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Sound investigation: effects of noise on marine animals across trophic levels

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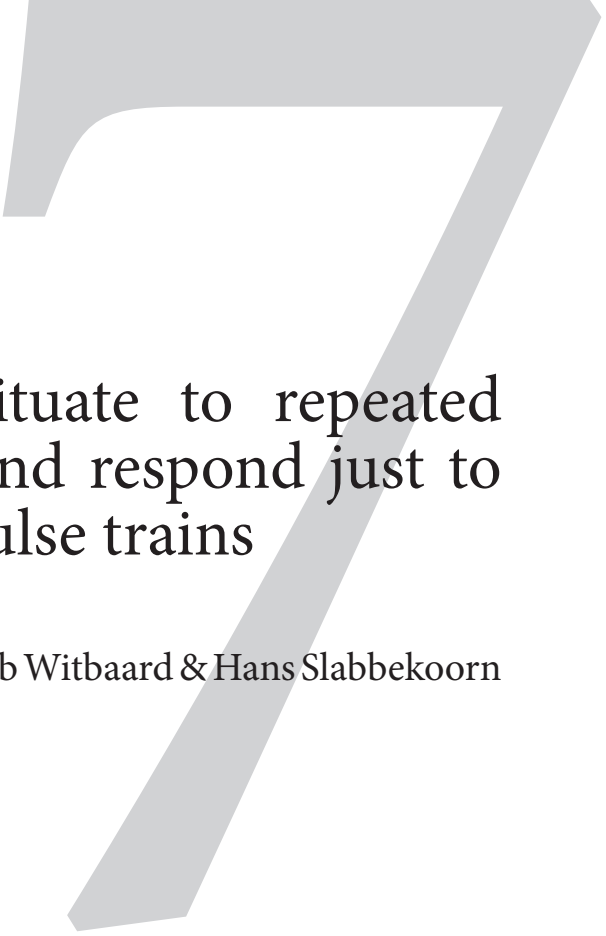


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Blue mussels habituate to repeated sound exposures and respond just to the on-set of fast pulse trains

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Abstract

Anthropogenic sound has been shown to affect marine animals across taxa. However, bivalves and other invertebrates received limited attention and most studies across taxa focussed on immediate, rather than long-term, effects of sound. Most bivalves adopt a sessile or sedentary lifestyle and are therefore expected to be subject to frequent exposure to the same sounds for long periods or repeatedly. For this reason, bivalves are an especially relevant taxonomic group to study long-term effects of sound. In the current study, we examined whether blue mussels (*Mytilus edulis*) habituate to repeated sound exposures and whether they recover quicker from a single pulse exposure than from a pulse train. We equipped individual mussels with sensors to monitor their valve gape and exposed them to repeated sound playback. We found that mussels responded to sound by partially closing their valves. This response was consistent and repeatable, but decayed over sequential exposures to the same sound stimulus, and was stronger again with exposure to a different sound. This pattern is clear evidence for acoustic habituation in a bivalve. Additionally, we found no differences in the initial response and recovery (time to return to baseline levels) between mussels that were exposed to single pulses and pulse trains. Our results therefore show that mussels are able to habituate to sound and suggest that mussels mostly respond to the onset of a pulse train. Future research is needed to determine whether mussels also habituate in situ to actual anthropogenic sound and whether a lack of a behavioural response to repeated sound also implies that other negative effects are also absent.

Introduction

Anthropogenic sound is omnipresent in the marine environment and has the potential to affect marine animals across taxa (Carroll et al., 2017; Slabbekoorn et al., 2010). Animals use and produce sound for orientation and communication and these functions can be undermined by masking or disturbing noise (Gordon et al., 2019; Wilson et al., 2014). Anthropogenic noise has been shown to impact animals in various ways, including disruption of movement patterns, foraging behaviour, communication, and metabolism (Codarin et al., 2009; Harding et al., 2018; Hawkins et al., 2014; Hubert et al., 2018). Many anthropogenic activities that produce sound, such as shipping, pile driving, seismic surveys, and dredging occur continuously or repeatedly and can last for months (Popper and Hawkins, 2019; Slabbekoorn et al., 2019). The effects of sound on invertebrates received limited attention, yet they make-up the majority of the biomass and are important for higher trophic levels (Morley et al., 2014; Solan et al., 2016). Immediate and short-term effects of sound exposure have been examined most. However, many marine animals, and especially invertebrates with a sessile life style or small home ranges, are likely exposed to the same sound repeatedly or for extended periods of time.

Bivalves are known to hear sound. Bivalves lack gas-filled cavities and are, for this reason, not expected to be sensitive to sound pressure, but rather to the particle motion aspect of sound. Their hearing is not fully understood, but members of 19 bivalve families, including the current study species, possess a specialized hearing organ, the abdominal sense organ (ASO; Haszprunar, 1983). Bivalve lineages that lack the ASO possess structurally similar organs, possibly also for hearing (Haszprunar, 1985; Zhadan, 2005). Removal of the ASO in two scallop species resulted in a major decrease in acoustic sensitivity, the remaining sensitivity was thought to be attributable to cells with short cilia on the mantle and tentacles (Zhadan, 2005). Statocysts have also been suggested to be involved in hearing (Charifi et al., 2017; Roberts et al., 2015). The hearing range of blue mussels is not known, but they have been shown to respond to tones from 5 to 410 Hz (no tones outside this range used, Roberts et al., 2015). Another bivalve, the Pacific oyster (*Magallana gigas*), has been shown to respond to tones of up to 600 Hz (Charifi et al., 2017), however, both location and development of the ASO is different in these species (Haszprunar, 1983). Several bivalves have also been reported to incidentally produce sound, typically associated with valve movement and expulsion of water and other substances (de Melo Júnior et al., 2020; Di Iorio et al., 2012).

Bivalves may use sound for various reasons. Planktonic larvae of bivalves settle

and metamorphose at the seafloor, – in most species – to start their sedentary or sessile life stage. Larvae of the eastern oyster (*Crassostrea virginica*) showed increased settlement behaviour in response to playbacks of recordings from oyster reefs when compared to adjacent soft bottom habitats (Lillis et al., 2013). Swash-riding clams (*Donax variabilis*) jump out of the sand and ride waves to migrate shorewards during ebb and flood tides, specifically with the largest waves (Ellers, 1995a). In the lab, these clams have been shown to jump out of the sand during the playbacks of wave sound, they were most responsive to the loudest waves and around high tides (Ellers, 1995b). It is clear that bivalves can hear, use, and respond to sound, so they are potentially also affected by anthropogenic sound.

Various studies examined the effects of anthropogenic or artificial sound exposures on bivalves. Bivalve physiology has been shown to be affected by noisy conditions through an increase in several biochemical stress parameters, single-strand breaks in DNA, reduced oxygen consumption, oxidative stress, and adjusted metabolism and hemolymph (a blood analogue) biochemistry parameters (Day et al., 2017; Peng et al., 2016; Vazzana et al., 2016; Wale et al., 2019). Increased mortality was found in scallops (*Pecten fumatus*), 14 and 120 days after seismic survey passes (Day et al., 2017). Pelagic bivalve larvae showed delayed development and body abnormalities after seismic sound exposure (de Soto et al., 2013) and displayed increased and faster settlement behaviour in response to vessel noise (Jolivet et al., 2016; Wilkens et al., 2012). Bivalves in their benthic stage have been shown to respond by both immediate closure of their valves as well as an increased mean valve gape, both a higher and lower filtration rate, increased and deeper digging behaviour, a reduction in ‘normal’ behaviour (close movements, coughs, and locomotion), and by retraction of the velum (Charifi et al., 2017; Day et al., 2017; Mosher, 1972; Peng et al., 2016; Roberts et al., 2015; Spiga et al., 2016; Wale et al., 2019). In these experiments, subjects were typically exposed to sound once for a relatively short period and it was not examined whether responses reduced over time. However, bivalves in situ are likely exposed to anthropogenic sound throughout their lives and may habituate to sound.

Habituation is a wide-spread phenomenon and is critical to understand the long-term impact of behavioural changes due to noise pollution. The term habituation is often misused in impact studies to explain the absence or decrease in response, yet it follows a strict definition (Bejder et al., 2006): Habituation is a decrease in behavioural response to repeated stimulus presentations, when this decrease cannot be attributed to sensory adaptation, sensory fatigue, or motor fatigue. The latter, alternative explanations of a decrease in behavioural response,

can be excluded by showing dishabituation or stimulus specificity (Rankin et al., 2009). Several marine fish species and cephalopods have been shown to decrease their behavioural or physiological stress response over repeated exposures (Johansson et al., 2016; Mooney et al., 2016; Nedelec et al., 2016; Radford et al., 2016; Samson et al., 2014). However, only for European seabass (*Dicentrarchus labrax*), the decreased responses have actually been ascribed to habituation by showing stimulus specificity (Neo et al., 2015a). We are not aware of evidence that bivalves are able to habituate to sound, however, two giant clam species (*Tridacna derasa* & *Tridacna maxima*) have been shown to habituate to repeated visual and tactile stimuli (Dehaudt et al., 2019; Wilkens, 1986).

Blue mussels (*Mytilus edulis*) are both a relevant and practical species to use in studies on the impact of sound exposures. Mussels are filter-feeders, provide habitat as reef builders, and are a common prey item for many species, and hereby play an important role in ecosystems (Borthagaray and Carranza, 2007; Jørgensen, 1990; Kautsky, 1981). Furthermore, mussels are an important commercial species (Eurostat, 2019a; Eurostat, 2019b). Mussels are relatively easy to collect because of coastal abundance. Their semi-sessile lifestyle makes them also a suitable species to study in the lab, as this lifestyle makes them less affected by confinement compared to many other species. Mussels have already been shown to respond to sound physiologically and behaviourally, including immediate and clear valve closure upon sound exposure (Roberts et al., 2015), this provided us with the opportunity to test the effects of repeated sound exposures.

In the current study, we conducted two experiments to test whether blue mussels can habituate to sound. In experiment 1, we exposed mussels sequentially to identical tones, followed by a single different tone. We examined whether the mussels' response in valve gape diminished over sequential exposures and whether they responded more strongly again to the different sound exposure. In experiment 2, we exposed mussels to three sequential pulse trains or to three single pulses, with identical onset times, and examined whether mussels returned to baseline valve gape levels more quickly after a single pulse. We aimed to answer the following questions: (1) Do mussels change their valve gape after the onset of a sound exposure? (2) Does the change in valve gape diminish over sequential sound exposures and can this be attributed to habituation? (3) Do mussels return to baseline valve gape levels more quickly after a single pulse than during or after a pulse train?

Materials and methods

Study subjects

We used 180 wild-caught blue mussels (*Mytilus edulis*). The mussels were collected from the poles of the Scheveningen Pier in the inter-tidal area of the North Sea coast in Scheveningen, the Netherlands. The experiments were conducted in April and May 2020. Due to the COVID-19 pandemic, experiments were run at the home of EB. The mussels were kept in a salt water aquarium (120 x 44 x 43 cm; L x W x H) at nearby restaurant 'Les Copains' in Delft, the Netherlands, for at least seven days before being used in an experiment. After being used in an experiment, we released the mussels back into the wild.

Experimental set-up

The trials were performed in a plastic container (53 x 39 x 35; L x W x H) with fresh water and an underwater speaker at the centre of the bottom. We hung four plastic one-litre bottles with 0.62 L of salt water at equal distances around the speaker. We cut the top ~ 6 cm of each bottle and hung them below bamboo sticks using wire, the bamboo sticks rested on the container edges (fig. 1). The bottles allowed us to test four mussels simultaneously while excluding chemical communication or physical contact between the individuals. For this reason, we also refreshed the water in the bottles with salt water from the stock tank before each trial and we added a few drops of phytoplankton (Reef phytoplankton, Seachem) to allow the mussels to feed.

We used a valve gape monitor to log the valve gape behaviour (Ballesta-Artero et al., 2017). The valve gape monitor consisted of multiple pairs of electromagnetic coils coated in epoxy and a plastic tube. The active coil of each pair, generated an electromagnetic field which resulted in a current in the responsive coil. The strength of the measured electromagnetic field was determined by the distance between the coils, and thus reflected gape opening at high resolution. We attached the coils of one pair on opposite valves of an individual mussel using a combination of hot glue and cyanoacrylate glue. Immediately after attaching the coils, the individual mussels were hung in the centre of one of the bottles and in the middle of the water column of the container. After we placed the last mussel in a bottle, we started the playback, which started with 25 min of silence before the first sound exposure (in the exposure conditions).

Sound exposure

During the experiments, we played back pure tones with silence intervals. For experiment 1, we used four different sound treatments with 150 and 300 Hz

tones and for experiment 2, we used two treatments with 150 Hz tones only. We chose these frequencies because mussels have been shown to respond to sound from 5 to 410 Hz (Roberts et al., 2015), and we aimed for two frequencies that were perceptually different enough to test stimulus specificity but still elicited a similar response. The sound treatments were created with Audacity (version 2.3.3) and played back with an underwater speaker (UW30, Lubell labs) from a recorder (DR-07, TASCAM), through an amplifier (M033N, Kemo). For both

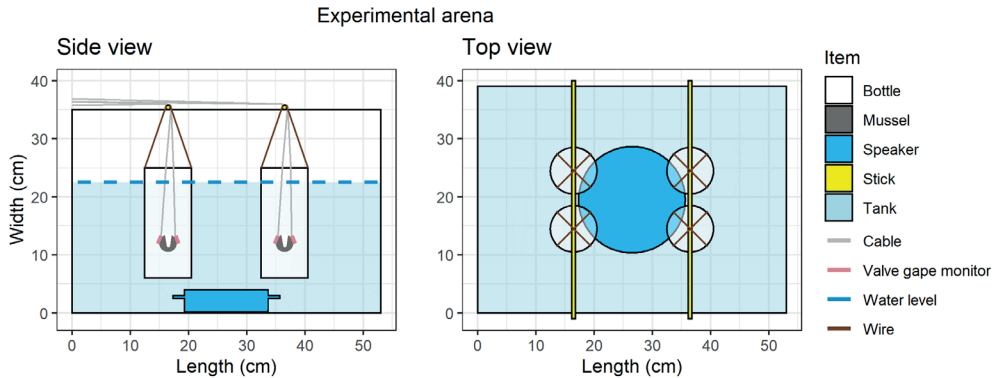


Fig. 1: Schematic views of the experimental arena (LxBxH = 54x48x35cm) from the front (Side view) and from above (Top view). During the experiments, the mussels were placed in plastic bottles hanging in the rectangular tank (see Side view). Four bottles hang at equal distances from the speaker (see Top view). The mussels were exposed to sound using a speaker on the bottom of the tank.

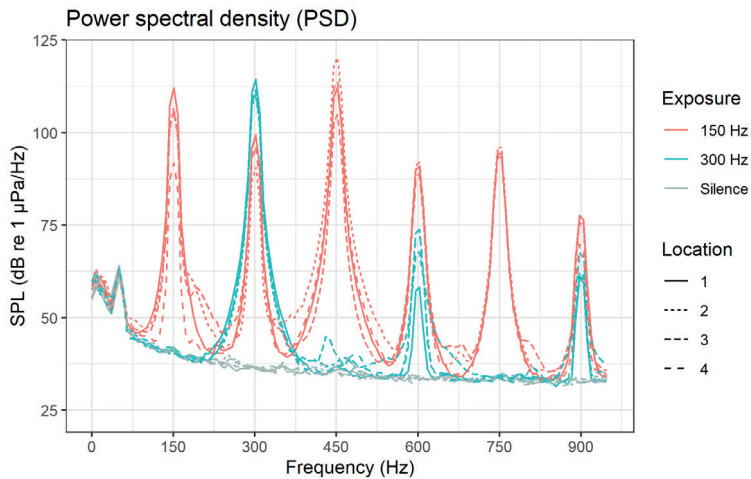


Fig. 2: Power spectral density (window length: 6144, window type: Hann) of recordings of the pure tones (red and blue) and silence playback (grey) at all mussel locations (line type). Higher harmonics of the fundamental frequency were present, probably due to speaker limitations.

experiments, we counterbalanced the order of the treatments. All trials were recorded with a calibrated hydrophone (96-min, HTI) and digital recorder (DR-100MKII, TASCAM) to confirm that all treatments had been played back correctly.

After the trials, we recorded both the pure tones that we used and the silent intervals in all four bottles, at the location of the mussel and generated power spectral density plots using a custom-made R-package (figure 2). The rms SPL (geometric mean of all locations in the 100-600 Hz bandwidth) was 138.4 dB re 1 μ Pa of the 150 Hz stimulus playback, 135.6 dB re 1 μ Pa of the 300 Hz stimulus, and 77.6 dB re 1 μ Pa for the silence playback. Both the sound levels across mussel locations and the harmonic structure (fundamental frequency plus higher harmonics at positive integer multiples) of both treatments were highly similar. Specifically, there was overlap between the higher harmonics of the 150 Hz treatment and the fundamental tone and higher harmonics of the 300 Hz treatment.

Sound propagation in tanks can be expected to differ substantially from sound propagation in the sea. The proximity of the tank walls and water surface affect the ratio between sound pressure and particle motion, and the directionality of particle motion (Campbell et al., 2019; Rogers et al., 2016). We placed the subjects as far as possible from the water surface and tank walls to minimize these effects, but the sound conditions are still expected to be substantially different from those in natural water bodies. This does not pose a problem for the current data, as our target was a proof of concept study into whether habituation to sound of any kind is possible in mussels and we did not aim to determine absolute response levels to a particular realistic anthropogenic sound.

Experiment 1

For experiment 1, we aimed to examine habituation of mussels to sequential sound exposures. To test this, we exposed individuals in the exposure conditions to 10 sequential pure tones of the same frequency, followed by a single pure tone of a different frequency. The first pure tone started after 25 min of playback of silence and each of the pure tones lasted for one minute and was followed by five min of silence, so, each trial lasted 91 min. We used a reciprocal design in which we aimed to expose half of the individuals in the exposure condition to 10 exposures of 150 Hz followed by a single 300 Hz exposure (figure 3A) and the other half to 10 exposures of 300 Hz followed by a single 150 Hz exposure (fig. 3C). We also ran control trials to examine whether the mussels in the treatment trials were responding to the sound exposures and to make sure that their responsiveness did not decrease over time in the experimental set-up

anyway, unrelated to the previous sound exposures. For the control condition, we replaced the first nine exposures of both exposure tracks with silence. In this way, we could expose half of the individuals to 79 min of silence followed by a single exposure of 150 Hz and a single sequential 300 Hz exposure (fig. 3B) and the other half first to 300 Hz and then a 150 Hz exposure (fig. 3D).

Experiment 2

For experiment 2, we aimed to examine the initial response and recovery time of mussels to single sound pulses and pulse trains. A pulse train consisted of 1 s pulses of 150 Hz separated with 9 s of silence (fig. 3E). In the single pulse

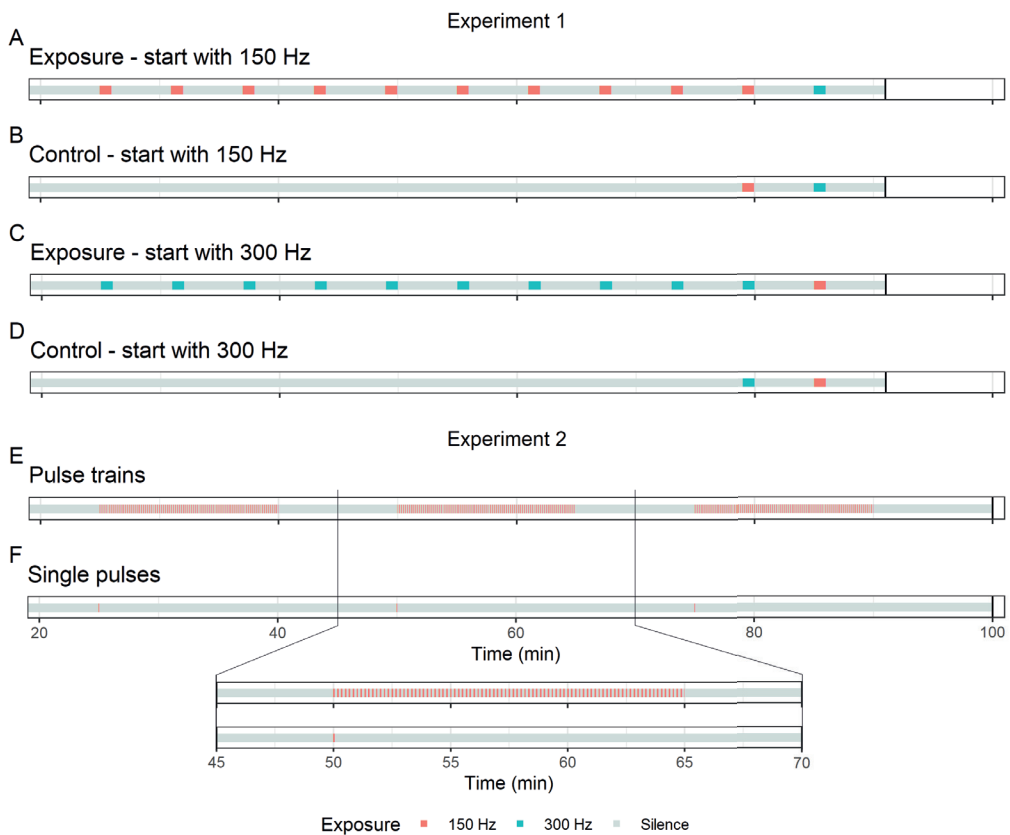


Fig. 3: Overview of the experimental playbacks in both experiments. In experiment 1, we exposed the mussels in the exposure condition to 11 sequential tones, this either started with 10 exposures of 150 Hz and ended with a single 300 Hz exposure (A) or the other way around (C). In the control conditions, we only exposed the mussels to the last two exposures (B & D). In experiment 2, we either exposed the mussels to three pulse trains (E) or three single pure tones (F). Note that this figure starts at 20 min, mussels in all conditions were only exposed to silence in the first 25 min. Duration of pulses is not to scale (E & F).

condition, we only exposed the individuals to the first pulse of a pulse train and replaced the others by silence (fig. 3f). We exposed individuals in the pulse train condition to three pulse trains of 15 min followed by 10 min of silence, the first pulse train started after 25 min of silence. In this way, both treatments lasted 100 min.

Behavioural observations

The valve gape monitor yielded on average 46 datapoints per minute of each individual. The raw data was the measured electromagnetic field strength that was converted to absolute distances using the calibration of the monitor. The size of the mussels and the location of the sensors on the mussels also influenced the absolute distance, therefore, we converted the absolute distance to the 'fraction open', with '0' being the minimal distance between the coils and '1' being the maximum distance between the coils during a complete trial of a single individual. We excluded individuals from the experiment if the absolute difference between the minimum and maximum opening distance during the entire trial was less than 1 mm (meaning the mussel barely opened, including before the start of the sound), if the mussels were not open for at least 25% at 23 min after the start of the trial (which is 2 minutes before the start of the first exposure in the exposure conditions in experiment 1 and both treatments of experiment 2), or when the coils got loose from the mussels. To examine the mussels' reaction to sound, we determined the mean fraction open the last 30 s before the onset of the sound and the first 30 s after the onset of the sound and subtracted fraction open before from the fraction open after; yielding our measure of Δ fraction open. For the second experiment, we also determined the time it took the mussels to return to at least 90% of the pre-exposure valve gape levels; the recovery time. For this, we used the fraction open before (30 s period) as pre-exposure level and used a moving average of 30 datapoints for the data after the onset of the sound to determine when the pre-exposure level was reached again (rounded to the nearest minute). If the mussel did not return to 90% of the pre-exposure levels within 25 min, we assigned 25 min as a recovery time for this individual.

For experiment 1, we analysed the data of 93 individuals; 49 in the exposure condition and 44 in the control condition. Another 15 individuals were excluded from the analysis because the valve gape sensors got detached from the mussel, the mussels did not open fast enough ($< 25\%$ at 23 min), or the mussel barely opened during the entire trial (difference between minimum and maximum distance < 1 mm). For experiment 2, we analysed the data of 61 individuals; 32 in the pulse train condition and 29 in the single pulse condition. Another 11 individuals were excluded from the analysis (reasons identical to experiment 1).

Statistics

We analysed the effect of the sound treatments on the change in valve gape (Δ fraction open) and the recovery time in R (R Core Team, 2016). For Δ fraction open, we used generalized linear models (GLMs) with a Gaussian error distribution and identity link-function. When treatment groups had unequal variance, we used a linear model using Generalized Least Squares instead (R-package nlme, Pinheiro et al., 2020). For recovery time (min), we used a zero-inflated regression (R-package pscl, Zeileis et al., 2008) with a binomial error distribution with logit link-function for the zero-inflation model and, a Poisson error distribution and log link-function for the count model. In experiment 1, we only used Δ fraction open as a response variable for all models and always used the fraction open before the sound exposure as an explanatory variable in the full model. Depending on the research question, we also used treatment type (exposure versus control) or both exposure number and treatment frequency (150 versus 300 Hz), and the interaction between them, in the full model. In experiment 2, we used Δ fraction open and recovery time as response variables in two separate models, both with the fraction open before the sound exposure, treatment type (pulse trains versus single pulses), exposure number, and the interaction between the latter two as explanatory variables in the full models. For each full model, we determined the AICc score of all possible explanatory variable combinations and selected the model with the lowest AICc as best model. If the explanatory variable of interest (relevant to the research question) was not part of the best model, we added it to the final model anyway to obtain an estimate and p-value. To determine the effect and significance of the covariates, we ran the final models.

Results

Experiment 1

Two example trials (fig. 4ab) show that these two mussels had closed their valves at the start of the trial, probably due to handling the individuals, and gradually opened during the first 25 min. Both individuals responded to the first few sound exposures by almost completely closing their valves. The magnitude of the response decreased over the first ten exposures in one of them (fig. 4a), whereas the other stopped responding altogether (fig. 4b). Both individuals responded again to the 11th tone of a different frequency than, but one of them did not respond as strongly as to the first exposure.

To test whether mussels changed their valve gape in response to the sound, we compared the Δ fraction open of the mussels in the exposure conditions at the

first exposure with the Δ fraction open at the same timestamp in the control condition (silence was played here). The mussels in the exposure condition significantly reduced their fraction open (they partially closed) when compared to the control mussels (Intercept: 0.32, Exposure: -0.35, p-value: < 0.01, fig. 5). Additionally, the more open their valves were before the onset of the sound, the more they closed (Intercept: 0.32, Before slope: -0.53, p-value < 0.01), this was the case in almost all models, so we do not mention it anymore hereafter. In the exposure conditions, their valve closure decayed (they closed less) over the first 10 sequential exposures (Intercept: -0.11, Exposure number slope: 0.03,

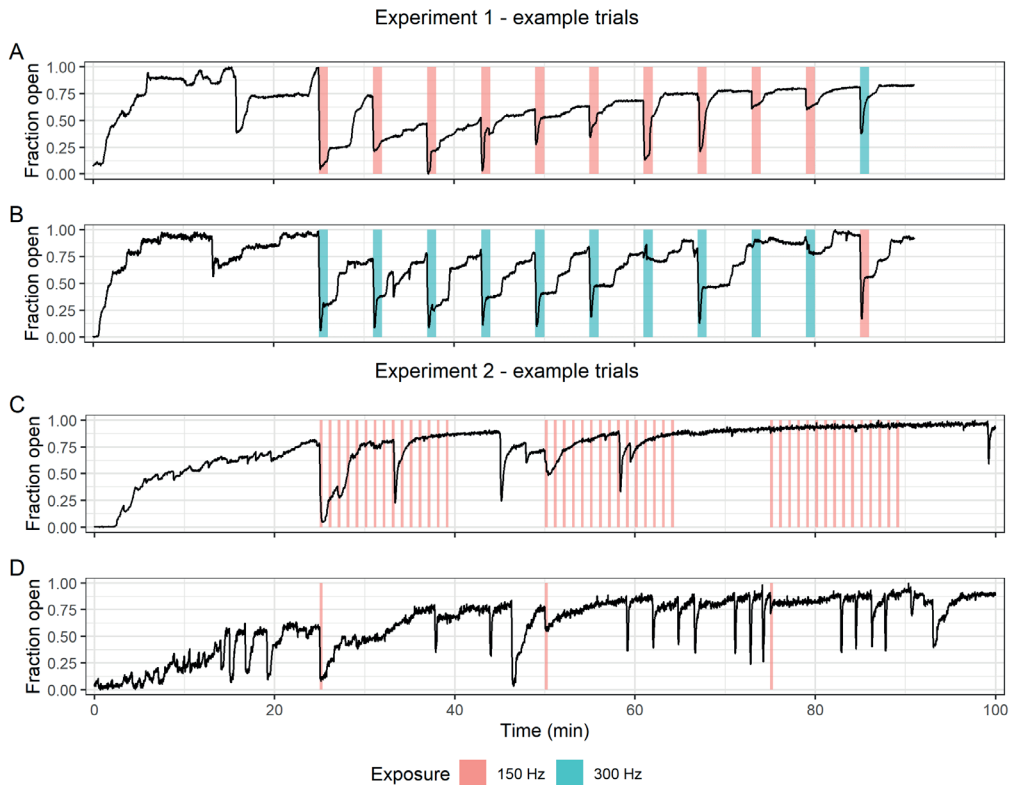


Fig. 4: Valve gape behaviour during an entire trial of four individuals. The individual in the top panel (A), responded to every single exposure, but the magnitude of the response decreased in the first 10 exposures and increased again at the 11th different exposure. The individual in the second panel (B) either responded quite strongly, or did not close at all (exposure number 7, 9 & 10), but responded strongly again to the last exposure with a pure tone of different frequency. The individual in the third panel (C) seems to have responded to the first pulse of the first and second pulse train and returned to pre-exposure levels before the end of the pulse trains. The individual in the bottom panel (D) seems to have responded to all three single pulses, but the magnitude of the response seems to have decreased. For display purposes, the pulse duration is not drawn to scale (C & D) and the amount of pulses is reduced (C).

p-value: < 0.01). The treatment frequency was not part of the best model, neither separately nor in interaction with exposure number. This meant that there were no differences in response to the pure tones nor a different pattern in the decay of the response. To test whether this decay in responsiveness can be attributed to habituation, we exposed the mussels in the exposure condition to an 11th tone of a different frequency and compared their response to this tone with their response to the 10th tone. Here, we found an interaction between the treatment frequency and exposure number; mussels that were first exposed to 150 Hz did not respond more strongly to a sequential 300 Hz tone (Intercept: 0.30, Exposure number slope: -0.03, p-value: 0.35). However, the mussels that were first exposed to 300 Hz tones, and subsequently to a 150 Hz tone, did close their valves more in response to the latter (Intercept: 0.30, Exposure number slope: -0.02, p-value:

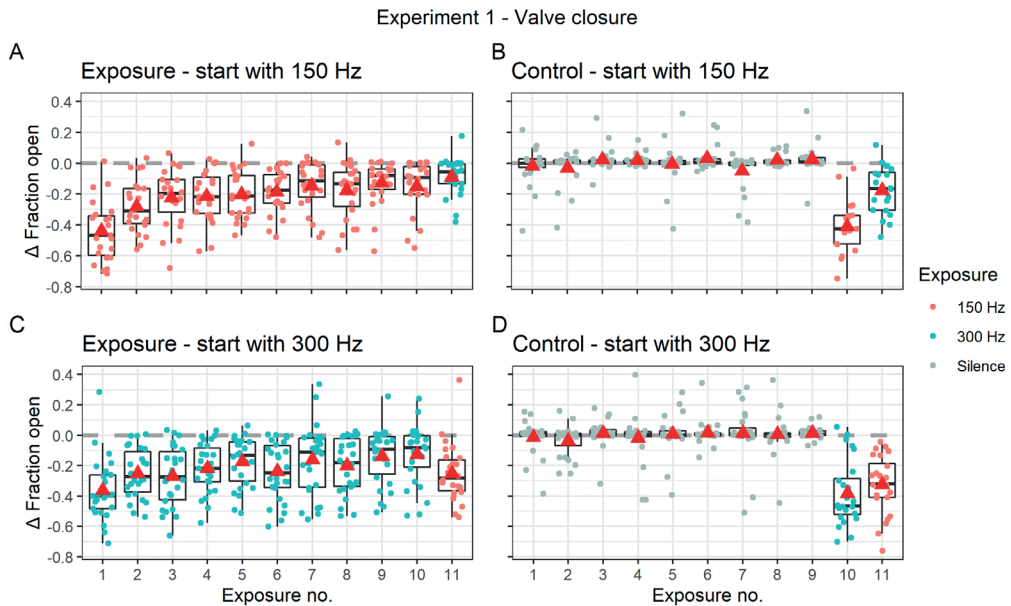


Fig. 5: The responses of all mussels that were exposed to any of the four treatments of experiment 1. A Δ fraction open below zero indicates (partial) closure of the valves. The coloured points indicate the individual responses to the sound exposures. The box-and-whisker plots indicate the median, first and third quartile and, minimum and maximum excluding outliers of all individuals per exposure, and the red triangles indicate the means. The dashed grey lines indicate 0, meaning no change in fraction open. The mussels significantly closed their valves in response to the first exposure (A & C) when compared to the same timestamp in the controls (B & D). There was a significant decay in response magnitude over the first 10 sequential exposures (A & C). Mussels that had been exposed to 10 sequential 300 Hz exposures responded more strongly again to the eleventh 150 Hz exposure (C), in the opposite order, this was not the case (A). There was no significant difference between the first exposure in the exposure treatments (A & C) and the first actual exposure (exposure number 10) in the control condition (B & D).

< 0.01). To verify that the reduced response of the mussels to sequential sound exposures cannot be explained by the increasing time in the experimental set-up, we compared the mussels' response to the first exposure in the exposure condition with the first exposure in the control condition (Exposure number 10). We found no difference between these responses (Intercept: 0.11, Treatment: -0.03, p-value: 0.34).

Experiment 2

Two example trials (fig. 4cd) show that these mussels responded to the sound exposure by almost completely closing their valves. Both mussels gradually returned to pre-exposure levels, the mussels that was exposed to the pulse train already during the sound exposure (fig. 4c). The response to the onset of the sound decreased over sequential exposures and one of them did not respond at all to the last exposure (fig. 4c).

Similar to the first experiment, there was a negative correlation between the mussels' response and the exposure number (Intercept: -0.04, Exposure number slope: 0.03, p-value: 0.02, fig. 6ab), the mussels reduced their valve closure with sequential sound exposures. The treatment type was not part of the best model, neither separately nor as interaction with exposure number, meaning that the response and decay in response was not significantly different for mussels that

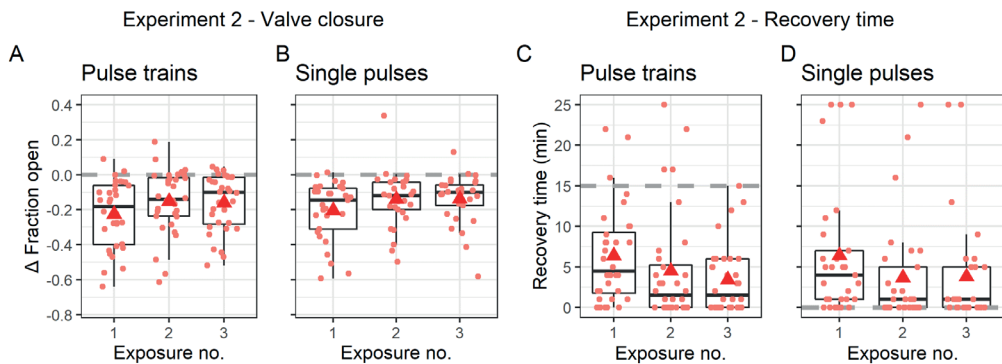


Fig. 6: The change in valve gape of all mussels that were exposed to one of the two playback treatments of experiment 2 (A & B). There was a significant reduction in response to the sound over the sequential sound exposures, but no differences between the reactions or decay in reaction in mussels that were exposed to pulse trains (A) or single pulses (B). Time it took the mussels in both treatments to return to 90% of the pre-exposure fraction open (C & D). There was a significant reduction in recovery time over the sequential sound exposures, but no differences between the recovery times or decay in recovery times in mussels that were exposed to pulse trains (C) or single pulses (D). The x-axes (Exposure no.) indicate the number of pulse trains (A & C) and the number of single pulses (B & D) in a trial.

were exposed to pulse trains or single pulses. Similarly, increasing numbers of mussels did not respond substantially (a Recovery time of 0 min) over sequential exposures (Intercept: 0.76, exposure number slope: 0.54, p-value: < 0.01, fig. 6cd). The mussels that responded (Recovery time ≥ 1 min), recovered quicker over sequential exposures (Intercept: 2.47, Exposure number slope: -0.12, p-value: < 0.01, fig. 6cd). Again, treatment type was not part of the best model, so, there were no differences between the recovery times or decay in recovery times to pulse trains and single pulses.

Discussion

In the current study, we exposed blue mussels to repeated sound exposures and examined their valve gape responses. Our results demonstrate that mussels responded to sound by partially closing their valve gape. Their response decreased over sequential sound exposures of 1 min, independent of stimulus frequency, and, in one of the exposure conditions, they responded stronger again to a different sound exposure. This result shows that mussels can habituate to sound. Their recovery time after a single pulse of 1 s, was not shorter than their recovery during and after a pulse train of 15 min. This result indicates that mussels mostly responded to the onset of the pulse train, with little effect of the rest of the pulses in the train.

Response to sound

The blue mussels in the current study responded to the sound exposures by (partially) closing their valves. Mussels have been shown to close their valves in response to a variety of environmental conditions, including fluctuations in temperature and salinity, air exposure, potentially poisonous chemicals and conspecific homogenate. So, valve closure seems to be a general defence response (Bayne et al., 1976; Curtis et al., 2000; Robson et al., 2010). The valve closure in the current study is in line with previous research in which mussels also responded to tonal sound by closing their valves (Roberts et al., 2015). In contrast, mussels that were exposed to ship noise for 1 h had a larger mean absolute valve opening than mussels in the control condition (Wale et al., 2019). However, only a limited number of individuals were tested in the latter study ($n = 6$ and 8 , for noise and control condition respectively). Nevertheless, it may be that mussels respond differently to tonal and shipping sound, or initially respond by valve closure and later compensate for this.

The consequences of partial valve closure for shorter or longer periods due to sound exposure are not straightforward, as becomes clear from a brief review of the few relevant studies. Two earlier studies examined the filtration rate of

mussels during sound exposures. Mussels that were exposed to 50 min of pile driving were shown to increase their filtration rate (Spiga et al., 2016), whereas mussels exposed to 3 h of ship noise were observed to reduce filtration rate (Wale et al., 2019). These different results may be due to the use of different stimuli, but again, they may also be due to the limited number of individuals tested in the latter study ($n = 5$ for both treatment and control). It is, nevertheless, interesting that Wale et al. (2019) found both a larger valve gape and a reduced filtration rate during shipping sound, in the same study, but in different individuals. This suggests that valve gape is not necessarily positively correlated with filtration rate. In another study, without any particular stressor, Jørgensen et al. (1988) did find a positive correlation between valve gape and filtration rate. But large variation in the correlation between valve gape and both exhalant siphon area and pumping rate is apparently not uncommon (Maire et al., 2007). More research is obviously needed to understand the impact of sound on mussel behaviour and physiology. It would be revealing to test the same individuals to the different types of acoustic stimuli and by examining valve gape (and potentially also exhalant siphon area) and consequences for filtration rate simultaneously.

Habituation to sequential sound exposures

In experiment 1, the mussel valve closure in response to the sound exposures decayed over sequential exposures. The mussels in the control conditions were not exposed to the first nine exposures and responded as strongly to the 10th exposure as the mussels in the exposure conditions to the first exposure, this shows that the decrease in responsiveness is not caused by an increasing time in the experimental set-up. The mussels in the exposure condition that were first exposed to 10 sounds of 300 Hz followed by one of 150 Hz increased their response again to the 150 Hz. This test of stimulus specificity provided essential proof for that the decreased response can be attributed to habituation and not to sensory adaptation, sensory fatigue or motor fatigue (Rankin et al., 2009). Mussels that were first exposed to 10 sounds of 150 Hz did not show stimulus specificity, as they did not increase their response to the final test sound of 300 Hz. This result can probably be explained by the higher harmonic tones at 300 Hz and 600 Hz of the 150 Hz sound (fig. 2). As 300 Hz and 600 Hz tones were also present in the spectrum of the last 300 Hz exposure, this test sound was potentially not novel enough to elicit a stronger response again. The response and decay in response to the first 10 exposures was not different between 150 and 300 Hz, so the differences in response to the 11th tone cannot be explained by a higher sensitivity to either of the stimuli. In contrast, the presence of 150 Hz, besides shared energy at 300Hz and 600Hz, made the 150 Hz sound exposure apparently novel enough to elevate response strength, which reveals spectral discrimination at this resolution, which is as far as we know also a novelty for

this invertebrate taxon.

We were able to show that blue mussels are able to habituate to sound, which is important for understanding potential impact. The fact that mussels can habituate in general is in line with previous studies that have shown that oysters (other bivalves) habituate to visual and tactile stimuli (Dehaudt et al., 2019; Wilkens, 1986). Habituation may be advantageous to stimuli that are continuously or repeatedly present without being associated with harmful consequences (Bejder et al., 2009; Rankin et al., 2009). However, habituation of a particular behavioural response does not necessarily mean habituation in all behavioural responses (Neo et al., 2018). Also, behavioural habituation does not necessarily mean the absence of a negative effect of disturbance (Bejder et al., 2009). Sound may still cause physiological stress (Wale et al., 2019), masking (Wysocki and Ladich, 2005), and attention shifts (Chan et al., 2010; Walsh et al., 2017). Nevertheless, investigating the potential for mitigating impact through habituation, is critical to understand the effects of long-term and repeated sound exposures on marine life.

Response to pulse trains

No differences were found in the mussels' initial response and recovery time to a pulse trains and single pulses. This may indicate that the response and rate of recovery was mostly determined by the first pulse of the 15 min pulse train. While this may explain the disturbance potency of anthropogenic sounds that are more sudden and fluctuating in time (Vetter et al., 2015; Wysocki et al., 2006; Zhao et al., 2019), it may also open up possibilities to alternative mitigation strategies beyond just stopping noisy activities. We chose the current pulse rate (1 s pulse, 9 s silence) because it falls in the range of pulse rates of seismic surveys, with a pulse every 5 - 15 s depending on the type of survey (McCauley et al., 2000; Slabbekoorn et al., 2019). For pile driving, typically a faster pulse rate is used; a pulse every 1 - 4 s (Hall, 2013; Matuschek and Betke, 2009). Both seismic surveys as pile driving also have larger breaks in between separate pulse trains, to turn the seismic ship, to adjust a pile, start with a new pile, or because of marine mammal sightings or bad weather. Different pulse rates may elicit different behavioural responses (Neo et al., 2015b), but not necessarily (Hubert et al., 2020). In both rats (*Rattus norvegicus*) and humans, faster pulse rates resulted in a faster decrease of startle-like responses (Davis, 1970; Gatchel, 1975). It may be that a lower pulse rate than currently used would have delayed the recovery time. We believe that more studies are warranted into the importance of inter-pulse interval and inter-pulse train interval in determining the habituation rate and recovery time to explore mitigation potential.

Conclusions

Our study examined the behavioural response, habituation tendency, and recovery time of blue mussels to artificial sound exposures. We found that mussels responded to sound by partially closing their valves. This response decayed over sequential sound exposures and the mussels responded stronger again to a different sound stimulus. We thereby provide unambiguous evidence that the decay in response can be attributed to habituation. We did not find differences in the initial response and recovery between exposure to a pulse train and a single pulse, which revealed a strong bias in salience towards the on-set of pulse trains, and maybe to sound condition changes in general. Future studies are needed to examine the effects of variation in the sound stimulus and interval duration. It appears that the response and habituation of mussels to anthropogenic noise has potential for mitigating impact and this should also be investigated in their natural environment.

Ethical statement

We adhered to the Guidelines for the treatment of animals in behavioural research and teaching (ASAB, 2018). There are no legal requirements for studies involving bivalves in the Netherlands.

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Data accessibility

All data used for the analyses reported in this article are available from the Zenodo Repository, DOI:10.5281/zenodo.4068351.

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