure.

Combining an assessment of mortality rates, analysis of behavioral responses in the field during real-world exposure, and quantification of physiological responses to exposure to seismic air gun signals will provide a conclusive understanding of how scallops are affected and advance our understanding of the potential impact exploration of the seabed may have for bivalve populations.

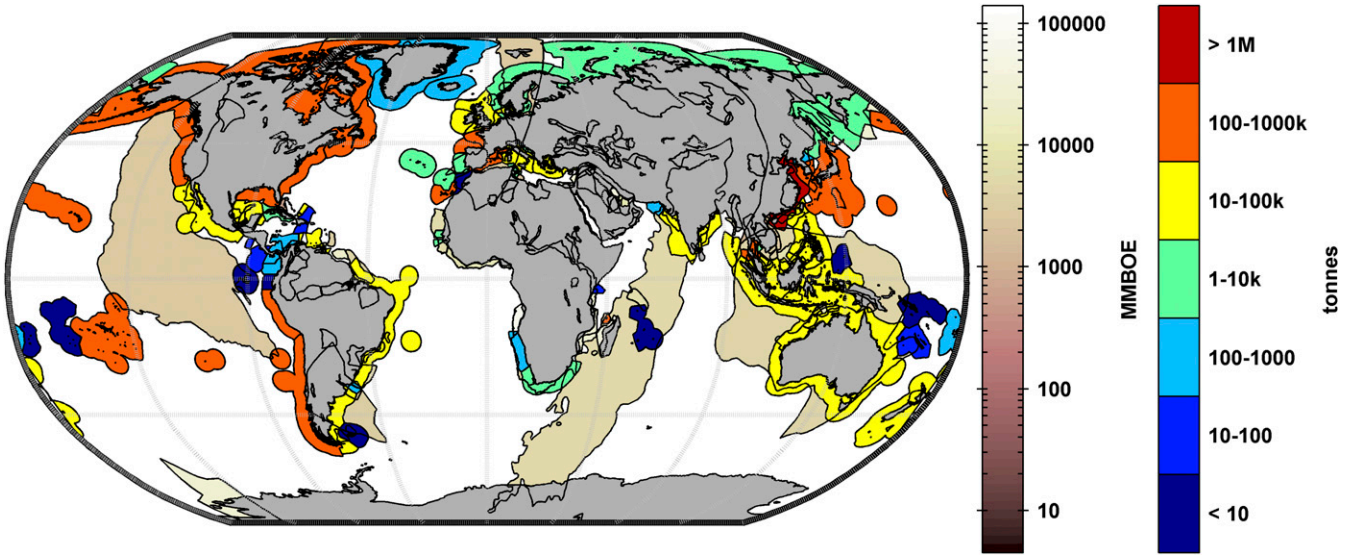

Fig. 1. Estimated global undiscovered oil [million barrels of oil equivalent (MMBOE) scale] and bivalve (mussels, oysters, scallops, clams) production (tonnes scale). Undiscovered oil estimates were sourced from US Geological Survey (71). Bivalve capture and aquaculture fishery production data were sourced from Food and Agriculture Organization (18).

Results

Seismic Exposure. The calculated sound exposure levels (SEL) and measured ground roll acceleration for the different scallop experimental regimes are given in Table 1, with the best fit curves used to determine values shown in Fig. S1. Compared with the modeled levels of a hypothetical 3,065-in^3 full-scale array (Table 2 and Fig. S2), the scallops exposed to one pass in the present study experienced exposures equivalent to a large commercial array passing within a 114- to 875-m range, the scallops exposed to two passes experienced the equivalent of a full scale array at 114- to 500-m range, and the scallops exposed to four passes received the equivalent of a full scale array passing at 114- to 275-m range. These range bounds were derived from the spread of comparative ranges when comparing our experimental air gun to the hypothetical air gun array, for single-shot SEL, cumulative SEL, and maximum values of the maximum magnitude of the single-shot ground acceleration vector. Lower range bounds were set by ground acceleration, and upper range bounds were set by cumulative SEL.

Mortality. The cumulative mortality at the conclusion of the 2013 experiment (i.e., day 120 postexposure) was 3.8% for control zero-pass scallops, 9.4% for one-pass scallops, 11.3% for two-pass scallops, and 14.8% for four-pass scallops. In the 2014 experiment, cumulative mortality rates at day 120 were similar ($P = 0.48$), with 3.6% in the control zero-pass treatment, 11.3% in one-pass treatment, 16.1% in two-pass treatment, and 17.5% in four-pass treatment.

The number of passes scallops were subjected to significantly increased the cumulative number of mortalities ($P = 0.009$). Exposure also significantly increased the probability of mortality over time ($P < 0.001$), with daily odds of mortality 0.1%, 1.2%, and 1.3% higher in scallops exposed to one, two, and four passes, respectively, relative to that of controls.

In the 2015 experiment, mortality rates were 5% in control scallops and 20% in exposed scallops at day 14. At day 120, both treatments, the control zero-pass scallops and the four-pass scallops, were found to have suffered 100% mortality. This loss was not attributed to seismic exposure, as the control group also suffered complete mortality.

Behavior and Reflexes. Qualitative analysis of video showed no evidence to support the hypothesis that seismic exposure promoted energetically expensive behaviors. In the 2014 experiment, out of 51 observed individual scallops, only four instances of swimming were observed between two individuals, which were brief (<5 s) and appeared either as responses to movements of other scallops or to adjust positioning. In the 2015 experiment, none of the 19 scallops were observed to swim. There was also no evidence of extended valve closure, as only two individuals, one each in 2014 and 2015, were observed to remain closed throughout observation. This observation was further tested by comparing tentacle state (extended, partially retracted, retracted) during preexposure, intraexposure, and postexposure time periods (Table S1), with no significant relationship between exposure and tentacle state found.

Quantitative analysis of behavior during exposure showed a significant reduction ($P < 0.001$; Table S2) in the occurrence of classic behaviors. This reduction was specifically in response to exposure, as no differences were observed in the periods before ($P = 0.38$) or following ($P = 0.14$) exposure.

In addition, a novel, nonclassic behavior, best described as a velar flinch, was observed (see Movie S1). This behavior was characterized by a rapid retraction of the velum and was distinct from the classic "cough" or "close" in that the upper valve, tentacles, and mantle were maintained in their "normal" resting state, unchanged relative to their position before the air gun signal. Rather, the velum was rapidly sucked in and then returned to position, with the whole behavior lasting less than 1 s. The behavior was observed exclusively in response to air gun signals at a maximum range of ∼350 m and continued to occur as the vessel approached. It was commonly observed just before the audible air gun signal, likely in response to the ground roll detected by the geophones. Velar flinches were the only observed behavior categorized as nonclassic and were significantly more frequent ($P = 0.002$) in two-pass scallops and four-pass scallops, in which they were observed in 100% and 75% of individuals, respectively, than in one-pass scallops, in which they were observed in 50% of individuals.

Recessing time showed a significant response to exposure in all three experiments, with increasing levels of exposure resulting in increasingly rapid recessing. For the 2013 experiment [$\chi^2(3) = 18.06$, $n = 131$, $P < 0.001$], four-pass scallops were found to recess significantly faster than both zero-pass and one-pass treatments (Fig. 2*A*). For the 2014 experiment, the recessing test was performed twice: immediately after exposure, as in the 2013 experiment, and again just before the day 120 sampling point. For the first test (Fig. 2*B*), there was a significant difference in time to recessing [$\chi^2(3) = 16.33$, $n = 146$, $P < 0.001$], with four-pass and two-pass scallops recessing significantly faster than zero-pass scallops. In the second recessing test for the 2014 experiment (Fig. 2*C*), conducted before day 120, four-pass scallops recessed significantly faster than zero-pass scallops [$\chi^2(3) = 8.66$, $n = 55$, $P = 0.034$]. For the 2015 experiment (Fig. 2*D*), the recessing test was only performed immediately after air gun exposure, due to mortality of all scallops before the 120-d sample point, and, again, the recessing rate was significantly different [$\chi^2(1) = 13.30$,

Table 1. Calculated exposure values for the scallop experiments

Experiment	Max PP	Shots within 3 dB max PP	Shots > 190 PP	Max SEL	Shots within 3 dB max SEL	Shots > 180 SEL	Max SEL_{cum}	Median SEL_{cum}	No. of shots	Min GR	Max GR
E-1 45-in^3 pass 1	191	40	14	181	3	1	189	189	167	0.29	37.22
E-1 45-in^3 passes 1 and 2	191	63	23	181	5	1	191	191	226	0.29	37.27
E-1 45-in^3 passes 1 to 4	191	148	52	181	8	2	194	194	393	0.29	37.57
E-2 150-in^3 pass 1	212	2	40	187	2	5	193	192	128	0.27	31.60
E-2 150-in^3 passes 1 and 2	213	2	71	188	2	8	195	194	195	0.27	35.37
E-2 150-in^3 passes 1 to 4	213	3	151	188	4	19	198	198	309	0.27	36.39
E-3 150-in^3 pass 1	213	1	26	188	1	3	191	188	54	0.68	35.54
E-3 150-in^3 passes 1 and 2	213	2	61	188	2	6	195	193	115	0.68	36.60
E-3 150-in^3 passes 1 to 4	213	2	140	188	2	6	197	196	251	0.67	36.60

Given are maximum (Max) peak to peak [PP, in decibels relative to (dB re) 1 μPa]; number of signals within 3 dB of maximum PP at any cage; number of signals at any cage > 190 dB re 1 μPa PP; maximum SEL (dB re 1 μPa^2·s); maximum shots/cage within 3 dB of maximum SEL; maximum shots/cage with signals > 180 dB re 1 μPa^2·s SEL; maximum cumulative SEL (SEL_{cum}, dB re 1 μPa^2·s); median SEL_{cum} across cages; number of shots/treatment; estimated minimum (Min GR) magnitude ground roll (GR)/treatment as measured on the seabed via geophone (meters per second squared); and estimated maximum (Max GR) magnitude ground roll/treatment as measured on the seabed via geophone (meters per second squared).

Table 2. Comparison of experimental scallop exposures and estimated equivalent range of hypothetical seismic survey, giving experimental regime, estimated exposure received during that experiment, and the estimated range this exposure occurred from a commercial array

Experiment	Max SEL	Range$_E$, m	Median SEL$_{cum}$	Range$_E$, m	Max GR (linear)	Range$_E$, m
2013 pass 1	181	250	189	725	37.2	114
2013 pass 2	181	250	191	500	37.3	114
2013 pass 4	181	250	194	275	37.6	118
2013 passes 1+2			196	200		
2013 passes 1+2+4			197	175		
2014 pass 1	187	150	192	400	31.6	129
2014 pass 2	188	150	194	275	35.4	117
2014 pass 4	188	150	198	200	36.4	120
2014 passes 1+2			196	175		
2014 passes 1+2+4			200	100		
2015 pass 1	188	150	188	875	35.5	117
2015 pass 2	188	150	193	325	36.6	115
2015 pass 4	188	150	196	175	36.6	115
2015 passes 1+2			194	275		
2015 passes 1+2+4			198	125		

The experiments are labeled by year (2013, 2014, and 2015) with one, two, and four passes within an experiment, with cumulative SEL from multiple passes indicated by pass 1+2 and pass 1+2+4. The estimated exposures are of maximum SEL experience; median cumulative SEL; and maximum magnitude of ground roll acceleration. Units are as follows: SEL and SEL$_{cum}$, dB re 1 $\mu Pa^2 \cdot s$; ground roll (GR), meters per second squared.

$n = 65$, $P < 0.001$], with four-pass scallops recessing more quickly.

Hemolymph Physiology and Biochemistry. In the winter 2013 experiment (Fig. 3*A*) on dredge-collected scallops, both exposure and sample time had a significant effect on hemocyte counts [$F(6,182) = 3.54$, $P = 0.002$], with control scallops showing significantly higher counts than exposed scallops immediately following exposure, with the number of hemocytes of zero-pass control scallops 73% and 75% greater than that of two- and four-pass scallops, respectively. At day 14, a slight decline in mean hemocyte numbers in zero-pass control scallops resulted in no significant differences found between control and exposed treatments. At day 120, the mean total hemocyte count of zero-pass control, one-pass, and two-pass scallops increased significantly from the levels recorded at days 0 and 14, whereas four-pass scallops remained at a similar level. The degree of increase differed, however, as zero-pass control scallops had 60 to 90% more hemocytes at day 120 than the three exposed treatments.

In the winter 2014 experiment using hand-collected scallops, the interaction between exposure level and sample time was again significant [$F(6,174) = 17.69$, $P < 0.001$], although, in this experiment, no difference was found in the number of hemocytes between zero-pass controls and any of the exposure treatments at day 0. At day 14, four-pass scallops had a 41% higher total hemocyte count than zero-pass controls, a difference that was significant. A similar response was observed in the 2015 experiment, which was conducted in the summer using hand-collected scallops, with four-pass scallops showing 21% higher total hemocyte count [$t(33.3) = 2.44$, $P = 0.03$] at day 14. At day 120 in the 2014 experiment, zero-pass control scallops maintained a similar hemocyte count to the previous sample points, whereas one-pass, two-pass, and four-pass scallops showed a significant decline to levels 40 to 50% of that of the control treatment.

Comparison of hemolymph pH (Fig. 3*B*) values for the winter 2013 experiment using scallops collected via dredge showed a significant interaction with air gun passes and sample time [$F(6,189) = 2.307$, $P = 0.036$], with pairwise comparison indicating that, at day 0, one-pass scallops had a significantly higher pH than the zero-, two-, and four-pass scallops, which was the only difference between treatments at the three sample points in the experiment. The general trend observed across the three sample points was alkaline pH values at day 0 (>8.00 for all scallops), to moderately alkaline values at day 14 (between 7.85 and 7.91 for all scallops), to normal levels by day 120 (between 7.46 and 7.63 for all scallops).

For the hand-collected scallops in the winter 2014 experiment, pH showed a significant interaction between air gun passes and sample time [$F(6,182) = 4.544$, $P < 0.001$]. Compared with control scallops, one-pass, two-pass, and four-pass scallops had a significantly more alkaline pH at day 0. At day 14, four-pass

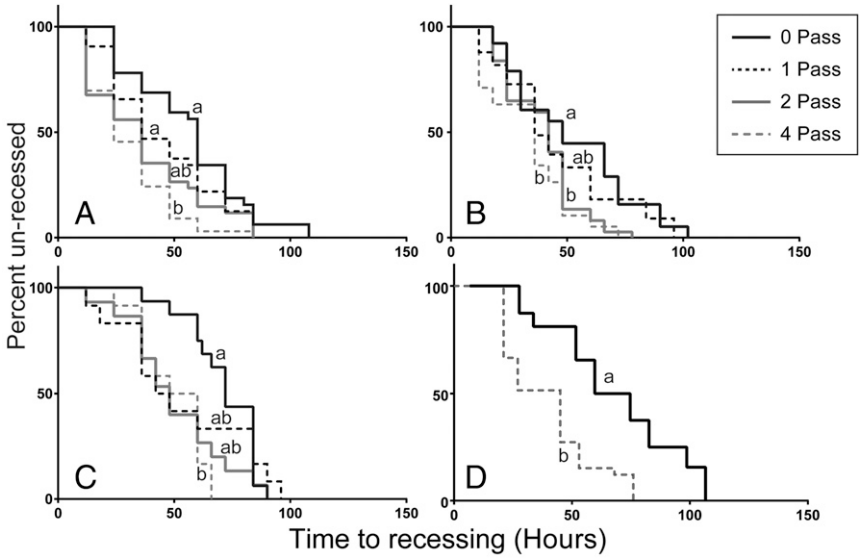

Fig. 2. Effect of seismic exposure on the recessing reflex of scallops, in the (*A*) winter 2013 45-in^3 experiment, (*B*) winter 2014 150-in^3 experiment, (*C*) winter 2014 150-in^3 experiment at day 120, and (*D*) summer 2015 150-in^3 experiment. Within each experiment, significantly different curves as determined using logrank (Mantel–Cox) tests are indicated by differing letters.

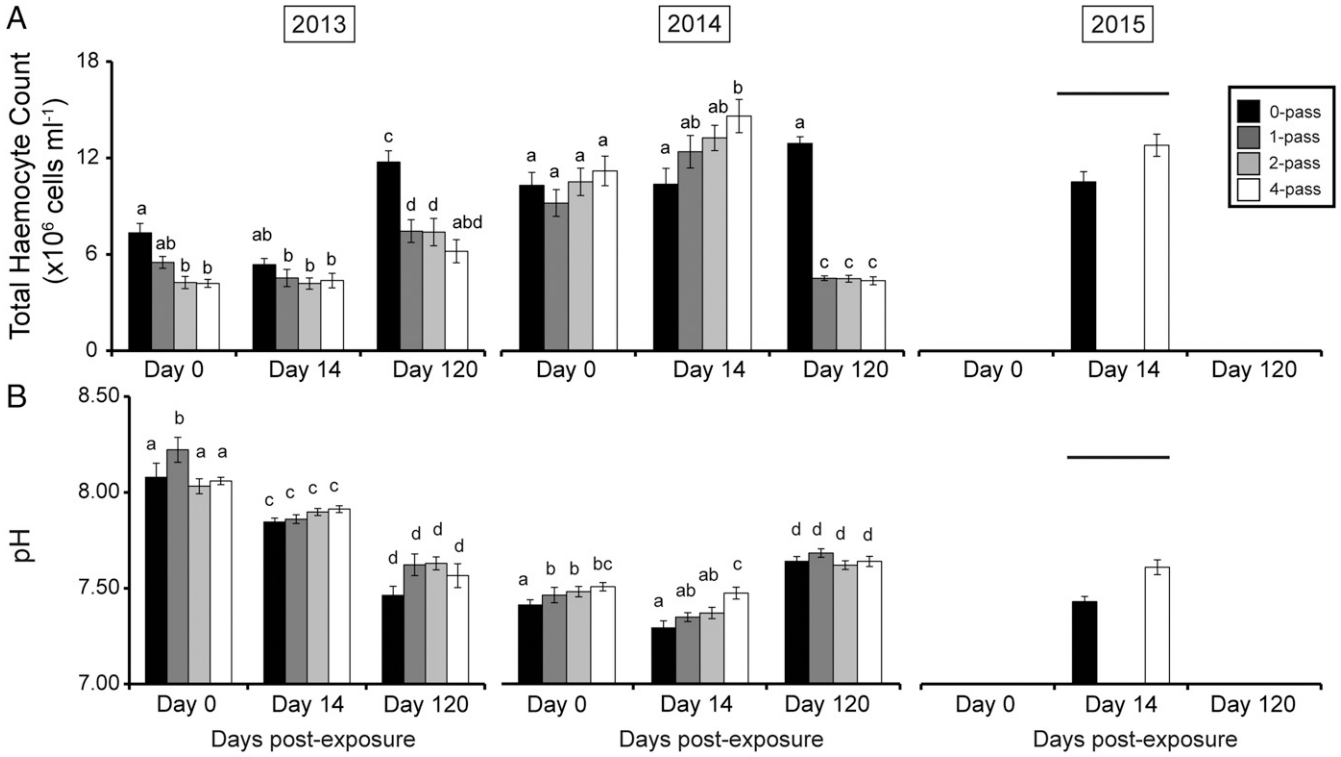

Fig. 3. Effect of seismic exposure on scallop hemolymph biochemistry, with (*A*) mean ± SEM total hemocyte counts and (*B*) mean ± SEM hemolymph pH by exposure level in the winter 2013 45-in^3 air gun experiment, the winter 2014 150-in^3 air gun experiment, and the summer 2015 150-in^3 air gun experiment. For each experiment, significant differences in response to an interaction between exposure time and sample time are indicated with differing lowercase letters, and significant differences between exposure level are indicated with a horizontal bar.

scallops had a significantly more alkaline pH than zero-pass control, one-pass, and two-pass scallops. No differences were found between control scallops and any of the exposed treatments at day 120.

At the lone sample point from the summer 2015 hand-collected scallop experiment, day 14, hemolymph pH was significantly more alkaline in four-pass scallops than in zero-pass scallops [$t(29.65) = -3.8253$].

Assays of humoral electrolyte and mineral ion levels, which were conducted on samples collected in the winter 2014 experiment, showed a range of responses to air gun exposure (see Table 3 for a summary of all responses, and see Table S3 for mean values and statistics for all assays).

Hemolymph sodium (Na) concentration showed a significant response to both exposure [$F(3,179) = 8.220$, $P < 0.001$] and sample time [$F(2,179) = 4.407$, $P = 0.014$], but not the interaction of the terms [$F(6,179) = 1.930$, $P = 0.078$], with exposure resulting in increased Na concentrations. Sample time showed significantly lower Na concentrations in day 0 samples compared with day 14 and day 120 samples. Exposure level significantly affected potassium (K) concentrations [$F(3,179) = 8.303$, $P < 0.001$], with four-pass scallops showing significantly higher K levels than zero-pass, one-pass, and two-pass scallops. Although there were differences in Na and K, there was no significant difference in Na:K ratio, in response to either exposure [$F(3,179) = 2.373$, $P = 0.072$] or sample time [$F(2,179) = 0.126$, $P = 0.88$].

There was a significant interaction between exposure and sample time for hemolymph concentration of magnesium [$F(6,179) = 3.668$, $P = 0.0018$], which tended to show a reduction in exposed scallops relative to controls; bicarbonate [$F(6,174) = 2.274$, $P = 0.039$], which was reduced in response to higher levels of exposure in the short and medium term; and calcium [$F(6,180) = 2.891$, $P = 0.010$], which was elevated in exposed scallops.

Chloride ions (Cl) differed significantly as a result of exposure [$F(3,179) = 4.1$, $P = 0.007$], with four-pass scallops showing elevated levels of Cl compared with zero-pass, one-pass, and two-pass scallops.

Phosphorus levels differed significantly as a result of exposure [$F(3,179) = 4.791$, $P = 0.003$], although, in this case, two-pass scallops had phosphorus levels significantly lower than zero-, one-, and four-pass scallops.

Organic molecules (see Table S4 for all mean values and statistics) showed a more limited response, with significant differences only in total protein and glucose levels. For protein, exposure and sample time displayed a significant interaction [$F(6,178) = 2.579$, $P = 0.020$], although no significant differences were found among relevant treatments and sample times following post hoc analysis. For glucose, exposure had a significant effect [$F(3,180) = 5.37$, $P = 0.01$), with zero-pass scallops showing higher glucose levels than one-pass and four-pass scallops.

Discussion

A common criticism of animal exposure experiments with air gun sources is that they do not represent "real" seismic sources or that the exposure either exceeds or is lower than that of a "real" seismic source. In the present study, a single air gun was used in open-water, field conditions to expose scallops in a natural habitat setting to signals emulating a larger commercial seismic array. With an exposure regime based on multiple passes, scallops received SELs and ground excitation comparable to that of a large commercial source passing within a few hundred meters, based on comparisons with commercial arrays and modeling of multiple passes of commercial sources (33).

Table 3. Summary of mineral ion and organic molecule concentrations in scallop hemolymph following exposure in the winter 2014, 150-in^3 experiment

Hemolymph parameter	1-pass	2-pass	4-pass
Sodium (Na^+)	+	+	+
Potassium (K^+)			+
Chloride (Cl^-)			+
Magnesium (Mg^{2+})	–	–	–
Bicarbonate (HCO_{3-})			–
Calcium (Ca^{2+})			+
Phosphorus (P^{3-})		–	+
Protein			
Glucose	–	–	–

Significant changes relative to control treatment are shown, with increased levels indicated by a plus (+) and decreases indicated by a minus (–).

Here we found that seismic exposure, particularly repeated exposure, significantly increased mortality in the scallop *P. fumatus* compared with the 4 to 5% mortality rate in control scallops. The observed mortality rates in all three experiments, which ranged from 9 to 11% in one-pass scallops to 13 to 20% in four-pass scallops, were not representative of a mass mortality event (34) and were at the low end of the range of the naturally occurring mortality rate documented in the wild, which ranges from 11 to 51% with a 6-y mean of 38% and a well-established correlation to fishery stress (35–37). However, given the arbitrary endpoint of 120 d postexposure, mortality could have potentially continued to increase in the exposed treatments.

One way in which seismic air gun exposure could result in mass mortality is by driving scallops to energetically expensive behaviors such as extensive swimming or long periods of valve closure, although neither qualitative analysis of behavior during exposure nor quantitative analysis of tentacle state (38, 39) offered support for this hypothesis. However, exposed scallops showed two disruptions to behavioral patterns. First, exposed scallops demonstrated a marked reduction in classic behaviors during exposure. Second, exposed scallops exhibited a novel velar flinch behavior. This novel flinching behavior was only observed in direct response to air gun signals, often slightly before the signal was audible, suggesting that the behavior is in response to the faster-traveling groundborne energy. Whether these changes in behavior might have an ecological impact on scallops is not clear. The velar flinch cannot be interpreted as a sign of stress or negative impact on its own, although it is clearly an acute response to exposure. The reduction in classic behaviors, potentially an indication of a reduced capacity to respond to other stimuli, was apparent only during exposure. As there was no difference in behavior between control and exposed scallops in the postexposure observation period, any long-term manifestation of this behavioral response is unlikely.

The scallops' recessing reflex, in which a scallop uses jets of water to create a depression in the sediment while also covering itself with sediment, was also impacted by exposure. Recessing appears to be the "natural" state for scallops in the *Pecten* genus (28, 29), assisting feeding, conferring protection from predators, preventing shell fouling, and reducing hydrodynamic profile (40). Typically, scallops demonstrate slowed recessing in response to stress, resulting from energy depletion during exposure (40–42); however, seismic exposure elicited the opposite effect, with repeated exposure increasing the rate of recessing. Furthermore, in these previous studies, the recessing time in stress-affected scallops had returned to normal within 1 d to 3 d (40–42), whereas, in the present study, the impacted response persisted to at least 120 d after exposure. Given that energy depletion caused the slowed recessing previously observed, it is not surprising that scallops exposed to air gun signals did not respond similarly, as swimming or valve closing behavior was not observed during air gun exposure. However, the more rapid recessing of exposed scallops cannot easily be explained.

We hypothesize that exposure impacted elements of the scallop sensory system, with the statocyst and the abdominal sense organ as potential candidates. The paired statocysts are the primary mechanosensory organ in scallops, as in many invertebrates, that provide a sense of balance through reception of gravity (43). The abdominal sense organ, a sickle-shaped pocket in the mantle fold densely populated with sensory hairs, has been indicated to play a role in mechanoreception and directional sensitivity (44), based on its high sensitivity to water- and groundborne vibrations (45, 46). If the abnormal reflex results found in this study are indicative of damage to mechanosensory organs, exposed scallops may face considerable ecological ramifications. Disruption of the statocyst nerve caused scallops to lose the ability to control the vertical component of their swimming (47), thus compromising a primary mechanism of predator avoidance. The abdominal sense organ has also been suggested to contribute to predator detection, with the detection of waterborne vibrations originating from above the scallop filling in a blind spot of the visual, tactile, and chemoreceptive systems (48).

The physiological response to exposure was explored through the hemolymph of the scallop, which is the invertebrate analog to vertebrate blood and performs many of the same functions, including gas exchange, nutrient and waste transport, osmoregulation, and immune function (45). Although these hemolymph parameters are useful for interpreting health, care must be taken in comparisons over time, as bivalve hemolymph tends to show considerable variation in hemolymph parameters in response to biotic (reproductive cycle, nutritional condition) and abiotic (temperature, food availability) factors (30, 49–51). This variability is particularly relevant to the 2015 experiment in this study, which was conducted in the summer, whereas the 2013 and 2014 experiments were conducted in the winter. This seasonal difference, along with having only one sampling point in the 2015 experiment, makes comparing the 2015 experiment to the winter experiments difficult, due to the influence of water temperature, nutritional condition, and reproductive state.

The effect of exposure on the cellular component of the hemolymph, which is responsible for mediating immune function, was quantified through total hemocyte counts. We found that our hypothesis regarding hemocyte counts, that exposure would cause a short-lived increase in the number of circulating hemocytes in a response similar to that of other stressors, was largely unsupported. In 2013, scallops had comparatively low hemocyte levels early in the experiment (i.e., days 0 and 14), with exposed treatments receiving multiple passes showing depressed hemocyte counts compared with controls. Conversely, in 2014, exposed scallops showed elevated hemocyte numbers at day 14 compared with controls, consistent with the typical bivalve response to stress (30). The dissimilarities between experiments in hemocyte response at initial sample points can likely be attributed to the differences in collection methods, as scallops for the 2013 experiment were collected via dredging and scallops for the 2014 and 2015 experiments were hand-collected by divers. The response to exposure of the 2013 scallops probably includes a latent response to the stresses resulting from dredging and repeated transportation (41), with the comparatively low levels of hemocytes and the immediate hemocyte response observed in exposed scallops in the 2013 experiment suggesting a synergy between dredging stress and seismic exposure that accelerated the overall response.

In both the 2013 and 2014 experiments, hemocyte numbers had decreased by day 120 in exposed scallops. Although conclusions regarding immune function cannot be directly drawn from hemocyte count results, the depressed levels in exposed

scallops suggest that the scallops in this study were immunocompromised, one of the most important drivers of mortality in bivalves (52, 53), and direct assays of immune function (i.e., differential hemocyte counts; assays of phagocytosis, hemocyte membrane stability, etc.) are an important next step for understanding the impacts of exposure.

Like hemocyte counts, hemolymph pH indicated that the scallops in the 2013 experiment showed stress from dredging. In that experiment, hemolymph pH values were high at days 0 and 14 in control and all exposed treatments compared with those from the subsequent experiments, with levels of >8.00 and >7.85, respectively. By day 120 postexposure, pH levels in all treatments, including controls, had returned to the expected range (between 7.46 and 7.63), with no difference between any treatments. These results followed our hypothesis of stable hemolymph biochemistry; however, the stress response would have masked any experimental response. In the 2014 and 2015 experiments, the prediction of stable pH was unsupported, as exposure resulted in elevated pH levels, or alkalosis. Reports of alkalosis in marine invertebrates are rare in the literature. The only report of alkalosis in a marine bivalve, the Pacific oyster *Crassostrea gigas* (54), resulted from extensive handling, shell drilling, cannulation, and repeated drawing of hemolymph. More broadly, alkalosis in marine invertebrates, primarily cephalopods, has been reported to occur in response to functional and environmental hypoxia as metabolism shifts to anaerobic pathways (55–60). There are considerable differences between these reported cases of alkalosis and its occurrence observed here. In the present study, scallops were in normoxic seawater throughout the experiment and demonstrated a decrease in hemolymph bicarbonate, rather than the increase typical of other molluscs, suggesting that mobilization of bicarbonate from the shell is unlikely to be a factor in the response. Furthermore, alkalosis in scallops was persistent for at least 14 d, far longer than the scale of hours previously reported in any invertebrate. The mechanisms of this response warrant further study, as they likely differ from those previously investigated, given the substantially different circumstances between this study and previous reports of alkalosis.

Exposure to air gun signals also resulted in considerable and persistent osmoregulation disruption, as the a priori hypothesis of stable hemolymph biochemistry was again unsupported. Owing to adaptation to the stable nature of their sublittoral habitat, scallops show a limited capacity for regulation of hemolymph ion concentration (61, 62). However, a broad scope of changes was observed, with every mineral and electrolyte assayed showing a significant alteration in response to exposure. Changes in hemolymph ion concentration have been observed in abalone (*Haliotis diversicolor supertexta*) in response to osmotic stress (63) and hypoxia (64), but not in response to thermal stress (65). It is notable that hemolymph electrolyte ions in stressed abalone stabilized within days, whereas the scallops in the present study showed changes over chronic time scales. Cellular damage decreased hemolymph sodium and chloride concentration and increased potassium concentration in mussels (*Mytilus edulis*) in response to the interaction of anoxia, metal toxicity, temperature, and salinity (66). It is difficult to conclude whether cellular damage played a role in the scallops' response to exposure, as damage causing cellular leakage would be expected to increase the hemolymph concentration of all hypotonic cellular ions. However, although some hypotonic ions (e.g., potassium) increased as would be expected, others (e.g., magnesium) decreased following exposure. Whatever the mechanism, these imbalances in hemolymph electrolyte ions can disrupt the membrane potential, affecting a range of biological functions, such as proton pump function, active transport across the cell membrane, and enzyme function within the cell (66).

Exploration for petroleum and the development of shellfish fisheries are necessary processes for these extractive industries that need to coexist in the marine environment. In this study, exposure to seismic surveys left scallops behaviorally and physiologically impacted and in a state such that any additional stress (e.g., dredging, warm water conditions, predation stress) could lead to further impairment or mortality (67). These results indicate a need for further study into the impacts of seismic signals, and, more generally, anthropogenic aquatic noise. In all cases, the mechanistic underpinnings and ecological impacts of these responses to exposure require further characterization to understand the overall economic and ecological implications. To avoid future conflict, a comprehensive understanding of how these industries may impact each other will be required in order to facilitate effective management.

Methods

Animal Care and Experimental Design. The present study comprised three experiments exposing the commercial scallop (*P. fumatus*) to signals from a Sercel G Gun II operated at 2,000 psi. The first experiment, referred to as the 2013 experiment hereafter, was conducted in July 2013 (austral winter) using 240 adult scallops that were collected by a commercial scallop dredge fishing vessel from the fishery near Ille des Phoques, Tasmania (42°21′20.62″S 148°10′3.25″E; Fig. 4). For this experiment, the air gun was fitted with a 45-in^3 chamber for exposure. Scallops were randomly assigned to four treatments of exposure levels defined by the number of passes of the seismic air gun—zero passes (control), one pass, two passes, and four passes—and color-coded and numbered tags (Glue On Shellfish Tags; Hallprint Fish Tags) were used to identify the treatment and individual. To determine whether time was a factor in any observed response, scallops from each of these treatments were sampled at three different points following exposure: 0 d, 14 d, and 120 d. Thus, 12 scallops (i.e., three sample days × four treatment levels) were placed into each of the 20 enclosures at the field site.

For the second experiment, referred to as the 2014 experiment hereafter, was conducted in July 2014 (austral winter) using a 150-in^3 chamber on the air gun, and 240 adult scallops were hand-collected by divers from Coles Bay, Tasmania (42°07′45.07″S, 148°16′03.83″E; Fig. 4). Treatment groups and sample times were identical to those of the 2013 experiment.

The final experiment, the 2015 experiment, was conducted in March 2015 (austral summer), again using a 150-in^3 chamber on the air gun. For this

Fig. 4. Scallop experiment locations: 1, IMAS; 2, Blackjack Rocks, field site; 3, Spring Bay Seafoods, Triabunna, mussel lease where day 120 scallops were held; 4, Ille de Phoques, collection site for 2013, 45-in^3 experiment; and 5, Coles Bay, collection site for 2014 and 2015, 150-in^3 experiments. Map data obtained from Google Earth.

experiment, the number of treatment groups was reduced to two (zero-pass and four-pass) and the number of sample points was reduced to two (14 d, 120 d), so 80 adult scallops were used. Scallops were again hand-collected by divers from Coles Bay, Tasmania, at the same site as the 2014 experiment, with four scallops (i.e., two sample days × two treatment levels) placed into each enclosure.

Before and following experimental field work, scallops used in this study were held at the Institute for Marine and Antarctic Studies (IMAS), Taroona, Tasmania (42° 56′59.13″S, 147° 21′16.60″ E; Fig. 4), in a 3,400-L (2 m × 2 m × 0.85 m) tank with *ca.* 10 cm of sand substrate with ambient temperature (*ca.* 13 °C in 2013 and 2014 experiments, *ca.* 17 °C in 2015 experiment) seawater supplied by a flow-through system. Scallops were held at IMAS for 1 wk before transport to the field site. Scallops were transported to IMAS in plastic bins lined and covered with burlap sacks wetted with seawater (68).

The field site for all three experiments, near Blackjack Rocks north of Betsey Island, Tasmania (43°02′16.37″S, 147°28′30.14″E; Fig. 4), was a sand flat at 10 m to 12 m depth. Scallops were transported to the site in a large bin (1.2 m × 0.75 m × 1 m) of seawater. A deck hose was used to pump fresh seawater into the bin to maintain O_2 levels. At the field site, divers placed scallops into 1.5-m-tall cylindrical enclosures (Fig. S3) constructed of 2-cm mesh with a 1.2-m-diameter floating ring at the top and skirted by a ring of heavy-gauge chain at the bottom. Enclosure bottoms were not meshed, allowing for scallops to be in contact with the sandy seabed. Scallops were held in the enclosures for a 2-d acclimation period before the experiment.

In the 2014 and 2015 experiments, video cameras were placed into 10 randomly selected scallop enclosures at the start of the experiment to allow for behavioral analysis of scallops during the control pass and before, during, and following each air gun exposure pass.

All research was conducted in accordance with University of Tasmania Animal Ethics Committee Permit A13328. Fieldwork was conducted in accordance with Tasmania Department of Primary Industries, Parks, Water and Environment Permits 13011 and 14038.

Exposure and Air Gun Measurements. At the beginning of the experimental procedure, the air gun vessel was positioned ∼1 km from the scallop enclosures, with the air gun deployed and towed at a depth of 5.1 m. First, a control (zero-pass) run was conducted, in which the air gun was deployed and charged but not fired. The vessel approached the scallop enclosures at a speed of 1.85 $m{\cdot}s^{-1}$ (3.5 kn) following a predetermined path that was used for all runs (Fig. S4). Following the control run, divers collected the scallops assigned to the control treatment based on tag color. Upon retrieval, scallops were randomly assigned to sample points (0 d, 14 d, or 120 d postexposure in 2013 and 2014; 14 d or 120 d postexposure in 2015) and placed into a large bin of seawater continually supplied with fresh seawater via the deck hose. After zero-pass control scallops were collected, the air gun vessel returned to the starting point and began firing the air gun, with one shot every 11.6s, while following the approach toward the scallop enclosures. At the conclusion of the run, divers collected the scallops assigned to the one-pass treatment, which were assigned to sample times and placed into the bin of seawater and returned to IMAS. The same process was followed for passes two through four. Following each air gun pass, the air cylinders used to power the air gun required recharging via onboard scuba compressors. As this process lasted for several hours, the number of air gun runs that could be conducted was limited, so the zero-pass and one-pass runs were conducted in 1 d and passes two through four were conducted the following day.

Upon return to IMAS, day 0 scallops were placed into a plastic crate that remained immersed in the holding tank. Scallops were haphazardly selected from this crate for sampling, until all were done. Scallops not sampled on day 0 were used in recessing tests (details in *Sampling*). Following the recessing test, day 14 scallops were held at IMAS, whereas day 120 scallops were haphazardly distributed into two lantern nets. The nets were placed into bins lined and covered with burlap sacks wetted with seawater and transported to Spring Bay Seafoods in Triabunna, Tasmania (42°35′59.65″S, 148°01′01.83″E; Fig. 4), where the lantern nets were deployed on mussel aquaculture lines that were held submerged at a depth of 10 m. The nets were left undisturbed until they were recovered and returned to the holding tanks at IMAS 1 wk before sampling at day 120, to allow for acclimation following transport.

Hydrophones placed on the seabed monitored the normal ambient noise and the air gun signals (sound pressure) received by the scallops, and geophones were placed to measure groundborne vibrations (velocity) before and during the experiment. Sea noise loggers were located next to scallop enclosures at each end of the scallop lines. A near-field logger was also deployed 0.5 m off the air gun ports to allow source levels to be quantified. A detailed description of the methods used to determine sound levels is given in *Supporting Information*.

To determine how the exposure regimes in this experiment equated to exposure to full-scale seismic surveys, measured levels were compared with those of a modeled 3,065-in^3 3D array source operating in 50-m water depth, based on data collected from previous surveys (1, 33). Additional details of this model are provided in *Supporting Information*.

Sampling. Mortality was assessed throughout the postexposure holding period through observation of abnormal positioning (i.e., inverted on the substrate, not recessed for an extended period, leaned up against the side of the tank, etc.) during periodic (i.e., at least three times per week) monitoring of the scallop tank or through discovery during sampling. Any observed mortalities were rounded to the next sampling point, i.e., a dead scallop discovered at day 10 was considered dead at the day 14 sample point. For the day 120 scallops, once scallops were transported to the mussel lease, mortality was not assessed until the scallops were returned to IMAS before sampling. Mortality rate was determined by comparing the total number of dead scallops from each treatment. Only mortalities observed following recovery were considered; that is, losses due to predation or unrecovered animals were not counted in the analysis.

Video recordings made during seismic exposure in the 2014 and 2015 experiments were used to analyze scallop behavior. Recordings were divided into three categories: preexposure, intraexposure, and postexposure segments, with the first 5 mins of preexposure time and the last 5 mins of postexposure time disregarded due to the influence of divers deploying and retrieving video cameras. For each segment, all visible scallops were observed and two sets of behavioral data were recorded. First, the observed behaviors were classified into two categories, classic and nonclassic, with the former encompassing visual behaviors, e.g., reflexive closure response to shadow or movement; "coughs" used to irrigate the mantle cavity; valve closures characterized by mantle velum retraction and valve adduction; and locomotory behaviors, such as swimming or repositioning (69). Any behaviors not encompassed within the classic behavior category were classified as nonclassic. The second analyzed behavior was tentacle extension, which was used as a method to evaluate valve closure (39), with tentacles recorded as either "extended," "partially extended," or "retracted" for the duration of the preexposure, intraexposure, and postexposure time categories for the 2014 and 2015 experiments. Extended and partially extended tentacles were considered to indicate that valves were open, and tentacle retraction indicated valve closure. It is important to note that the preexposure periods differ somewhat between the one-pass treatment and the higher exposure treatments, in that the one-pass scallops were naïve to any air gun exposure during the preexposure period, whereas two- and four-pass scallops had been exposed during the intraexposure period of the previous treatments.

At each of the three sample points (at days 0, 14, and 120 postexposure in the 2013 and 2014 experiments and at day 14 in the 2015 experiment), all individuals within the four treatment levels (two treatment levels in the 2015 experiment) were terminally sampled. First, the adductor muscle was detached from the upper valve, and then the upper valve was removed. Then, 2.5 mL of hemolymph was drawn from each scallop from the pericardial sinus using a prechilled syringe fitted with a 26-gauge needle. This sample was divided into two aliquots: a 500-μL aliquot for immediate analysis of pH (Testo 205 pH meter) and a 500-μL aliquot that was added to a centrifuge tube prefilled with 500 μL of anticoagulant (Lillie's formol calcium: 2% NaCl, 1% calcium acetate, 4% formaldehyde) for total hemocyte counts using an improved Neubauer hemocytometer under 40× magnification. In the 2014 experiment, an additional 1,500-μL aliquot was drawn and centrifuged at 3,000 × *g* for 3 min, after which 1,000 μL of supernatant was transferred into a cryovial and snap-frozen in liquid nitrogen for later biochemical analysis. This sample was shipped, frozen on dry ice, to Diagnostic Services at the Atlantic Veterinary College, University of Prince Edward Island, and analyzed using a Cobas c501 automated biochemistry analyzer (Roche Diagnostics Corporation) for a full blood profile consisting of the electrolytes (millimoles per liter = millimolars) sodium (Na), chloride (Cl), potassium (K), magnesium (Mg), and bicarbonate (bicarb); minerals (millimoles per liter) calcium (Ca) and phosphorus (P); metabolites (millimoles per liter = millimolars) glucose (Gluc), lactate (Lact), cholesterol (Chol), triglyceride (Trig), total protein (TP, in grams per liter) and uric acid (Uric, in micromoles per liter).

Beginning at day 0 upon return to IMAS following exposure, the scallops scheduled for destructive sampling at days 14 and 120 postexposure were used for a recessing reflex test (41, 42). Starting from when the scallops were placed into the holding tank following exposure, scallops were visually assessed for recessing three to four times daily at ∼6-h intervals, with

recessing defined as having an upper valve even with the substrate level. To ensure consistent assessment of recessing, the same researcher performed all assessments. Once a scallop was observed to have recessed into the substrate, it was collected, and the time taken to recess (in hours) was recorded. In all three experiments, the recessing test continued until all scallops had recessed, which was under 5 d in all cases. In the 2014 experiment, this test was also conducted a second time, before the day 120 sample point using scallops scheduled for destructive sampling at day 120 postexposure.

Final sample sizes for each component of the three experiments at each sample point, which exhibited some variation due to differences in mortality rates and animals lost to predation or missing tags, are given in Table S5.

Statistics. To evaluate cumulative mortality in the 2013 and 2014 experiments, mortalities from each treatment were compared using a binomial regression. The analysis was restricted to the 2013 and 2014 experiments, as all scallops died while deployed on the mussel lease before the day 120 sampling point for both zero- and four-pass treatments in the 2015 experiment.

Comparisons of behavioral analysis were conducted on the rate (incidences per unit time) of observations of classic and nonclassic behaviors. Rates were used, as the observational period differed between the scallops, thereby complicating the use of conventional count-based models. Nonclassic behavior was only observed in exposed scallops; hence the nonclassic and classic behavioral modes were analyzed separately using a generalized linear model with the number of exposure passes, the year, and temporal category (preexposure, intraexposure, and postexposure) as categorical explanatory variables.

Tentacle extension was compared for each treatment by summing the duration each individual scallop spent in each of the three states of tentacle extension. This sum was then converted into a proportion of total time of each temporal category, and multinomial regression was used to analyze the behavioral modes (two options, since the proportions add up to 1) as a function of the year, treatment, and phase.

Recessing reflex data from all three experiments was analyzed using Kaplan–Meier estimator analysis of the time-to-recess for each individual and compared using log-rank (Mantel–Cox) test with $\alpha = 0.05$, followed by multiple comparisons with Bonferroni correction and a family-wise error rate of 0.05.

Hemocyte count data from the 2013 and 2014 experiments were tested for assumptions of equality of variance (Levene's test) and normality (Shapiro–Wilk test) before analysis using two-way ANOVAs with air gun passes and sample time as factors and $\alpha = 0.05$, followed by a Tukey honest significant difference (HSD) post hoc test for significant results. Data from 2013 required log transformation, and data from 2014 required square root transformation. Data from the 2015 experiment were compared using a Welch Two-Sample *t* test between zero- and four-pass treatments at the 14-d sample point.

Hemolymph pH data for the 2013 experiment did not meet normality assumptions, so a two-way randomized permutation test ANOVA (70) with 5,000 iterations was used, with passes and sample time as factors and $\alpha = 0.05$, followed by post hoc Tukey HSD tests for any significant results. For the 2014 experiment, data met ANOVA assumptions, so a two-way ANOVA with passes and sample time as factors and $\alpha = 0.05$ was used. Significant results were analyzed using a Tukey HSD post hoc test. For the 2015 experiment, pH was compared using a Welch *t* test between zero- and four-pass treatments at the 14-d sample point.

Biochemistry data from the 2014 experiment was analyzed using two-way ANOVA for parametric data and randomized permutation test two-way ANOVA for nonparametric data, with passes and sample time as factors and $\alpha = 0.05$, followed by post hoc using Tukey HSD tests to evaluate significant results.

Except where noted otherwise, all statistical comparisons were conducted using R v3.1.3 (The R Foundation for Statistical Computing).

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