OCS Study
BOEM 2024-061

Behavioral Effects of Sound Sources from Offshore Renewable Energy Construction on the Black Sea Bass (*Centropristis striata*) and Longfin Squid (*Doryteuthis pealeii*): A Field-based Study

U.S. Department of the Interior
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Sterling, VA

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November 2024

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Prepared under Obligation M20AC10009
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Bureau of Ocean Energy Management
Sterling, VA

DISCLAIMER

This study was funded, in part, by the U.S. Department of the Interior, Bureau of Ocean Energy Management (BOEM), Environmental Studies Program, Washington, DC, through Interagency Agreement Number M20AC10009 with the National Oceanic and Atmospheric Administration National Marine Fisheries Service Northeast Fisheries Science Center. This report has been technically reviewed by BOEM, and it has been approved for publication. The views and conclusions contained in this document are those of the authors and should not be interpreted as representing the opinions or policies of BOEM, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

REPORT AVAILABILITY

CITATION

Mooney TA, Cones S, Jarriel S, Formel N, Ferguson S, Aoki N, Stanley JA, Jezequel Y. (Woods Hole Oceanographic Institution, Woods Hole, MA). 2024. Behavioral effects of sound sources from offshore renewable energy construction on the black sea bass (*Centropristis striata*) and longfin squid (*Doryteuthis pealeii*): a field-based study. Sterling (VA): U.S. Department of the Interior, Bureau of Ocean Energy Management. 106 p. Obligation No.: M20AC10009. Report No.: BOEM 2024-061.

ACKNOWLEDGMENTS

Many people have assisted on this project through three years and without their help this project would not have been possible. We thank Scott Bennet, Erin Gaglias, and the R/V *Gemma* Captains for providing and collecting squid. We thank the W.S. Schultz Company for conducting the pile driving, especially Matt and Ben Karson. We thank Edward O'Brien and Kimberly Malkoski for facilitating SCUBA operations throughout the experiment. We also thank Rick Galat, Kerry Strom, Stephanie Madsen, and other members of the WHOI Facilities team for coordinating dock space and vessel traffic. We express gratitude to Roger Hanlon for his discussions on our squid results and data interpretations. We appreciate the support and suggestions of Brian Hooker and Jacob Levenson, and BOEM Center for Marine Acoustics.

Contents

List of Figures

List of Tables

Abbreviations and Acronyms

Short form	Long form
BOEM	Bureau of Ocean Energy Management
IH	impact hammer
MBL	Marine Biological Laboratory
PD	Pile driving
ODBA	overall dynamic body acceleration
OSW	offshore windfarm
PSD	power spectral density
SPL	sound pressure level (dB re 1 uPa)
SPL_{rms}	Root-mean-squared sound pressure level (dB re 1 uPa)
SPL_{pp} SPL_{zpk}	Peak-to-peak sound pressure levels zero-to-peak sound pressure levels
SEL_{cum}	Cumulative sound exposure level (dB re (1 μPa)^2.s)
SEL_{ss}	Single strike sound exposure level (dB re (1 μPa)^2.s)
PAL_{zpk}	zero-to-peak particle acceleration levels
VH	vibratory hammer
WHOI	Woods Hole Oceanographic Institution

Overview

This study project was a Cooperative Agreement between BOEM and the Woods Hole Oceanographic Institution. This overall project had two parts. Part one was a laboratory study awarded as an interagency agreement with the National Oceanic & Atmospheric Administration's (NOAA's) Northeast Fisheries Science Center in 2017. This current report describes the companion field study. Several papers were published in peer-reviewed journals, as a result of this project's BOEM funding. Three are reproduced in this report, along with links to the initial publications.

The objectives of the study were to examine the effects of offshore windfarm construction noise on two key commercially and ecological important taxa, squid and black sea bass, using field-based controlled exposures to actual *in situ* pile driving and associated vessel noise.

The principal target for the investigation is commercially important fish in the North and Mid-Atlantic Planning Areas, principally black sea bass and longfin squid. The percussive action of pile-driving offshore wind foundations has the potential to induce physical or behavior impact to fish. This study will evaluate that potential in a field setting.

Because this study principally addresses fisheries resource impacts from offshore wind energy development it addresses not only impacts to the fishery resource itself, but on the U.S. private and public sectors that rely on the resource for commercial and recreational use, respectively. This study would evaluate the physical and physiological impact to fish and/or mollusks during construction of an offshore wind energy facility.

Among the research questions to be addressed were: Does sound generated during offshore wind construction affect important fish species like black sea bass and squid? At what amplitude do pile-driving or other project sounds induce a behavioral response? At what amplitude do these sounds lead to physiological damage to the auditory system?

Auditory thresholds for some commercial fish species have been established but, for some species, such as black sea bass, data are lacking. Black sea bass support valuable commercial fisheries in the North, Mid, and South Atlantic Planning Areas. Black sea bass show affinity for certain habitats within the wind energy lease areas and are thus not a temporary resident of these lease areas (Guida et al 2017). Commercial and recreational fishermen have expressed concern that noise produced during sub-bottom surveys, pile-driving, and operation of renewable energy facilities may have a negative effect on the behavior of black sea bass ranging from catchability to long-term reproductive success. This species is known to detect mid-frequency acoustics (80–1000 Hz) which may be used as environmental indicators but their sensitivities to anthropogenic sounds such as pile driving noise, and their behavioral responses to them, is not understood (Stanley et al., 2020). Sounds that could lead to acute or chronic sub-lethal effects may be generated as a result of offshore wind development. Black sea bass could be vulnerable because they are known to use acoustic cues to communicate and because their habitats overlap within renewable energy lease areas. If feasible, other priority species, such as squid, identified in the Normandeau 2012 (BOEM Contract # M11PC00031) may be evaluated.

Researchers conducted experiments and observations of longfin squid, black sea bass, and the surrounding ecosystem at set distances before, during, and after pile driving events (or control, no-sound days). The project followed a before-during-and-after gradient design, where potential significant changes from baseline in the variables of interest are assessed using statistical methods that allow for the exploration of changes in spatial relationships over time.

Overall objectives of this study are listed below.

1. Characterize the relevant acoustic pile driving signals in pressure and particle motion (in the water column and on the benthos) at varying distances during offshore construction. Carry-out field-based controlled exposures.

2. Quantify the movement, energetic patterns and potential displacement of free-swimming squid and black sea bass using high-resolution, movement and behavior tags and moored echosounders. The goal is to evaluate potential changes in swimming energetics, swimming patterns and overall displacement from an area.

3. In caged and location-controlled animals, examine distance and sound-level dependent impacts to:

    a. Representative, sexually mature reproductive adults schooling and shoaling of squid and black sea bass, including impacts to communication, group cohesion/predator avoidance, mating behaviors and breeding. The goal is to evaluate critical behavioral impacts to schools and small populations of animals, impacts would influence future populations through disturbance to breeding, intraspecific mating communication, and susceptibility to predators. Multiple spatial scales (distances) and sound levels will be addressed.

    b. Potential hearing loss for in situ exposed animals. The goal is to determine if there are physiological, auditory impacts due to acoustic pressure or particle motion; impacts which could influence sensory systems and balance.

    c. Development and potential premature hatch of immobile squid embryos and egg mops. Initially, in the first year of the study, controlled environment pilot studies will be carried out to better understand the temporal and spatial scope needed to transfer the methods to the field. The goal here is to measure whether pile driving and construction noise exposure will induce premature hatching and late-stage developing embryos, thus a potential impact on future cohorts.

4. Address the overall influence on the biological community around squid and black sea bass and their habitats, using moored echosounders. The goal is to evaluate whether potential predator or prey availability are also influenced by pile driving. Such data are key, particularly for squids, given their important role a central trophic link in marine food webs.

Results from this study will help in BOEM’s noise impact assessments to commercial fish species under the National Environmental Policy Act and the Essential Fish Habitat provisions of the Magnuson-Stevens Fishery Conservation and Management Act. The outcome will be a better understanding of the physical, physiological, and behavioral impacts to fish associated with offshore wind construction activity.

1 Introduction

Sound is a vital sensory cue for a diverse array of marine animals. Emerging, or sometimes underappreciated, evidence underscores that acoustic ecology, and by extension, noise impacts, are not confined to marine mammals. Fish and aquatic invertebrates are also sound-sensitive (Gedamke et al., 2016). In recent decades, appreciation and understanding of sound detection and use by these taxa has grown and more research has quantified sound-mediated abilities. In fishes, this includes species that differ in the degree or type of auditory specializations (Popper and Hawkins, 2018, 2019; Popper et al., 2019); in invertebrates, sound-sensitivity has been noted across mollusks, arthropods, and cnidarians (e.g., Jézéquel et al., 2021; Lillis et al., 2016; Mooney et al., 2010; Packard et al., 1990; Roberts et al., 2015b; Stanley et al., 2011).

Yet we are only just beginning to understand how these taxa detect, use and are impacted to use sound. Many details about the hearing abilities and ecological functions of hearing in these taxa remain unknown (Popper and Hawkins, 2018). Little is understood regarding how noise pollution from anthropogenic activities may adversely affect the physiology and behavior of marine fishes and invertebrates, their sound-detection abilities, and how such changes may lead to population-level consequences (Hawkins et al., 2015). The ocean is rapidly changing, in part because of increasing human-produced noise. It is timely to conduct research focused on model invertebrate species and habitats wherein the sound cues available to these species, and responses of invertebrates to sound can be thoroughly quantified and better understood.

1.2 Noise Impacts on Fish and Invertebrates

In the past century, anthropogenic noise has become increasingly prevalent in underwater environments due to expanding human use of the oceans. For example, shipping activity and associated noise has increased dramatically over recent decades and is predicted to further increase in this decade (McDonald et al., 2006; Kaplan and Solomon, 2016). Other common anthropogenic noise sources include sonar from resource exploration, geophysical research, and military operations, noise from construction of marine platforms, and operational noise from marine energy platforms such as wind turbines (Slabbekoorn et al., 2010; Mooney et al., 2020). Given the measured (or expected) overlap in animals' sound sensitivities and the frequencies of anthropogenic noise, many fishes and invertebrates may detect and respond to anthropogenic sounds (Roberts and Elliott, 2017; Murchy et al., 2019; Mooney et al., 2020). Yet, to date, research has been limited, especially for field-based studies.

With respect to fishes, studies investigating the lethal and permanent effects caused by pile driving noise have revealed a variety of results in multiple species, ranging from mortality to damage to hearing tissues and other organs (Popper and Hastings, 2009). For example, many injury types were observed hybrid striped bass white bass (*Morone chrysops/saxatilis*) when exposed to simulated pile driving signal using High Intensity Controlled Impedance Fluid Filled wave tube (Casper et al., 2013b). Injury number and severity increased with fish size. Similar results were also found in lake sturgeon (*Acipenser fulvescens*) and Nile tilapia (*Oreochromis niloticus*), with injury occurring at the lowest levels tested 204 SEL_{cum} (dB re 1 $\mu Pa^2.s$) and 174 SEL_{ss} (dB re 1 $\mu Pa^2.s$), and single strike exposure levels only slightly higher than those recorded 1.5 km away from real wind turbine construction (Table 1, HDR 2020). More severe and greater number of injuries occurred at the loudest cumulative and single-strike sound exposure levels (216 SEL_{cum} and 186 SEL_{ss}).

Using vastly different methods, caged northern anchovy (*Engraulis mordax*) and common sole larvae (*Solea solea*) showed no increase in mortality or pathology compared to control groups when exposed to 4-min of pile driving (9.75 m from a 0.61 m diameter pile) and simulated pile driving sound levels [up to 210 dB re 1 μPa^2 zero-to-peak (z-pk)] (Abbott, 2005; Bolle et al., 2012). Comparative studies show that fishes with physoclistous swim bladders are more susceptible to injury from impulsive noise sources, including pile driving, than fishes with physostomous swim bladders (Halvorsen et al., 2012; Casper et al., 2013b).

In terms of damage to hearing systems, hybrid striped bass and Mozambique tilapia (*Oreochromis mossambicus*) exposed to 960 pile driving strikes showed barotrauma and damaged inner ear hair cells when exposed to the highest levels in the study (216 dB re 1 μPa^2.s) (Casper et al., 2013a; Casper et al., 2013b).

With respect to physiology and behavior, the European seabass (*Dicentrarchus labrax*) is among the most widely studied of the fishes and have been found to change their schooling structure and dynamics, becoming less cohesive, directionally ordered and poorly correlated in speed and directional changes when exposed to playbacks of pile driving (SEL$_{cum}$ 154 dB re 1 μPa^2.s). Exposure significantly disrupted the organization of their shoals and abilities to coordinate their movements with one another, behaviors which are ecologically beneficial for information exchange and reducing predation risk ((Herbert-Read et al., 2017). Additionally, when exposed to impulsive, low-frequency noise (200-1000 Hz, mean SPL$_{zpk}$ 180-192 dB re 1 μPa and SVL$_{zpk}$ 124-125 dB re 1 nm/s) seabass exhibit increased swimming speeds and depths, reduced inter-fish distances, increased startle responses, and increased movement away from the sound source (Neo et al., 2016). Trials with the use of a ‘ramp-up’ procedure (amplitude fade-in’s applied as a mitigation strategy) also elicited immediate diving responses similar to normal exposures (Neo et al., 2016).

Studies on invertebrate responses to various anthropogenic noise sources have revealed a suite of behavioral changes, including startle responses of squid ((Fewtrell and McCauley, 2012; Stanley et al., 2023), hermit crabs (Roberts et al., 2016), and mussels (Roberts et al., 2015), impaired feeding and resource searching of crabs (Wale et al., 2013; Roberts and Laidre, 2019), delayed metamorphosis of pre-settlement crabs (Pine et al., 2012), impaired escape behaviors in rock lobsters (Day et al., 2019), and physiological changes indicating stress or reduced capacity for homeostasis in scallops and crabs (Wale et al., 2013; Day et al., 2017). Temporary reductions in hearing sensitivity and masking effects (reduced detectability of ecologically relevant cues, in part due to lower signal-to-noise ratios) during noise have been demonstrated in some marine mammals and fishes (Popper et al., 2005; Caiger et al., 2012; Kastelein et al., 2015), and prior to this work only one study has addressed potential hearing loss in a marine invertebrate (exposed to vessel noise) underscoring the need for replication and addressing additional noise types.

It is notable that fishes and many invertebrates detect particle motion, which refers to the “back-and-forth” vibratory movement of particles in a sound field (Hawkins and Popper 2018). Some fish (with compressible air cavities such as an air-filled inner ear) detect sound pressure, but pressure detection has not been described in marine invertebrates. Invertebrates, which generally lack compressible, air-filled cavities, appear only sensitive to particle motion.

Table 1. Received levels (sound pressure and particle acceleration) of real wind farm construction pile driving at various distances reported in literature, all of which had no sound mitigation strategies employed.

Offshore wind farm	Distance from sound source (m	SPL$_{zpk}$ (dB re 1 μPa)	SPL$_{pp}$ (dB re 1 μPa)	SEL$_{ss}$ dB re (1 μPa)^2.s	PAL$_{zpk}$ (dB re 1 μm/s^2	Foundation
Moray Firth wind farm (Bailey et al., 2010)	100 m	-	205	-	-	1.8 m tubular steel pile (jacket)
	~ 990 m	-	~190-195	-	-	
Block Island Wind Farm (BIWF) (Amaral et al., 2020)	7500 m	~150 - 167	-	~130 - 144	-	1.5 m tubular steel pile (jacket)
Windfarm in the German Bight, North Sea (Sigray et al., 2022)	880 m	-	-	-	129	Not mentioned
Coastal Virginia Offshore Wind (CVOW) (HDR 2020)	1500 m	182	188	165	71.5 – 76.8 (30 – 83 Hz) (Potty et al., 2023)	7.8 m monopile
	3000 m	176 - 177	182 – 183	158 – 159	-	
	7500 m	169 - 170	175-176	151 - 152	-	

Literature values of received levels from real wind turbine construction.

1.3 Renewable Energy

Offshore wind energy development has been identified as a priority in European and U.S. waters. In the U.S., much of this development occurs in Essential Fish Habitat for federally managed and commercially important fish species. Designing effective monitoring and mitigation programs to assess potential wind farm influences requires the evaluation of assessments of impacts to taxa and communities. Baseline data and experiments are critical; without these it is impossible to test predicted impacts and effectively examine potential changes. One of the vital areas of potential effects, as identified by U.S. agencies, is acoustic disturbance from ‘noise’.

With the range of structures and activities involved during OWF construction there also comes a variety of potential noise sources and levels. One of the most significant activities during the construction is the installation of piled foundations (International Organization for Standardization, 2017). This is most often achieved using impact or vibrational/percussive hammers which can produce a wide range of peak source sound levels. Sound is generated by contact of the pile to the water and striking of the hammer to the pile creating acoustic waves radiating out from the pile via multiple paths through the water column and substrate, resulting in a loud, high-energy, impulsive sound with sharp rise times (review - Andersson et al., 2016). Underwater sound levels (both particle motion and sound pressure) and detection distances will vary substantially by site, and is dependent on many factors including substrate characteristics, depth, pile diameter, size of impact hammer and how it is measured. However, pressures measured from field examples are in the order of 220 dB re. 1 μPa at a range of ~10 m, 200 dB re. 1 μPa at a range of 300 m from 0.75 m and 5 m diameter piles respectively (Reinhall and Dahl, 2011). The predominant energy is below 500 Hz, with some energy extending past 1 kHz, and with sharp rise times to maximum energy. The measured frequency range directly overlaps with the auditory bandwidth of many fish and invertebrate species across multiple lifestyles (e.g., pelagic, epibenthic, demersal) including cod, salmon, black sea bass, flatfish and squid, to name a few (Chapman and Sand, 1974; Hawkins and Chapman, 1975; Mooney et al., 2010; Popper et al., 2019). Several studies have investigated the effects of construction noise exposure. These studies use a range of methods and species and have consequently found a wide of results and impacts which range from severe physical injury to no effect (see Mooney et al. 2020 for a review), making it difficult to extrapolate across taxa. This works seeks to fill those gaps by focusing our studies on two key taxa of commercial and ecological importance.

1.4 Key Study Taxa

1.4.1 Black sea bass (*Centropristis striata*) biology

The black sea bass *Centropritis striata* (Linnaeus 1758) is a principal fisheries species in the western North Atlantic. This is a warm temperate taxon that shows an attraction toward structurally complex habitats such as rocky reefs, cobble and rock fields, stone coral patches, exposed stiff clay, and mussel beds (Steimle, 1999). Black sea bass occur along the entire eastern seaboard of North America. However, the species exists as three populations or stocks: northern, southern, and Gulf of America (previously the Gulf of Mexico). For the northern stock, which is the focus of this project, Cape Cod is typically the northernmost endpoint, with this population undergoing a seasonal migration, moving north and inshore from southern and deeper waters respectively in late spring (Steimle, 1999). This stock also supports a valuable commercial and recreational fishery. There is some circumstantial evidence that *C. striata* communicate acoustically (Fish and Mowbray, 1970), and potentially during spawning events. Additionally, there is one study that elicited young of the year in this species to approach a predetermined feeding space when presented with a 280 Hz pure tone (Lindell et al., 2012). Recent efforts have indicated that the auditory thresholds and hearing range of *C. striata* overlaps that of pile driving, suggesting that noise impacts may occur (Stanley et al., 2023).

1.4.2 Cephalopods and the longfin squid (*Doryteuthis pealeii*)

Cephalopods are mollusks belonging to the class Cephalopoda, which also includes cuttlefishes, octopuses and nautiluses. The longfin squid *D. pealeii*, which is the study species for Studies 3 – 5, has been the study subject of decades of neurophysiological and behavioral research. It has long been a model species for biomedical and neurobiological work, and much of the basic knowledge of nerve fiber mechanisms has been obtained from the giant axon of this species (Gilbert et al., 1990). Behaviors of *D. pealeii* have been extensively observed and quantified, including anti-predator defense behaviors and strategies (Staudinger et al., 2011; Crook et al., 2014), and reproductive behaviors around egg spawning beds (Shashar and Hanlon, 2013). A detailed ethogram describing their behavioral repertoire has been published (Hanlon et al., 1999).

Compared to other marine invertebrate phyla, the morphology of the cephalopod sound detection sensory organ, the statocyst, is relatively well understood (Budelmann, 1979; Hanlon and Budelmann, 1987). However, bioacoustic research on cephalopods is in its infancy; little is known about their sensitivities to sound outside a handful of studies [e.g., (Packard et al., 1990; Budelmann and Williamson, 1994; Mooney et al., 2010; Samson et al., 2014). There is a clear avenue to leverage the broad wealth of foundational neurophysiological, behavioral, and ecological research on cephalopods toward examining these animals' acoustic sensitivities.

Cephalopods are of considerable commercial value, making up a 6% share (USD) of global exports and about 4.5% of global capture production from 2012–2018 (FAO., 2021). Select species hold great commercial value in certain regions. For instance, the longfin squid (*D. pealeii*) fishery in New England has had annual landed values of about $30 million since 2010 (NMFS, 2019). Cephalopoda is further considered an ecological keystone taxon because many species occupy central positions in marine food webs (Boyle and Rodhouse, 2005). Cephalopods comprise large portions of the diets of many marine mammals, seabirds, and predatory fish. For example, squid can constitute over 50% of regional seabird diets and up to 95% of odontocete diets [ibid]. In turn, many cephalopods are opportunistic predators that feed on a wide variety of prey throughout their lifetime, such as copepods (consumed by pelagic pre-adults), benthic crustaceans and bivalves, and fishes (Boyle and Rodhouse, 2005; Hunsicker et al., 2010). The widespread ecological roles and the commercial values of cephalopods incentivize their use in research to better understand marine invertebrates' ecological uses of sound and how anthropogenic noise pollution may affect individual fitness and populations.

2 General Methodology

2.1. Animals, Collection, and Husbandry

2.1.1. Black sea bass (*Centropristis striata*)

Centropristis striata for Study 1 were collected under Scientific Commercial Permit 175150, administered by the Commonwealth of Massachusetts Department of Fish and Game.

Adult black sea bass were collected in Vineyard Sound, Massachusetts by line fishing and transferred to the Environmental Systems Laboratory (ESL) or dock-based tanks at the Woods Hole Oceanographic Institution (WHOI), Woods Hole, Massachusetts. Fish were held in flow-through holding tanks of at least 1.2 m/3400 L in diameter and were kept at low stocking levels so that water inflow to tanks could be kept at low levels to minimize chronic tank noise disturbance. Fish were held at constant temperatures (see individual studies). All tanks were subject to a natural light cycle. Fish were fed every 2 days to satiation, with bits of squid (*Doryteuthis pealeii*). All experiments and animal care were undertaken in accordance with WHOI's Institutional Animal Care and Use Committee under ID number BI24843.00 to TAM. Any fish deemed unhealthy, owing to capture or otherwise, was not used in the experiments. After fish were used in the experiment they were released back into Great Harbor and the waters from which they came.

2.1.2 Longfin squid (*Doryteuthis pealeii*)

While there were some small differences between studies, methods of collection and husbandry were generally similar and were as follows: squid were collected in Vineyard Sound via trawl, by the Marine Biological Laboratory (Woods Hole, Massachusetts). As the collection vessel returned to dock, squid with minimal physical damage (e.g., few or no skin lesions) were gently hand-transferred to coolers filled with ambient temperature Vineyard Sound seawater. The squid were driven on the WHOI dock where husbandry and experiments were conducted. Upon arrival, squid were immediately and gently hand-transferred into circular holding tanks of at least 1.2 m diameter (3400 L), with ambient flowing seawater (Figure 1). All tanks were subject to a natural light cycle. Sexes were kept mixed, and densities were below one squid per 680 L. During experimental periods, means + SD of environmental measurements across holding tanks were as follows: temperature: 20.63 + 1.06 °C. Squid were hand-fed daily with *Fundulus* spp. collected from local estuaries (WHOI IACUC approval to TAM) and local grass shrimp. Squid were held in these conditions for at least 24 hours before being tested, and experimenters took care to minimize sound in and near the holding tanks.

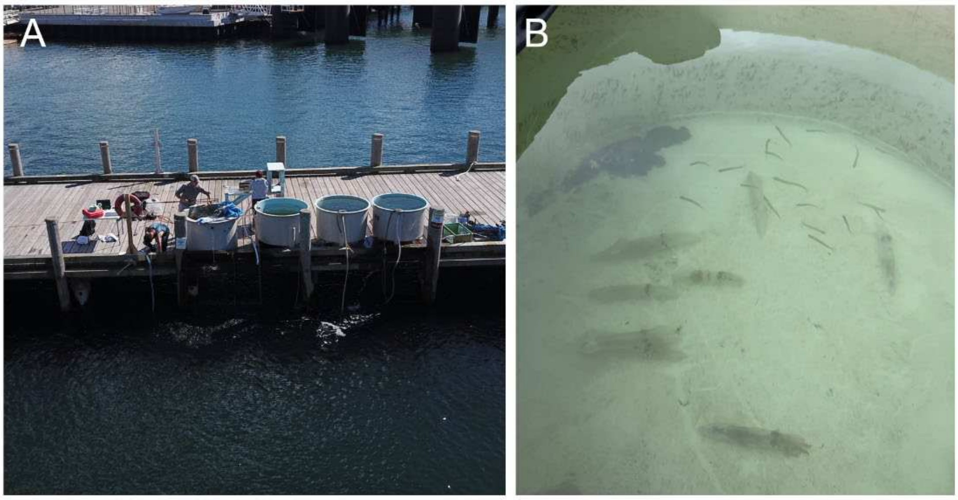

Figure 1. Picture of the holding tanks used for squid husbandry in 2021 on the pile driving site. Tanks were supplied with continuous seawater from the experimental site. A. shows the tanks on the dock before deployment. B. shows a close up of the squid in a tank underwater.

2.2. Location and characteristics of the pile driving

Pile driving was conducted for 11 days per year in the fall of 2021 and 2022 off a dock owned by the Woods Hole Oceanographic Institution. Generally, at the start of each pile driving day a cylindrical steel pile (length: 10 m, diameter: 0.3 m, wall thickness: 0.02 m) was positioned into the sediment using a vibratory hammer (VH, weight: 212 kg, H&M model 135) at 1150 blows per minute. Squid were then introduced into cages (see below for details) and given 15 minutes to acclimate. Exposures began as (1) a steel impact hammer (IH, weight: 1500 kg) was dropped at 1.2 m height at a rate of ~10 strikes per minute until the bottom edge of the steel pile was approximately 5 m into the substrate, taking approximately 15 min. (2) The VH was then used to pull the pile out of the substrate and to reposition the pile in an adjacent location for another round of impact hammering. This process was repeated five times per experiment day, which lasted for three to four hours.

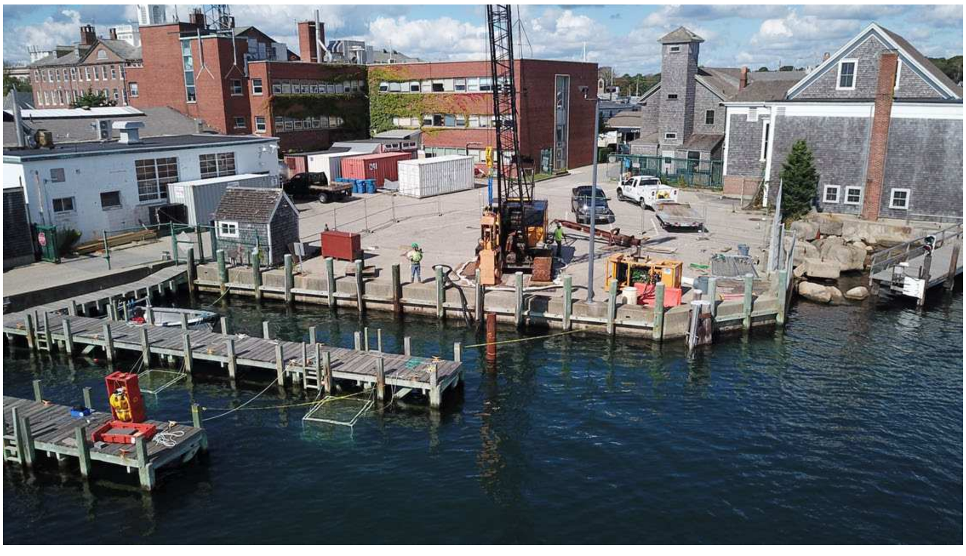

Figure 2. Aerial picture of the pile driving site off WHOI’s dock showing the crane and cages at the near site.

Figure 3. Picture of the impact (A) and vibratory (B) hammers used to drive the pile.

2.3. Overview of the Experimental Design

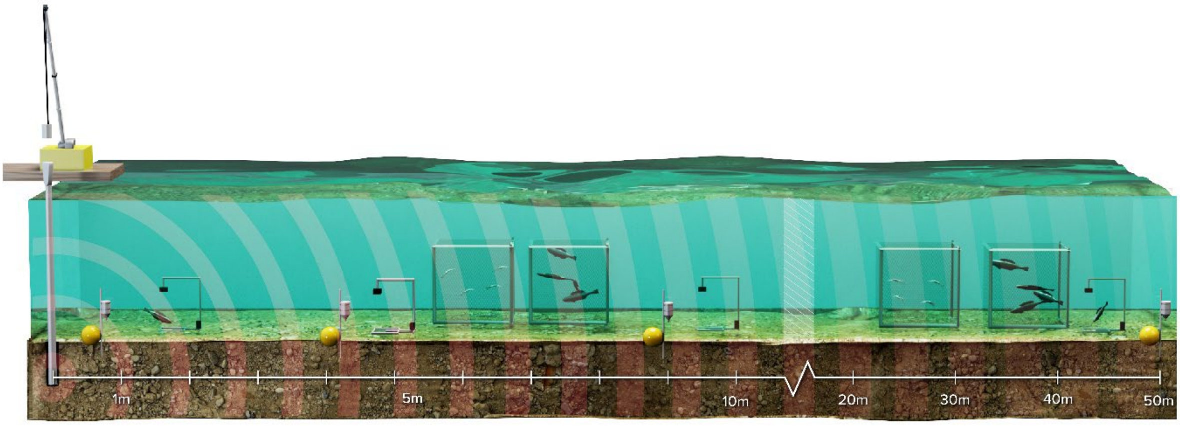

Figure 4. Overview of all experiments and recording devices deployed for pile driving. Hydrophones, depicted on rebar stakes, and geophones, represented by yellow orbs, are shown closest to the pile and were deployed at four approximate distances away.

The next setup as you move away from the pile are bait cams (BRUVs), PVC setups with two cameras. Squid cages (left) and black sea bass cages (right) were deployed at two approximate distances from the pile. Note that for both taxa, there were two cages in the near group and two cages in the far group (only one for each group is shown).

Together, this pile driving methodology, while conceptually simple, provided the unique ability to address multiple species, at multiple locations that were increasing distances from the pile driving, and allowed for a Before-During-and-After Gradient experimental design [e.g., (Methratta, 2020)], all in a field-based setting. Consequently, the setup was flexible, logistically manageable and cost-efficient enough to allows us transferability and flexibility between animal species, comparability between years, the monitoring with and leveraging of multiple acoustic sensors to quantify both pressure and particle motion in multiple ways. Its replicability and accessibility allowed us to use standard methods such as cages and baited remote cameras, but also innovate, via novel squid and scallop tags. In effect, it has become a key testbed for innovation and quantifying the pile driving impacts on key marine taxa. The resulting efforts on black sea bass and squid are described below.

3 Species 1: Black Sea Bass (*Centropristis striata*)

3.1 Study 1: Black Sea Bass (*Centropristis striata*) Exhibit Short Term Behavioral Changes in Response to In-situ Pile-driving

3.1.1 Background

In response to the growing clean energy demands around the world, offshore wind farms (OSWs) have advanced globally in recent years as a more sustainable alternative energy source (Musial et al., 2023). While OSWs have shown initial promise and utility, the impacts on local fauna from construction, maintenance, operation, and decommission ideally would be addressed as managers seek to mitigate potential impacts (Mooney et al., 2020). The construction phase has received the most attention and concern, as it requires steel piles up to around 10 m in diameter to be driven into the seabed through repeated hammering (Negro et al., 2017). This produces high intensity impulsive sounds that can propagate up to tens of kilometers through the water (Brandt et al., 2011; Amaral et al., 2020).

Previous studies have connected this intense percussive sound to behavioral and physical detriment to fishes. In controlled laboratory studies, behavioral impacts included disruption to group cohesion (Herbert-Read et al., 2017), lowered anti-predator avoidance (Spiga et al., 2017), and reduced feeding (Stanley et al., 2023), although habituation was also noted in several species (Kastelein et al., 2017, Spiga et al. 2017). With intense exposures, tissue damage around the swim bladder can also occur (Halvorsen et al., 2012). In the field, physiological and behavioral detriment has also been observed with the onset of pile driving sound in several marine species. Heightened stress, indicated by oxygen uptake, following pile driving sound commencement has been reported in European sea bass (*Dicentrarchus labrax*) (Debusschere et al., 2016, Spiga et al., 2017) and black seabream (*Spondyliosoma cantharus*) (Bruintjes et. al., 2017). Similar field-based studies have also demonstrated behavioral changes, such as reduced swimming and conspecific interaction (Debusschere et al., 2017), reduced anti-predator behavior (Spiga et al., 2017), and abrupt changes in swimming direction and swimming speed with the onset of pile driving stimulus (Mueller-Blenkle et al., 2010).

Free-ranging animals have more ability to remove themselves from the stressor, although species with high site fidelity may not leave (Iafrate et al., 2016), and any displacement from their preferred habitat may have consequences of its own. Few studies have determined pile driving-induced displacement among fishes, and evidence suggests that among these, effects are short term and limited in distance (van der Knaap et. al., 2022). Along the northeast Atlantic seaboard of the United States, 18 active leases for OSWs are in various stages of planning, construction, or operation as of May 2023 (Musial et al., 2023). This region is also home to a number of economically valuable species, including black sea bass (*Centropristis striata*). The northern stock of black sea bass can be found all along the Mid-Atlantic bight, overlapping with the majority of leased OSWs (Moser & Shepherd 2009, boem.gov). They seasonally migrate offshore during the winter and have a high degree of site fidelity when returning to coastal areas for summer (Moser & Shepherd 2009; Miller et. al. 2016).

In addition, black sea bass hearing ranges overlap with the highest amplitude frequencies produced by pile driving sound (Stanley et al., 2020), implying these animals may detect these sounds, and potentially respond via some disruption in normal behavior. As black sea bass have shown avoidance responses to other acoustic disturbances, such as vessel traffic (Secor et al. 2021), it stands to reason that their behavior may be affected by pile driving sound. Tank studies of *C. striata* have reported a decrease in active behaviors with the introduction of pile driving playback, replaced by sinking, pivoting, and resting, (Stanley et al., 2023). This behavioral change was particularly strong upon initial exposure, diminishing throughout a 15-minute period (Stanley et al., 2023). The playback treatment also corresponded with a decrease in feeding (Stanley et al., 2023).

Despite this preliminary work, field-based studies examining pile driving-induced impacts on black sea bass are lacking. This is a crucial next step, as the sensory environment, behaviors, and sound propagation in tanks can be challenging to accurately extrapolate to *in-situ* circumstances (Jones et al., 2019; Stanley et al., 2023). To verify and expand on these results in a controlled field environment, we used video analysis to track behavioral changes of caged black sea bass before and during actual pile driving in a nearshore marine environment. Behavior and position in the cages were tracked through multiple impact hammer (transient, impulsive and high-intensity) and vibratory hammer (continuous low-intensity) sequences in day. Then, to evaluate multi-day consequences, the same fish were exposed to an identical treatment on a second, consecutive day. Finally, potential dose-dependent responses were examined with treatments at two different general distances and received levels – near the pile (~4-13m, peak-to-peak sound pressure level ~ 203-213 dB re 1µPa) and farther from the pile (~60m, peak-to-peak sound pressure level ~ 178 dB re 1µPa).

3.1.2 Materials and methods

3.1.2.1 Fish acquisition and maintenance

A total of 46 adults black sea bass (*Centropristis striata*) were wild-caught via line fishing from Vineyard Sound (Massachusetts, USA) during September and October 2022 under MA Scientific Collecting Permit. Black sea bass exhibit differences in hearing sensitivity with age and size (Stanley et al., 2020), and thus to constrain this variable we used medium-sized, sub-adult to adult individuals (range: 16 cm – 35 cm total length), compromising between higher hearing sensitivity of smaller fish and the visibility of fish in our camera systems. Any fish that was deemed not healthy, owing to capture or otherwise, was not used in experiments. Fish were held in groups of 3-6 animals within 1.2 m or 2.0 m diameter fiberglass tanks constantly supplied with unfiltered ambient seawater directly from Vineyard Sound (temperature range 21.0 - 22.5 °C). After capture, fish were kept in tanks for a minimum of 24 hours before being used in the study. All holding tanks were exposed to natural light cycles and fish were fed every other day with pieces of local squid. Feeding was synchronized to feed fish just before going into experimental cages, and then no feeding occurred for the two experimental days. This study was carried out in accordance with the principles of the Basel Declaration and recommendations and approval of the WHOI Institutional Animal Care and Use Committee scientific protocol to TAM.

3.1.2.2. Location and pile driving characteristics

Pile driving (PD) was conducted for 11 days between September 20th and October 12th, 2022, off a dock at the Woods Hole Oceanographic Institution (Woods Hole, Massachusetts). Construction methods were identical to previous experiments (see Cones et al. 2022, Jézéquel et al. 2022 for additional details), and will only be briefly described here. A combination of two techniques were used to repeatedly drive a cylindrical steel pile (length: 10 m; diameter: 0.3 m; wall thickness: 0.02 m) into the ground. First, a steel impact hammer (weight: 1500 kg) was dropped onto the pile from a height of 1.2 m at a rate of approximately 10 strikes/min, producing high intensity, impulsive sounds. Impact hammer (IH) sequences lasted 15 minutes, resulting in the steel pile penetrating at least 3 m into the seabed. Next, a vibratory hammer (weight: 212 kg, H&M model 135, 1150 blows per minute) was used to pull the pile out of the substrate and to reposition it in an adjacent location for another round of the IH pile driving. Vibratory hammer (VH) sequences are characterized by continuous, lower amplitude sounds. This process was repeated five times per experiment day, with PD activity commencing at 13:00 and lasting for ~3 h. Hence, in total, each *C. striata* individual was exposed to five IH and VH sequences per day, always starting with the IH exposure.

3.1.2.3 Experimental Design

A primary goal was to ensure a sufficient supply of black sea bass were located at a particular location and acoustic received level. Thus, black sea bass were contained in-situ, 3.4 m^3 cubic cages (1.5 × 1.5 × 1.5 m). These cages were constructed with a polyvinyl chloride (PVC) frame (3.8 cm diameter pipe) and enclosed with 1.5 cm knotless polyester mesh netting, resulting in an acoustically transparent enclosing structure. Because black sea bass tend to naturally aggregate around structure, a rectangular “milk” crate (0.33 x 0.33 x 0.28 m) was provided in each cage; it was attached to the cage to provide a general replica of benthic structure, but the open side faced down that the fish could not hide inside. Small holes were cut on the top and side of the cage to allow two underwater cameras (GoPro Hero 8 Black, San Mateo, California) to be attached on the outside for easy deployment but given unobstructed views. At least 30 minutes before PD began, the cameras, with external batteries for long term continuous recording, were attached to the cage with Velcro straps, one on the top center facing down and one on the side net, about 0.5 m above the bottom (Fig 5C). Once deployed, cages rested on the sea floor (4–6 m depth).

Because the hammered pile had to be repositioned after the piling was driving into the seabed, the precise location of the sound source varied slightly between and during the day (received levels are described below). Thus, to test for any potential dose-dependent responses, the two treatments were grouped as “near” to the pile and “far” from the pile, although exact measurements were still taken for each cage after every repositioning of the pile. Two cages were placed in both the “near” group (within 4.2 – 13.0 m), and the “far” group (56.6 – 62.6 m from the pile [Fig 5B]). The day before experiments, two black sea bass were quickly transferred from holding tanks to the cages via a hinged door on top of each cage. Upon latching the cage with fish inside, the cage was lowered to the seabed and the fish were allowed acclimatize in the cages overnight after handling. The “pre” control period started the following day, twenty minutes before the PD sound exposure started. This period was considered normal, unaltered behavior and used as a baseline to determine behavioral changes once PD commenced.

To evaluate habituation on a multi-day level, each pair of *C. striata* were exposed to two consecutive days of PD. Following the second exposure, experimental black sea bass were released back into the marine environment.

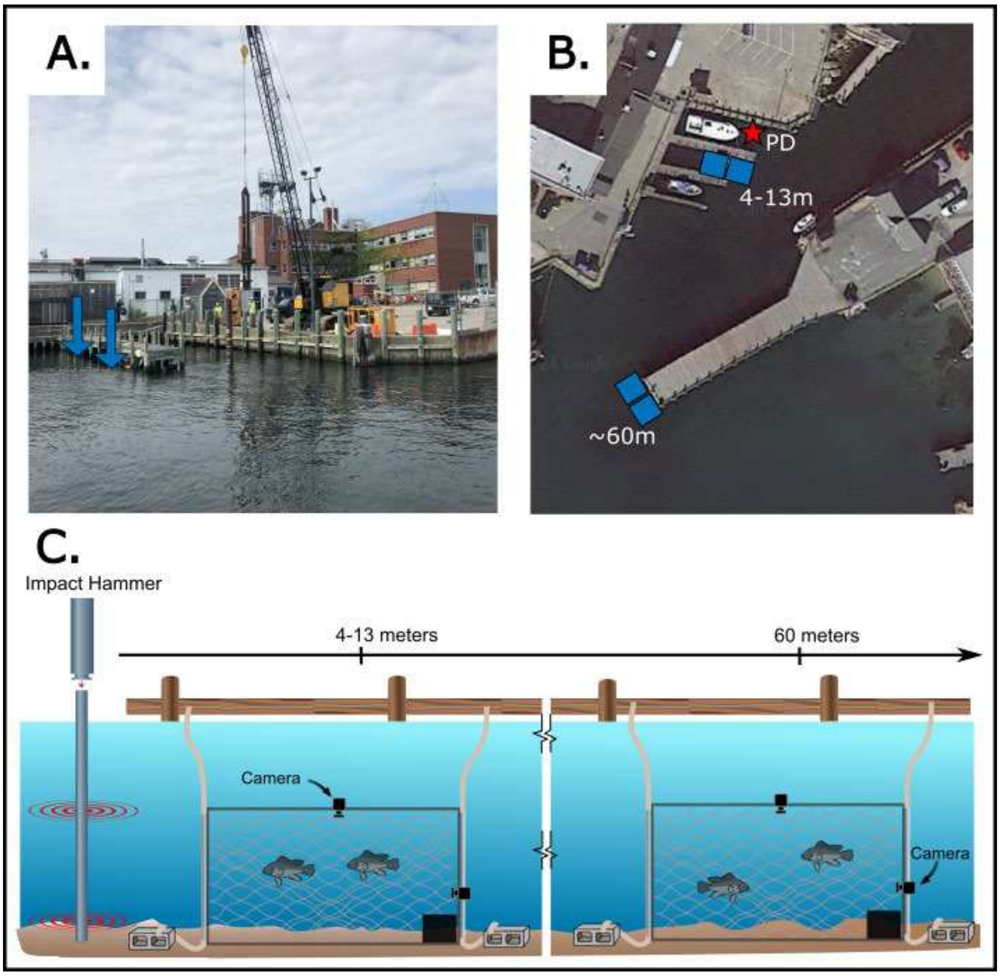

Figure 5. Diagram of in-situ pile driving set-up.

A) Side view of the pile driving apparatus, with the crane and impact hammer hovering over the pile. Blue arrows denote the two close cages of black sea bass. **B)** Top view of entire set up, with two close cages (blue squares) located within 4-13m of the pile (red star), and two far cages located around 60m from the pile. **C)** Design of the cages with two black sea bass inside each (8 fish total per experiment). Note that each treatment (near and far) consisted of two identical cages, while only one is shown for each. Each cage was outfitted with two GoPro's, one on the top and one on the side net. Additionally, a square crate was secured inside each cage for habitat.

3.1.2.4 Data analysis

3.1.2.4.1 Sound recordings

Sound pressure arising from pile driving was recorded using two different types of calibrated recorders located at various distances extending from the pile. Similar to the cages, precise distance of each recorder varied during and between days as the pile shifted, but were re-measured before each IH sequence.

Four Snap recorders (Loggerhead Instruments, Sarasota, Florida) were located approximately 5 m, 7 m, 8 m, and 9 m from pile hammering (averaged distances, sensitivity: -209.7, -210.5, -210.0, and -209.8 dB re 1µPa/V respectively), providing high resolution received levels where the received levels were expected to decrease rapidly. Two farther sites were measured with SoundTrap 600 STDs (Ocean Instruments, Aukland, New Zealand). These were placed at approximately 13 m and 55 m from the sound source (sensitivity: -176.0 and -188.8 dB re 1µPa/V respectively).

For the IH sequences, intensity was assessed by computing 0-peak sound pressure levels (SPL_{zpk}; dB re 1 µPa) and peak-to-peak sound pressure levels (SPL_{pp}). Next, single strike sound exposure levels (SEL_{ss}; dB re (1 µPa)^2.s) were calculated by integrating SPL_{zpk} over the pulse length containing 90% of the signal energy, and cumulative sound exposure levels (SEL_{cum}; dB re (1 µPa)^2.s) were calculated using the following equation:

$$SEL_{cum} = SEL_{ss} + 10 \times \log 10(N)$$

where N is the number of impulses.

The VH sound levels and propagation were also assessed at these same distances. Root-mean-squared sound pressure levels (SPL_{rms}) were calculated for a 5.5 min duration segment of VH sound.

3.1.2.4.2 Behavioral analysis

Videos were manually analyzed in BORIS (v8.20.4; Friard and Gamba 2016) for fish behavior and position in the water column following Stanley et al., 2023 on tank-bound animals. After an initial analysis of these field data, variables quantified included: swimming, resting, or hovering, and midwater or at the bottom, for behavior and position respectively (see Table 2 for full list of behaviors and descriptions). Hovering was relatively rare, so analysis was not conducted on that behavior specifically, although it was worth noting that it is an absence of swimming or resting. After comparing several methods with continuously analyzed videos, a subsampling schedule of 5-min on, 5-min off was determined to sufficiently capture both short-term reactions and long-duration behavioral trends. Intervals were framed around the onset of IH pile driving, beginning 20 min before the start of PD. For the pre- period, and every IH and VH sequence thereafter, two 5-min sequences were annotated, thus capturing the first 5 min and last 5 min of each IH sequence (Fig 6A). Due to occasional camera failure or poor water clarity, a subset of videos was analyzed, and within these videos the number of IH sequences able to be annotated varied. Shifts to the subsampling schedule occurred if IH paused, or in the event of external disturbances unrelated to PD that may affect behavior, such as a vessel passing by the far cages. Despite these adjustments, analysis intervals were always exactly 5 min long, with two observation intervals completed for each treatment.

As to not disturb behaviors (e.g., through tagging) fish were not marked in any way and often went off screen. Thus, it was not possible to assess behavioral responses by specific individuals throughout the experiment, thus no attempt was made to differentiate fish within a cage. If at any point the fish went off screen or were obstructed, their behavior was labeled "not visible".

Table 2. Description for each behavior and position used in video analysis.

Ethogram Glossary*			
Behavior	Definition	Position	Definition
Resting	maintains position on tank floor	At bottom	fish is on or within 15-20 cm of bottom
Swimming	using fins to move through the water	Midwater	fish is in the water column, not associated with bottom
Hovering	maintains position while suspended in the water column	-	
Not visible		fish is out of view of both camera angles; all other behaviors and positions are canceled out	

* Fish were annotated with both a behavior and a position when they were visible, if both could clearly be determined. If the fish went offscreen, they were labeled as “not visible”, canceling out all other behaviors or positions.

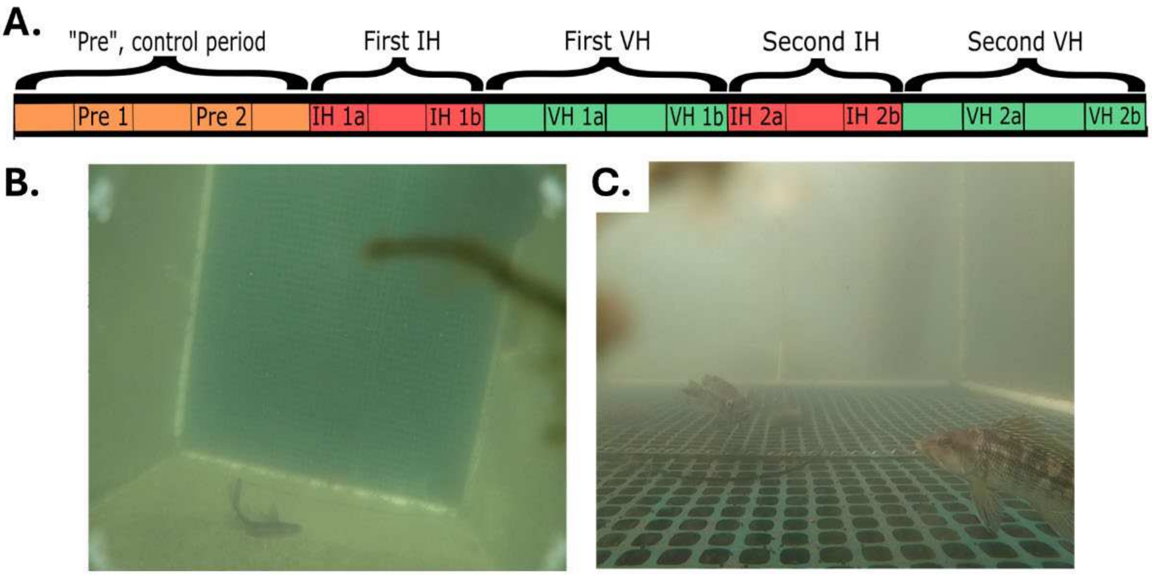

Figure 6. Video observation of black sea bass.

A) Observation schedule for video analysis, demonstrating the order of 5-min intervals annotated during pre (“Pre 1” and “Pre 2”), and first two IH and VH sequences. For days with longer video durations, the pattern continued. Each labeled box corresponded with a 5-min annotated interval, unlabeled boxes were also approximately 5-min, although it varied based on visibility and external factors, duration of VH, or any pauses in IH. B) Top view example, one black sea bass can be seen swimming in the midwater. C) Side view example, two fish can be seen at the bottom.

3.1.2.5 Statistical analyses

Black sea bass behavior was analyzed with two different metrics: duration of time spent in each behavioral state to compare between treatments (close vs far cages, day 1 vs day 2), and point sampling of percentage of fish in each behavioral state before and after the first IH onset. All data manipulation, statistical analysis, and figure production was done in MATLAB R2023A (Mathworks, Natick, Massachusetts).

Duration metrics were employed to assess behavioral changes between "Near" and "Far" cages throughout multiple IH sequences over a day, as well as gauge reactions to the IH between the first exposure and second day exposure. For each individual fish, duration was calculated for each behavioral state based on percent of visible time, after subtracting the duration of "not visible" time from total seconds (300 s per observation interval). Then, since fish were not uniquely identifiable, the two fish in each cage were averaged together forming one sample point. Averaging between two fish per sample not only increased the visibility of the targets, but also minimized impacts of individual variation in behavioral responses, allowing us to examine broader trends. If one fish was not observed the entire period, it was treated as null for that period and not included in any averaging. Cages were grouped by treatment (near vs far, day 1 vs day 2) and average durations were plotted for each observation interval through the experimental day (sample size for each treatment are summarized in Table 2). A paired t-test was then used to statistically compare each observation interval to their corresponding pre- control period (average of pre1 and pre2). Observation intervals with a sample size of less than three were not statistically evaluated or plotted.

To evaluate day 1 and day 2 treatments at the initial onset of IH, a magnitude of change was calculated between pre- and the first 5-m IH interval. Percent of visible time spent in each behavior was calculated as an average for pre1 and pre2 and subtracted from IH1. Thus, behaviors that increased under IH conditions are positive and behaviors that decreased are negative.

Durations of "not visible" times were averaged for each observation period by treatment. To test whether significant shifts in visibility throughout the day occurred and could influence results, a Kruskal-Wallis test (KW) was administered on the slightly non-normal data.

With the inconsistent visibility of fish inherent in the experimental design, a second analysis method was implemented to evaluate behavioral trends irrespective of duration and ensure patterns were not driven by a few visible fish. In addition to duration of behaviors, point sampling at a higher resolution (every 3 s) assessed the proportion of fish in each behavioral state before and during the onset of the first exposure. Only "Near" cages on the first day of exposure were included in this, to examine fine scale behavioral trends in individual fish with the most impacted treatment. Each visible black sea bass was treated as an independent sample for this analysis. A moving average line, taken every 4 samples (12 s), with standard deviation shading were overlayed. The proportion of fish exhibiting a given behavioral state before and after IH onset were statistically assessed with two-sample t-tests. To gauge the precise timing of greatest impact, we compared the pre- period (5-min) to the first minute, second minute, and third through fifth minutes of IH exposure.

Table 3. Number of cages for each treatment, representing sample size at the start of the experiment; each cage contained two fish.

_______	Near	Far
Day 1	6	4
Day 2	4	4

3.1.3 Results

3.1.3.1 Sound characterization

Over 6,000 individual hammer strikes were recorded throughout the 11 days of the experiment. Acoustic analysis from two individual IH sequences, as well as a representative VH sequence, are included here to illustrate the acoustic environment the black sea bass experienced throughout the day. Results are summarized in Table 4.

An example day of underwater sound pressure recordings from an entire PD experiment (including five IH and VH sequences) is shown in Figure 2A. Note that given the *in situ* and realistic nature of this actual PD, pulse amplitudes and inter-pulse time intervals did vary slightly within and across IH sequences as the pile was driven into the sediment (Fig 7). The variability in these metrics across impulses reflected that which a wild black sea bass may experience near an offshore PD site. The near site was always characterized by higher SPL compared to the far site (>30 dB difference). Here, we were interested in studying behavioral effects of PD sound at two distances from the pile, rather than studying responses dependent on these specific metrics.

Table 4. Acoustic levels of the IH and VH measured at three different distances from the pile.

Distance from sound source (m)	IH Measurements*				VH
Distance from sound source (m)	SPL$_{zpk}$ (dB re 1 µPa)	SPL$_{pp}$ (dB re 1 µPa)	SEL$_{ss}$ (dB re (1 µPa)^2.s)	SEL$_{cum}$ dB re (1 µPa)^2.s 113 strikes	SPL$_{rms}$ ** (dB re 1 µPa)
4.9 m	206.8 ± 1.6	212.6 ± 1.3	165.6 ± 0.8	186.1 ± 0.4	147.9
8.7 m	196.9 ± 1.9	203.2 ± 1.7	156.2 ± 1.4	176.7 ± 1.3	143.4
55.4 m	172.3 ± 2.8	178.3 ± 2.7	132.7 ± 2.2	153.2 ± 1.0	131.8

*Results for all SPL and SEL$_{ss}$ calculations are mean ± standard deviation for each strike over two IH sequences (9/26/22 and 9/27/22). SEL$_{cum}$ is the mean ± standard deviation for the two IH sequences.
**SPLrms was measured on a 5.5 min duration VH sequence following the first IH on 9/27/22.

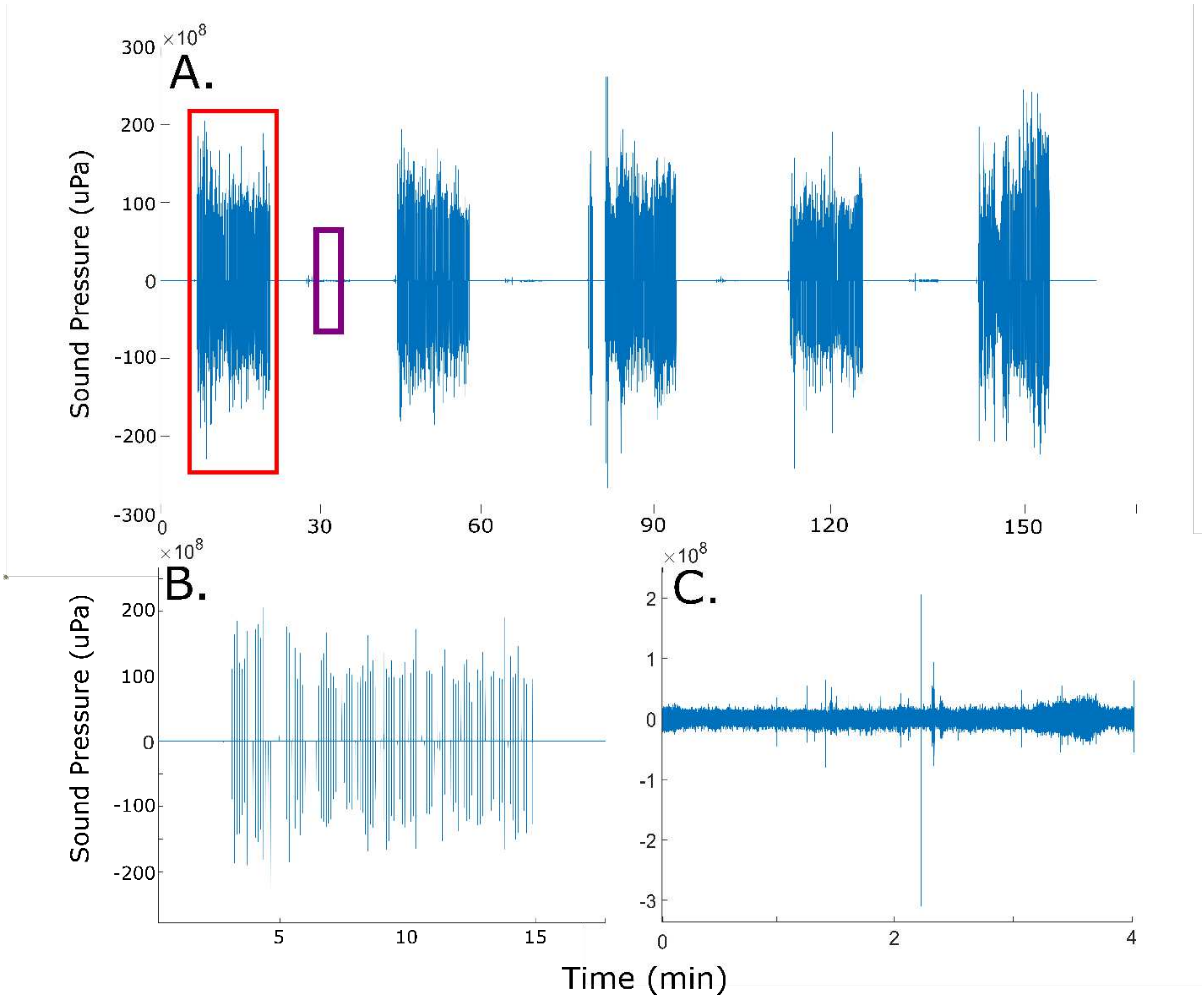

Figure 7. A) One full day of PD, showing sound pressure (μPa) of five bouts of high intensity IH separated by four lower intensity continuous VH sequences, all recorded 5.5 m from the pile driving. B) Zoomed in view of one IH sequence, boxed in red, from the same day and distance represented in sound pressure (μPa). Each strike is clearly visible as a short duration, high intensity impulse. C) A section of vibratory hammering, boxed in purple, characterized by continuous, lower intensity sound.

3.1.3.2 Behavioral responses

Overall, IH pile driving elicited increased resting behaviors and association with the bottom, resulting in declines in swimming and use of the water column. Animals returned to normal behavior levels, not significantly different from "pre", during each intermittent VH sequence, in every treatment. The strength of this response to IH varied based on distance to the pile and novelty of exposure.

For Day 1 exposures, time spent swimming significantly declined in both the close and far cages during the first 5 min of the first IH sequence (paired t-test, near: *t*(5) = 2.81, p = 0.0375, far: *t*(3) = 5.80, p = 0.0102, Fig 8). Resting correspondingly increased (paired t-test, near: t(5)= -3.08, p=0.0276; far: *t*(3) = -4.77, p = 0.0175). Further, at the near cages, the altered behavioral state persisted through the entire first IH, as the average of the first and last 5 min of IH significantly differed from pre levels in swimming (paired t-test, *t*(5) = 2.99, p = 0.0306) and resting duration (*t*(5) = -3.45, p = 0.0183, Fig 8).

The vertical shift out of the water column to the bottom was strongly influenced by proximity to the pile driving. During the first IH exposure, fish in the near cage spent significantly more time on the bottom in the first 5-min interval (paired t-test, $t(5) = -6.98$, $p < 0.0001$) and the entire IH sequence ($t(5) = -5.78$, $p = 0.0022$, Fig 9). Fish in the near cages also responded to the second IH sequence, spending significantly more time at the bottom vs mid water during the first 5 min (paired t-test, $t(5) = -4.08$, $p = 0.0095$), and persisting for the whole sequence ($t(5) = -2.92$, $p = 0.0332$). While the far cages observed similar trends in position, the changes were not significantly different.

For Day 2 exposures, both vertical position and behavior showed no significant changes between pre and IH states at either distance, although the same general trends in behavior were still observed. The discrepancy between the strength of reaction to the initial exposure over consecutive days was particularly remarkable at the near cages (Fig 10).

While there was some unavoidable fluctuation in visibility throughout the experiments, a KW test revealed that no observation periods were significantly outstanding in average “not visible” time (Table 4). This indicates that visibility did not largely factor into behavior results.

Further, an in-depth examination (3 sec interval) of the proportion of fish exhibiting each behavior before and after the first IH suggests that these patterns were not driven by just a few visible fish (Fig 11). For Day 1 exposures, the proportion of fish, out of 12 total, in near cages resting and at the bottom significantly increased in the first 5 min of IH1 compared to the second pre period (two sample t-test, resting: $t(219) = -18.6$, $p < 0.0001$, at bottom: $t(219) = -16.7$, $p < 0.0001$). This corresponded with declines in the number of fish swimming and in the mid-water upon commencement of IH (two sample t-test, swimming: $t(219) = 5.43$, $p < 0.0001$, midwater: $t(219) = 13.12$, $p < 0.0001$). Further, behavioral and position shifts were highly significant in the first minute, second minute, and third through fifth minute of IH, suggesting immediate onset of behavioral disruption (Fig 11).

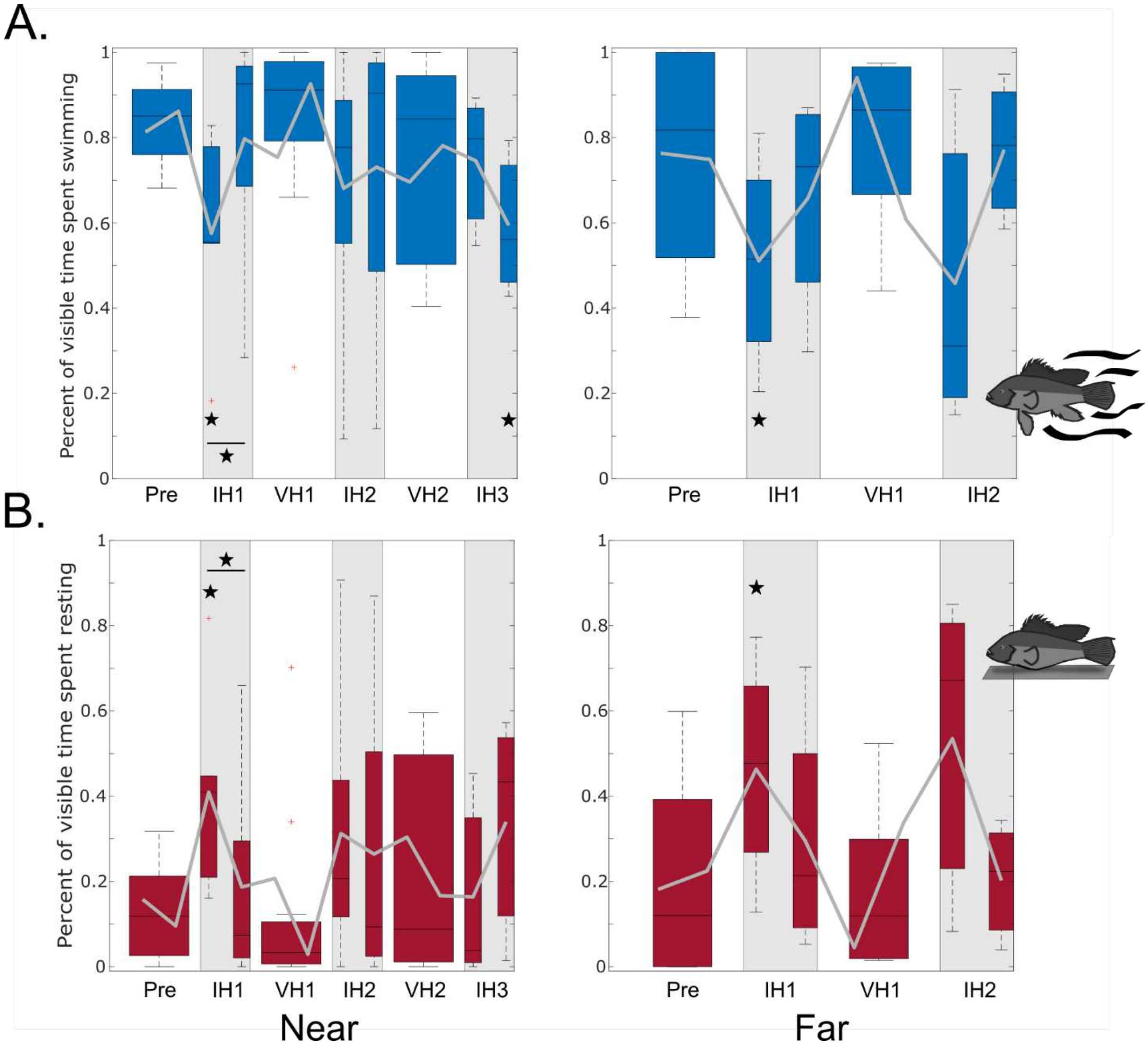

Figure 8. Duration of fish resting and swimming throughout multiple IH sequences for Day 1 pile driving.

Only periods of statistical relevance (n ≥ 3) are shown. Stars represent significant change in behavior between the two pre intervals averaged together and the IH observation interval, or intervals, of interest (paired t-test, ★ p < 0.05. ★★ p < 0.01, ★★★ p < 0.001).

A) Time spent resting significantly increased in near cages (left) during the first 5-min of IH as well as the entire first IH. The far cages (right) also saw an increase in resting that was significant during the first 5-min interval of IH 1. **B)** Swimming duration similarly decreased during the first IH for both close and far cages. An unexpected decline in swimming during the last 5-min of IH 3 also reported significance.

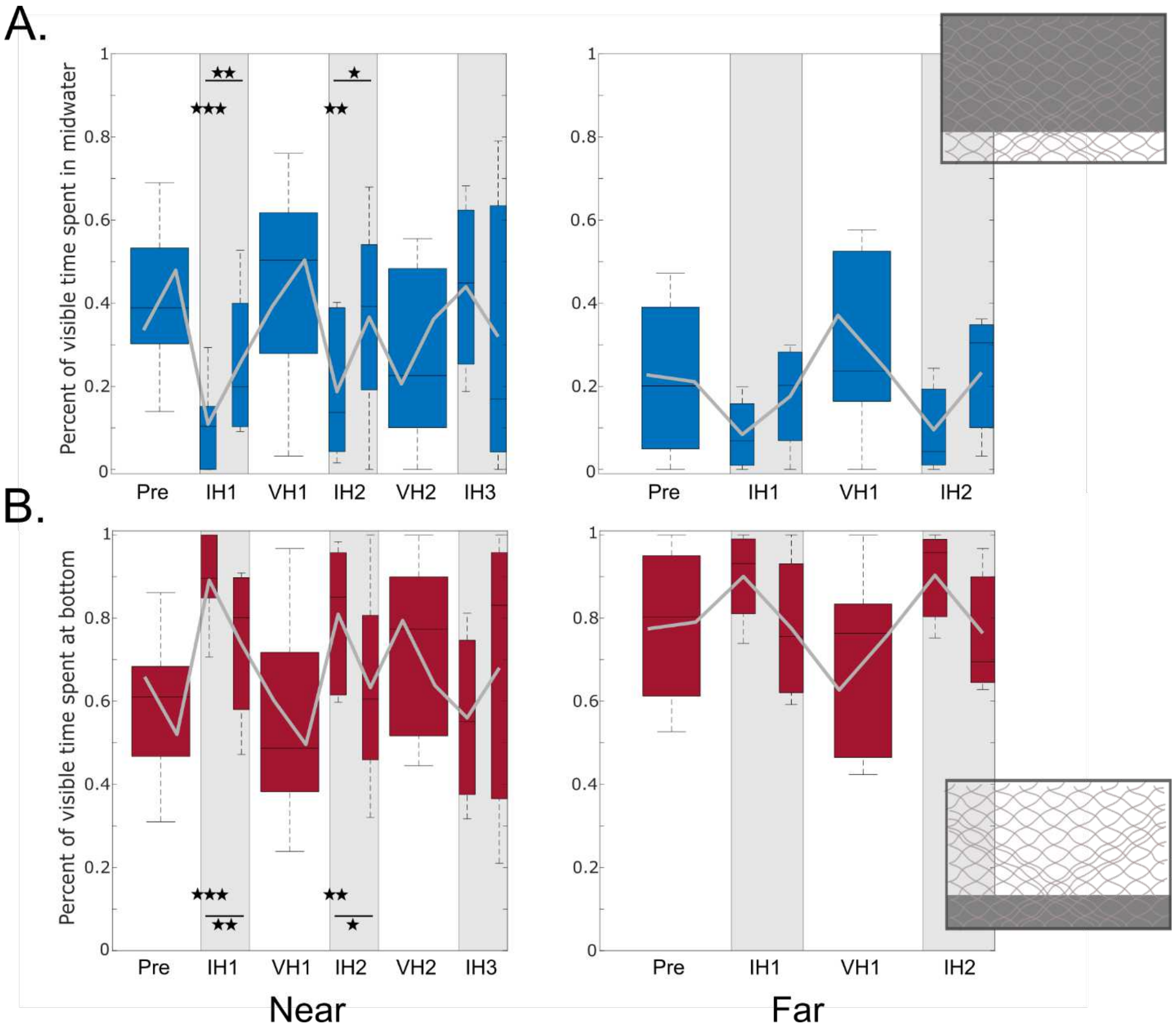

Figure 9. Duration of fish at the bottom and in midwater throughout multiple IH sequences for Day 1 PD exposures.

Only periods of statistical relevance (n ≥ 3) are shown. Stars represent significant change in position between the two pre intervals averaged together and the IH observation interval, or intervals, of interest (paired t-test, ★ $p < 0.05$. ★★ $p < 0.01$, ★★★ $p < 0.001$).

A) In close cages (left), fish spent significantly more time at the bottom during the first 5-min of IH 1 and 2, as well as the entire first and second IH (both 5-min intervals averaged together). Far cages (right) observed no significant changes to time spent at bottom. **B)** Similar to at bottom, fish spent significantly less time in the midwater during the first and second IH sequences, although far cages saw no significant change.

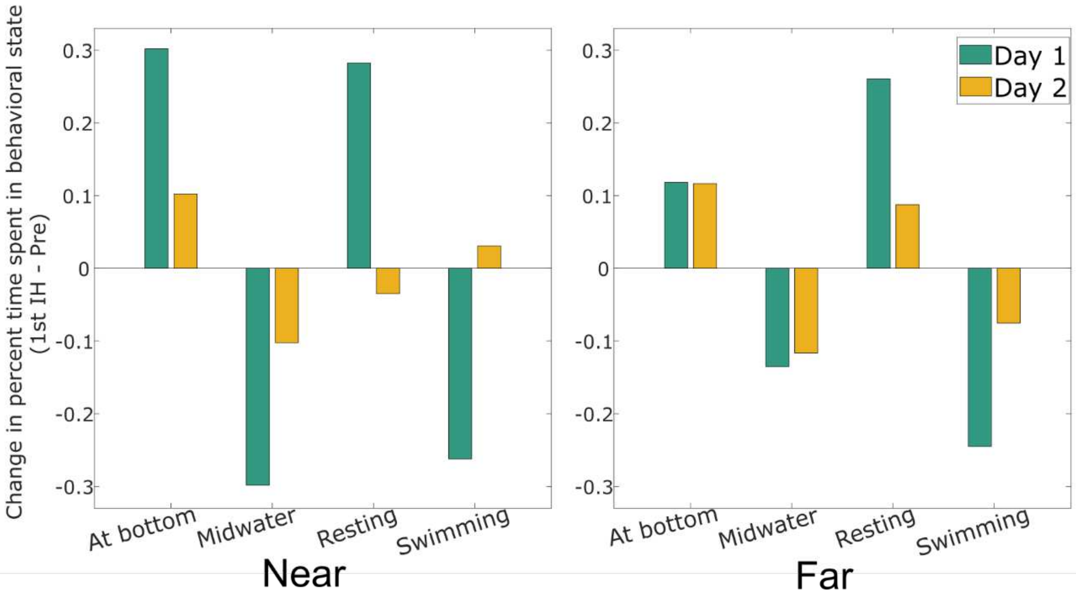

Figure 10. Magnitude of change in time spent in position or behavioral state upon onset of the first IH between the first day of exposure (green) and second consecutive day (yellow).

Positive values show an increase in behavior from pre to IH and negative values indicate a decrease. Despite strong reactions in all behaviors and positions to the first exposure on Day 1 in the Near cages, on the second day there was no significant reactions and distance seemed to have very little effect.

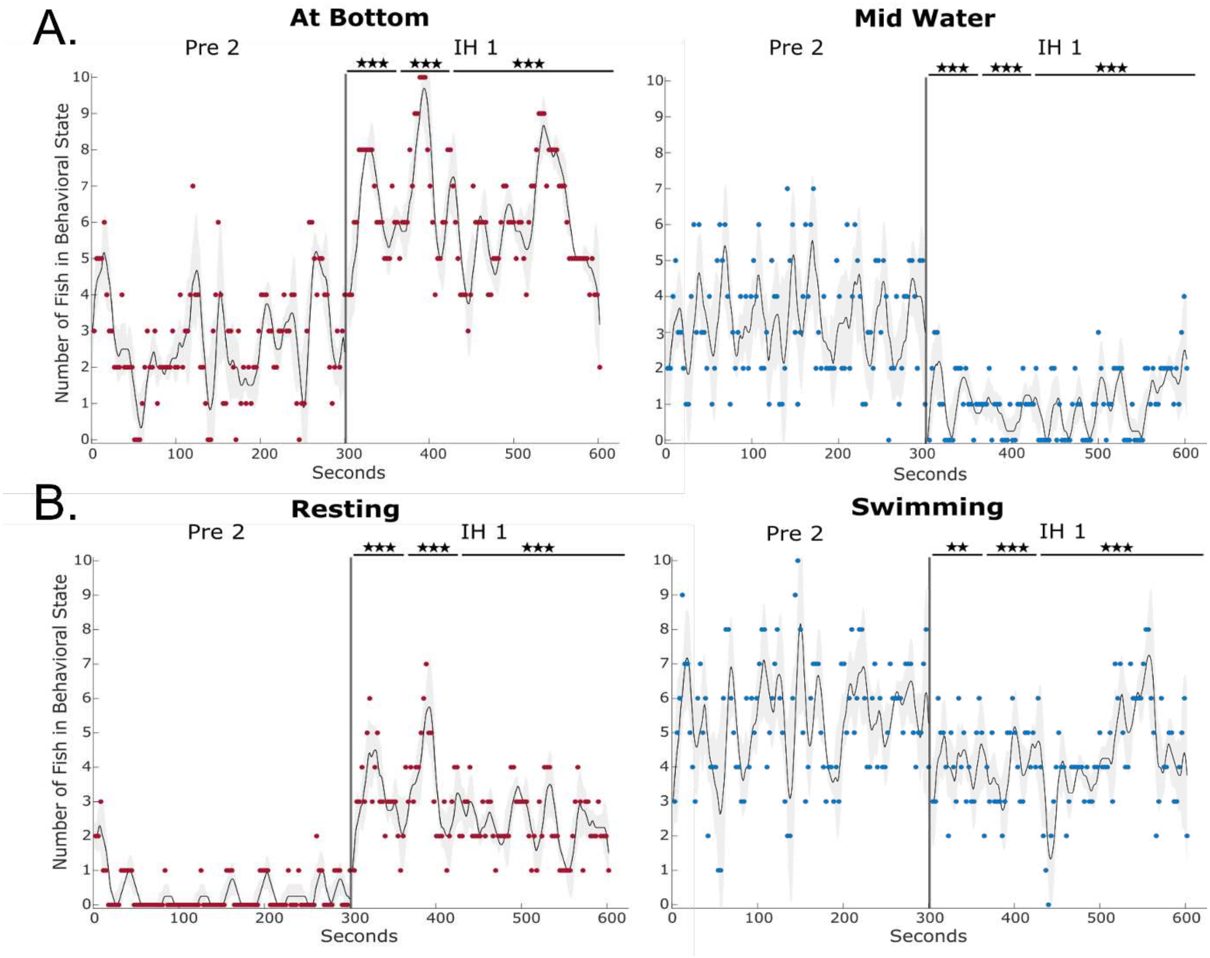

Figure 111. High resolution analysis of number of fish in each position and behavioral state before and upon onset of the first IH sequence in the near cages only.

Observations (points), made every 3 seconds, are overlayed with black interpolated moving average line (mean sampled every 12 s) and shaded moving standard deviation. The vertical bar at 300 s represents the onset of the first IH with the left side being the second 5 min pre period. Note that these two observation periods were not continuous in real time. Stars reflect significant differences between number of fish in a behavioral state between the 5-min pre interval and the first minute, second minute, and third through fifth minute of IH (two sample t-test, ★ $p < 0.05$. ★★ $p < 0.01$, ★★★ $p < 0.001$). **(A)** Position: Number of fish at the bottom (red, left) and midwater (blue, right) were randomly distributed during the control but bifurcate upon onset of IH, reflecting significant changes in positions throughout the first 5 minutes of IH. **(B)** Behavior: During the control, few animals were resting (red, left); the default state was generally swimming (blue, right). However, highly increases in resting and decreases in swimming during the first IH resulted in a more even balance of the two behaviors.

3.1.4 Discussion

This study examined black sea bass behavioral responses to actual pile driving in large *in-situ* cages, the first field study of its kind for this species. Black sea bass altered their behavior in response to pile driving (PD), moving out of the water column and spending increased time at the bottom in ‘crypsis’ like behavior, where there is defensive arousal, freezing, avoidance and likely increased vigilance, all defenses responses to a potential threat (but notably, not to the level of flight or sheltering); (Kennedy, 2022). This corresponds with results from tank experiments on black sea bass noise exposure, which also saw a reduction in active behaviors, although it was hypothesized to be similar to sheltering (Stanley et al., 2023).

Notably, this increased crypsis and apparent vigilance comes at a cost, particularly to foraging and social behaviors such as mating.

Extent of impact was farther than initially predicted, as fish in the far cages ($SEL_{ss} < 133$ dB re (1 μPa)^2.s, $SPL_{zpk} < 172$ dB re 1 μPa), observed a significant transition from a swimming to a resting state upon the onset of impact hammer pile driving (IH). These sound levels are similar to reported values up to 7.5 km from actual wind farm construction (Amaral et al., 2020, Table 1). Proximity to the sound source did, however, prolong habituation and strongly impacted the vertical position of fish in the cage. Far cages reported no significant effect of IH on use of the water column, meanwhile at the near cages significant relocations to the bottom persisted over multiple IH sequences. However, even at the most impacted site ($SEL_{ss} \approx 156$-166 dB re (1 μPa)^2.s, $SPL_{zpk} \approx 197$-207 dB re 1 μPa), fish returned to states similar to pre levels by the third IH sequence. Compared to field values, the received levels at our closest site would be similar to 100 m from large scale pile driving (Bailey et al., 2010, Table 1).

Decreased responses to sound exposure, whether through habituation or other mechanisms, carried into Day 2 of pile driving, as none of the reactions at close or far cages reported significant alterations from baseline. This is interesting, as nearly 20 hours passed between exposures, suggesting that multi-day pile driving, at these sound levels at least, may have minimal impact on behavior. Given that these sound exposure levels are on par with National Marine Fisheries Service Endangered Species Act criteria for physical injury onset to fishes of this size [$SPL_{0\text{-}pk} = 206$ dB;], one key factor that should be considered is potential sound-induced hearing loss. Sound-related auditory damage has been observed with impulsive sounds in the hybrid striped bass (*Morone chrysops*/*saxatilis*) and Mozambique tilapia (*Oreochromis mossambicus*), both showing barotrauma and damaged inner ear hair cells after exposure to 960 pile driving strikes ($SEL_{cum} = 216$ dB re (1 μPa)^2.s) (Casper et al., 2013). Hearing loss can also take several days to recover in certain fishes (Smith et al., 2004). Thus, such persisting threshold shifts could have been incurred here and contributed to the diminished behavioral response on Day 2. As further evidence, fish in the near cages, exposed to the highest sound levels, experienced a much stronger decline between Day 1 and Day 2 reactions to the onset of IH sound (Fig 6). Overall, while no significant differences in vertical position and behavior were found on Day 2, we did observe similar general trends as the first day (particularly in the far cages), suggesting that responses were waning or sensory systems were impacted. Perhaps an increased sample size on Day 2 would have elucidated such reactions to sound.

Anecdotally, there were no obvious pile driving-induced changes associated with horizontal movement (away from the sound source) or associated with the structure provided; such reactions were not analyzed in this study as no such responses were evident in the pilot data nor observed in subsequent analyses. With their high site fidelity (Secor et al., 2021) and temperature driven migrations (Wiernicki et al., 2020; Moser & Shepherd, 2009), it is uncertain whether black will attempt to relocate away from offshore windfarm (OSW) constructions and what implications this may have. This phenomenon requires more study on free-ranging animals, as cage and tank experiments may introduce an element of learned helplessness that can inhibit natural instinct to move away from the sound source.

With the tradeoff of being one step closer to a natural, realistic environment, controlled *in-situ* experiments come with more external factors and limitations that may play a role in the results. Surrounded by a natural environment, experimental black sea bass were exposed to stimulants beyond those investigated here, such as wild fish and local boat traffic. Indeed, interactions with fish both outside of the mesh cage and with their fellow experimental fish inside the cage were observed occasionally throughout the study. Having two fish in the same cage was designed to mitigate this unknown factor, as natural interactions could be more easily observed and assessed if they occurred within the cage. Although not explicitly analyzed, interactions appeared to occur more during periods corresponding with higher activity behavior (pre, VH, and later IH periods), consistent with what has been seen in similar studies on European sea bass (Debusschere et al., 2017).

This study took place in an active harbor, resulting in occasional vessel traffic throughout the experimental days, which black sea bass are known to react to (Secor et al., 2021). While most were small boats, several large passenger and car ferries (from 69 to 72 m long), operate nearby as well. Disturbance from the ferries was mitigated by removing any video sections that were audibly or visibly disrupted (immediate drop in visibility) from analysis, particularly at the far cages, which were closer to the ferry path. Further, fish were used to these noisy conditions, having been locally caught, and were given ample time to adjust to ambient sound levels in the harbor (18+ hours). Finally, the presence of boats throughout the study meant that vessel traffic overlapped with most observation periods at some point, yet cyclical shifts in behavior and position corresponding with the onset of IH were clear and related to distance from the pile.

This study was an important step towards scaling up to OSW construction impacts on black sea bass, but still pales in comparison in terms of sound levels produced, rate of IH strikes, and duration of PD. For reference, the pile used in our study is about 1/30th the diameter of monopiles being used in commercial installations (Kallehave et al., 2015), producing peak-to-peak sound levels at our close cages that match empirically measured levels over 100 m from OSW construction (Bailey et al., 2010, Table 1). While it appears that fish stop significantly responding to the IH by the first day, the combination of higher intensity, more rapid hammering, and extension over longer periods without breaks, may cause long term detriment to their fitness not predicted by this study.

Black sea bass are visual predators, active and up in the water column to hunt during the day (Secor et al., 2021). Daytime pile driving would thus have a higher impact on fitness as black sea bass may sacrifice foraging time to shelter or increase vigilance near the benthos. Effects of nighttime pile driving were not evaluated in this study, but we predict there would be less change in behavior and position with PD, as black sea bass are known to shelter on the bottom at night (Secor et al., 2021).

While this study, and most similar studies so far, only looked at the construction phase of OSWs, impacts during other phases of an OSW lifespan must also be evaluated moving forward. During the operational phase, OSWs produce low frequency continuous sound, with source levels approximately 10–20 dB lower than boat noise in the same frequency range (Tougaard et al., 2020), and are a new structure and potential habitat for the area. Given their association with reef habitat and proclivity for settling on artificial reefs (Low Jr & Waltz 1991), once constructed, OSWs may actually provide valuable habitat and shelter for black sea bass. In other parts of the world, several established windfarms (> 5 years of operation) have shown higher abundance and diversity of fish associated with them than surrounding areas or before construction (Stenberg et al., 2015; Hal et al., 2017). Indeed, a before-after control-impact (BACI) study around the first operating OSW in American waters, Block Island Windfarm, revealed that black sea bass numbers had actually increased the near the operating windfarm compared to previous levels and surrounding areas (Wilber et al., 2022).

In conclusion, this study found that impact hammer pile driving significantly altered behavior of caged, in-situ black sea bass, whereas the interspersed vibratory hammering did not. Similar to previous lab-based studies, *C. striata* exhibited fewer active behaviors, which are typical during the day for the visual predator, and instead adopted bottom-associated sheltering behavior. However, a return to "pre" behavior was observed within the first day and continued through the second day of exposure, suggesting that some level of multi-day habituation or persevering hearing loss occurred. Future work should focus on scaling up exposure to more realistic commercial levels and examining reactions of free-ranging black sea bass.

3.2 Study 2: In-situ Approach to Characterize Effects of Pile-driving on the Presence of Black Sea Bass (*Centropristis striata*)

3.2.1 Background

The expansion of renewable energy to meet electricity demands has resulted in numerous offshore wind farm projects across the globe. The large number of offshore wind farms (OSWs) proposed to be established along the east coast of the United States has raised concern, especially among commercial fishermen, about the impacts the farms will have on fisheries. The installation stage of OSWs is of special concern as the sounds generated when installing piles to secure the structures have been shown to have a range of impacts on marine fauna, from mortality to disruption of foraging or predator avoidance behaviors (Brandt et al., 2011; Mooney et al., 2020). The majority of acoustic studies focused on the impact of pile driving noise on marine species have been aquaria-based. Valuable information is derived from these controlled and confined studies, but aquaria studies have also been shown to create distorted sound fields which have the potential to influence animal behavior (Akamatsu et al., 2002; Jézéquel et al., 2022a).

Studying animal behavior at wind turbine installation sites is difficult with sites typically far from shore, resulting in higher costs and more complicated scheduling that can be easily interrupted by poor weather conditions. A solution to this problem is to use nearshore pile driving as a proxy for offshore pile driving [e.g., (Cones et al., 2022a; Jézéquel et al., 2022b)]. While the size of the equipment and piles installed for nearshore structures like docks and channel markers are typically on a smaller scale, the sounds produced can be a good approximation for true offshore wind, and are without the complications of aquaria boundaries (Jones et al., 2019). The unfettered sound field makes pile driving treatments more realistic and the animal behavior is closer to a natural state. It also becomes possible to measure effects at multiple distances, and do so concurrently, thus evaluating potential received level and distance based-dose impacts in a Before-during-and after-gradient design (Methratta, 2020).

There is a need to address how in situ pile driving impacts the vital behaviors of key fish species in areas impacted by planned wind farm construction. One fish species that has been studied in a number of tank experiments is the black sea bass (C*entropristis striata*) (Stanley et al., 2023). Black sea bass are of commercial and recreational importance along the eastern seaboard of North America as well as the Gulf of America (previously the Gulf of Mexico). In this study we examine the impact nearshore pile driving has on the abundance of baited black sea bass to assess potential fishery impacts from offshore wind farm installation in as natural an environment as possible.

3.2.2 Methods

3.2.2.1 Location and characteristics of the pile driving

Pile driving experiments took place from the 20th of September to 12th of October 2022 in an open area off Woods Hole Oceanographic Institution's pier (41.5239° N, -70.6708° W). It is a shallow water habitat with 3–5 m depth during low and high tide (respectively), with a flat bottom composed of homogeneous sand and silt. OWF foundations primarily consist of a mono-cylindrical steel structure driven into the seabed using hydraulic or diesel hammers that employ impact (IH) or vibratory (VH) pile driving. For the purpose of this study, we simulated the construction of a wind turbine with a cylindrical steel monopile (length: 10.0 m, diameter: 0.3 m, thickness: 0.02 m) with a steel plate welded at the bottom that was impact driven ~2.5 m off a dock. A crane (American 595) with a 20 m long boom lifted a VH (weight: 212 kg, H&M model 135) that was first used to secure the pile into the seabed at a rate of 1150 blows per min. Then, a steel IH (weight: 1500 kg) was manually dropped at a height of 1.2 m and released against the top of the pile at a rate of 8 to 12 strikes per min for a duration of ~15 min. The energy generated by the impact was ~16 kJ per strike. Once the pile was at a depth of up to 5 m below the water–sediment interface, the VH was used again to remove the pile and drive it at another location adjacent to the previous hole. Hence, one PD sequence comprised IH phase followed by an VH phase, separated by five minutes from each other, and was repeated five times for each exposure day. Eleven total pile driving days were carried out over the three-week study period, with pile driving being carried out over a three-hour period starting at ~1 pm each day.

3.2.2.2 Baited remote underwater video

Baited remote underwater video rigs (BRUV)(Fig 12) were constructed from PVC and employed two GoPro underwater cameras each (Hero Black, Frame rate: 60fps, Resolution: 720p, HD) with extended batteries to maximize recording time. A mesh bait bag was attached to the frame with one camera focused from 0.5m above, and one camera focused from 0.5 m to the side. BRUVs were labeled 1-4 to mark their deployments sites and general distances (1, 5, 10 and 50 m) from the pile driving (respectively). Each bait bag was loaded with 0.1 kg chopped squid. Cameras were turned on and BRUVs were deployed at their respective sites ~30 minutes prior to the start of pile driving. Exact pile position was measured using a digital laser distance meter (Rockseed, S2) for each event to determine precise distances from each BRUV to each pile driving event. While the precise distances of each site varied each IH sequence, similar to real pile driving, for simplicity they will be referred to as their planned distances – 1, 5, 10, and 50 m. The true range of distances for each site are as follows: 1 m (2.4 – 8.7m, mean: 4.9m), 5 m (6.7 – 10.6m, mean: 8.4m), 10 m (11 – 15.3m, mean: 12.9m), 50 m (53.1 – 57.5m, mean: 54.9m). After pile driving activities ceased each day camera and bait were collected to prepare for future deployments. Recording length ranged from 90 to 240 minutes over the experiment and was typically limited by camera battery life.

Control BRUV deployments consisted of identical BRUV camera, bait, and site configuration, without pile driving activity. Seven control days were collected for this experiment, one, prior to the pile driving on September 19, 2022 and six more were collected from September 19 to October 4, 2023.

Figure 12. (A) BRUV (PVC structure on the left). Note the small BSB just above the bait bag. A low sensitivity SNAP acoustic recorder can be seen in the background of the bait camera setup. (B) Views from the top and side camera of the bait camera setup with quite a few BSB of differing age classes in view. A number of other species were also observed including scup, flounder and menhaden.

3.2.2.3 Recorders

To record sound conditions throughout the experiment, two Snap (Sampling rate: 44.1 kHz, Loggerhead Instruments, Sarasota, Florida) and two SoundTrap ST600 (Sampling rate: 16 kHz, Loggerhead Instruments, New Zealand) acoustic recorders were deployed at the study site. Recorders were installed on rebar inserted into the substrate with recorders oriented upwards and hydrophones 0.5 m off the substrate. The Snaps were set for low gain to avoid clipping and deployed adjacent to the pile driving at the site of the 1 m and 5 m BRUVs (sensitivity: -209.7 and -210.0 dB re 1µPa/V respectively). The SoundTraps were set to high gain and deployed adjacent to the 10 m and 50m BRUVs (sensitivity: -188.8 and -176.0 dB re 1µPa/V, respectively).

For controls carried out in 2023, SoundTrap ST600s (Sampling rate: 48 kHz) were deployed at the 5 m and 50 m BRUV sites.

3.2.2.4 Video Analysis

Over the course of nineteen study days 457 hours of video were recorded to be analyzed for black sea bass counts. Initial video clips for each day were trimmed to synchronize timing, and then all clips were concatenated and subsampled at 10fps. A set of 730 randomly selected video frames were chosen from the finished videos and each frame was manually annotated using MATLAB’s Video Labeler (MATLAB R2023b), marking location and species of fish in each frame. While five fish species were identified in these annotations, including black sea bass, scup, tautog, northern puffer, and menhaden, only the study species, black sea bass will be covered here. For black sea bass the annotations identified the fish as adult or juvenile based on color and pattern distinctions unique to the two life stages. These annotations were used to train a YOLOv5 object detection algorithm, using randomized weights and a batch size of 16. Results of the YOLO training validation had an average precision with an intersection over union of 0.5 (AP@0.5) of 0.951 for adult black sea bass and 0.857 for juveniles (adult black sea bass are correctly identified 95.1% of the time the algorithm’s detections matched the manual annotations with at least a 50% overlap of the detection areas; 85.7% for the juveniles) Using the weights generated by this training run YOLOv5 was used to detect fish in the entire video dataset outputting a continuous count of black sea bass adults and juveniles every tenth of a second during control and pile driving periods. Each detection label included the position within the video frame, as well as the identity and confidence of the detection. The confidence score is a combination of confidence in the bounding area having an object of interest and confidence in that object of interest being what the algorithm identified. A real-time output of the algorithm is noted in Figure 13.

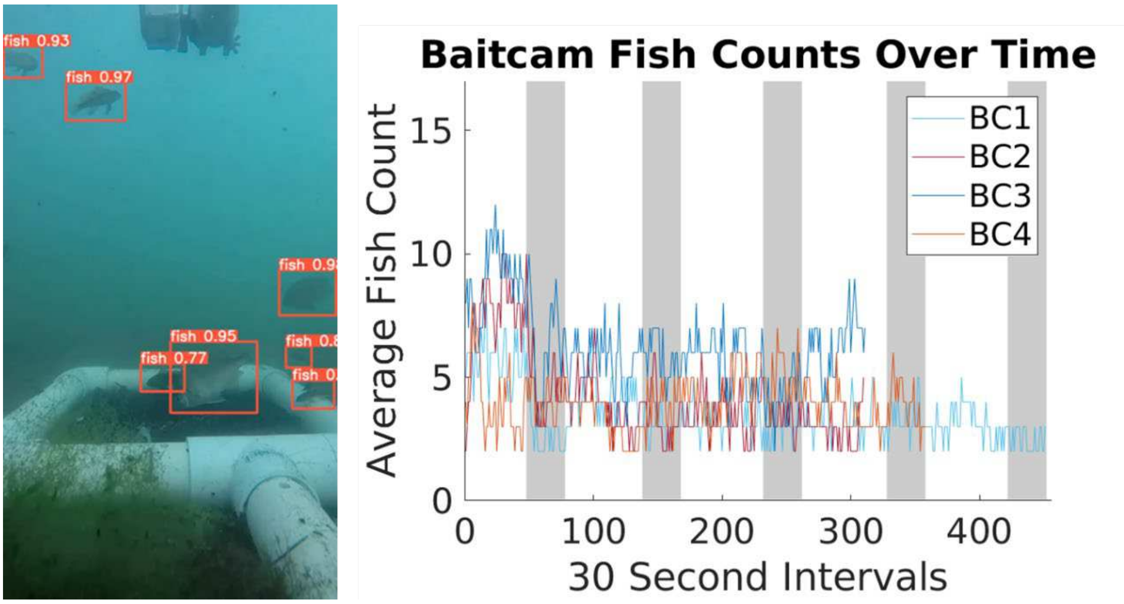

Figure 13. Left: Example of the output from the machine learning algorithm developed to detect fish in each video frame.

Red boxes denote the area a single fish is found in. The number identifies the confidence in object ID by the algorithm. Right: An example of average fish counts at each baitcam, determined using our new machine learning method. Data are plotted every 30-seconds for the duration of one day of pile driving. Gray areas denote the time the impact hammer was driving in the pile.

3.2.2.5 Statistical analysis

All statistics and data analysis were carried out in R Studio (2023.06.0, Posit Software) and all significance levels were set at $\alpha = 0.05$. A subset of the videos was manually examined to determine pile driving start and end times. This metadata was combined with the detection data and each datapoint was classified as either control, pile driving event one through five, pre-pile driving for each day, and the time between pile driving events to allow a finer examination of the data. To improve the quality of the dataset, any detections with a confidence score of 0.5 or less were removed. Further, single frame detections for each life-stage of black sea bass in a 10-frame period were set to zero to remove erroneous detections. These errors tended to occur as animals in view shifted or turned, so this step helped reduce the number of misleading detections. Otherwise, the maximum number detected (MaxN) for each life-stage for each second of video was recorded to examine fish abundance at each BRUV over time.

To examine the relationship between distance from pile driving and the effect on black sea bass abundance, fish detections from each BRUV were pooled as either control or treatment (i.e. pile driving events one through five) and analyzed by location. To meet the assumptions of ANOVA, the data were square root transformed and analyzed using a one-way ANOVA.

To examine the relationship between black sea bass abundance and short-term exposure to pile driving, fish detections from the 1 m and 5 m BRUVs were compared for each day during each pile driving event with a subset of the control data. The subset was determined by the average start time of each pile driving event and the average duration of pile driving. To account for incidental variability in black sea bass counts between days all data were normalized to a proportional abundance by day and location. The data did not meet the assumptions of normal distributions, so the non-parametric Spearman's rank correlation test was used to compare the effect of short-term pile driving exposure time on black sea bass counts. To examine the relationship between fish abundance and long-term (weeks) exposure to pile driving, fish detections from the 1 m and 5 m BRUV were used. Control and piledriving days were considered as "days since the first day of pile driving," or in the case of the controls, since the start of the control study. Linear regressions were plotted using normalized black sea bass counts and days of pile driving to determine the nature and strength of the relationship.

3.2.3. Results

We initially compared average fish observations for juveniles and adults during IH pile driving and control periods, compiling all IH sequences (Fig 14). Significant differences between controls and pile driving counts were found for juveniles at the two closest sites. No differences were noted for the adults.

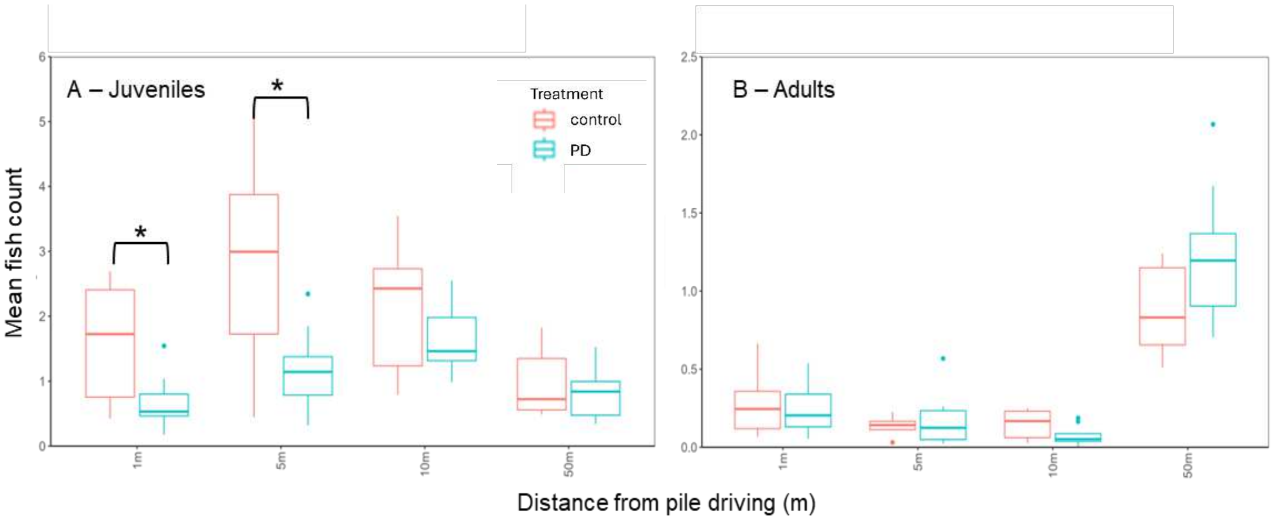

Figure 14. A. Comparing average fish observations for (A) juveniles and (B) adults during IH pile driving and control periods, compiling all IH sequences.

Significant differences between controls and pile driving counts were found for juveniles at the two closest sites. No differences were noted for the adults.

Average counts of juvenile and adult black sea bass were then assessed for the four BRUV observation stations, comparing control observations to animals across the sequence of an afternoon of pile driving (5 IH sequences). All experimental days were averaged. For the juveniles at the closest site, the average number of animals observed significantly decreased for IH sequences 2, 3, 4 and 5. At the 5 m site, the average number of juvenile animals observed significantly decreased for IH sequences 3, 4 and 5. There we no apparent differences in juvenile mean observations between any sequence and the control times at the 10 and 50 m sites. Somewhat oddly, adults showed a significant decrease in observed animals at the middle 10 m site for pile driving sequences 4 and 5; otherwise there were no differences between IH and control periods.

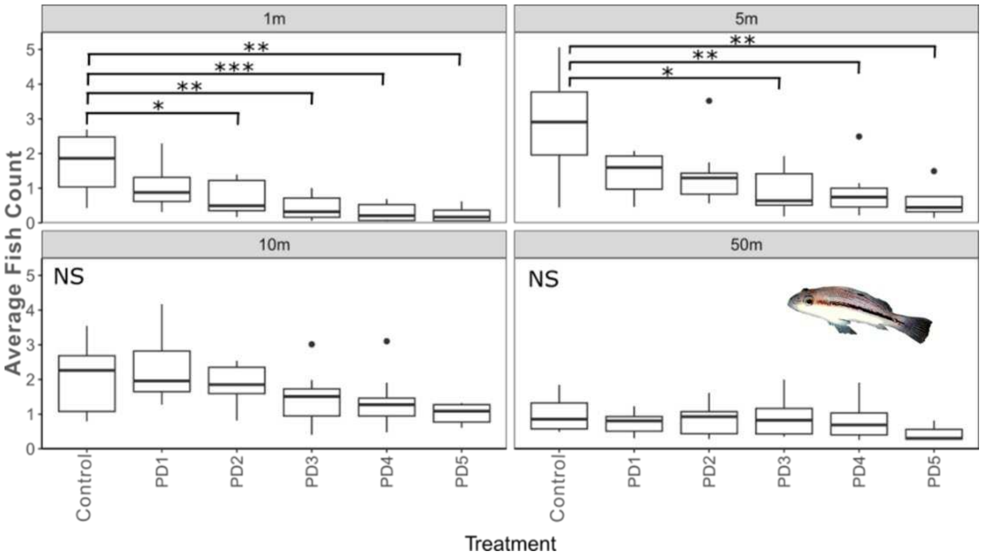

Figure 15. Average counts of juvenile black sea bass at the four BRUV observation stations (increasing distance from the pile driving), comparing control observations to animals across the sequence of an afternoon of pile driving (5 IH sequences).

All experimental days are averaged. At the closest site (2.4-8.7 m), average number of animals observed decreased for IH sequences 2, 3, 4 and 5. At the 5 m site (6.7-10.6 m), average number of animals observed decreased for IH sequences. 3, 4 and 5.

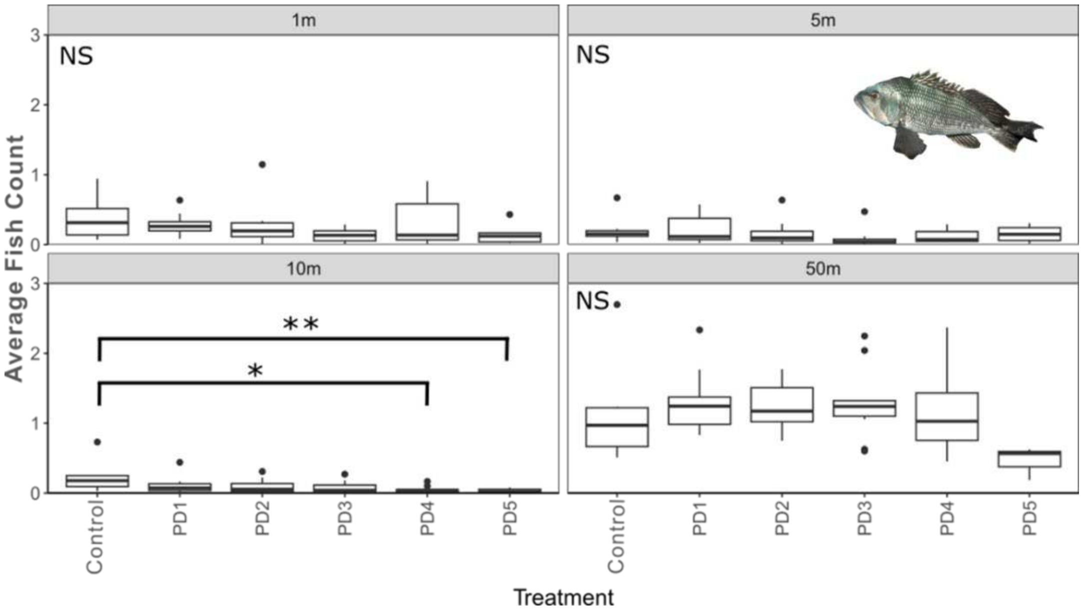

Figure 16. Average counts of adult black sea bass at the four BRUV observation stations, comparing control observations to animals across the sequence of an afternoon of pile driving.

All experimental days are averaged. No particular trends were evident. Only the 10 m distance showed significant differences between the control and the 4th and 5th sequence of pile driving.

Switching to a larger temporal scale, here we examined for changes in black sea bass counts across the entire 2–3 week pile driving period, comparing the pile driving in 2022 the same weeks (and same water temperatures) without pile driving in 2023 (Fig 17). In 2023, when no pile driving was present, there was no significant change in juvenile or adult animal counts, over the multi-week period ($p>0.05$). However, both the juvenile and adult populations showed significant declines over the course of the pile driving during the sound treatment year (2022). In both timeframes, this is pooling data from all BRUV observation stations ($p<0.018$, $r^2=0.14$ for juveniles; $p<0.015$, $r^2=0.09$ for adults; Fig 18).

For the adults, similar significant declines in animals observed were notable at BRUV observation sites 1 m, 5 m and 50 m (data not shown here), and all sites, for the juveniles. In the control year and by site, observations of juveniles and adults increased or showed no particular trends.

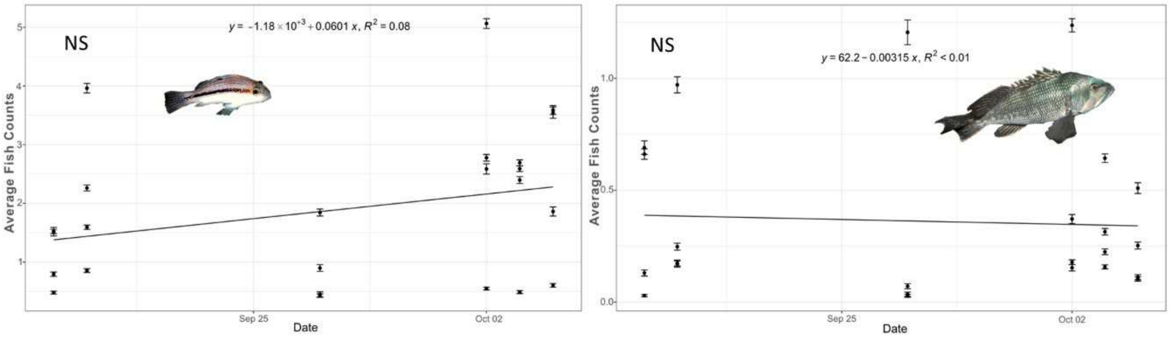

Figure 17. Average counts of juvenile (left) and adult (right) black sea bass at all BRUV observation stations during 2023, a year without pile driving.

There was no significant change in observed numbers of animals for either age group.

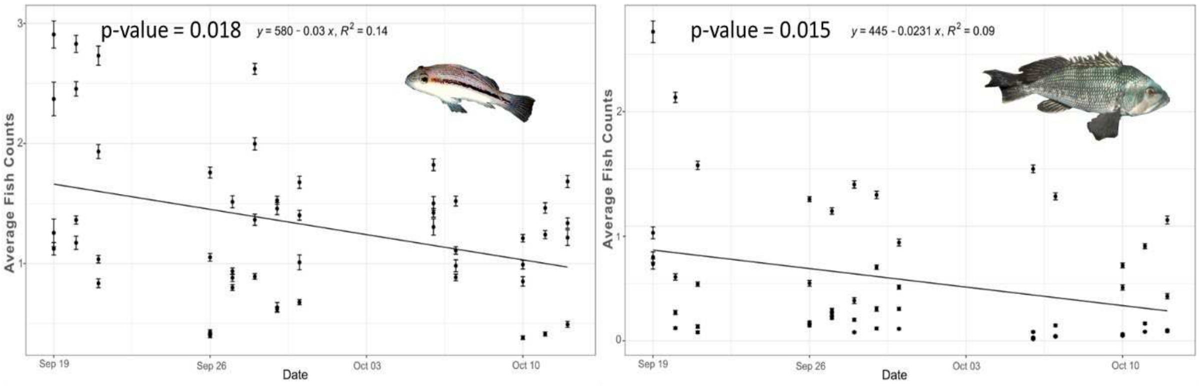

Figure 18. Average counts of juvenile (left) and adult (right) black sea bass at all BRUV observation stations during 2022, a year with pile driving.

Pooling all sites (shown), there were significant decreases in observed animals over the course of the sound exposure period.

3.2.4. Discussion

Here we provide results from a unique field study that evaluated how natural populations and age classes of black sea bass respond to actual marine pile driving. Animals were free-swimming and had the opportunity to forage during the experiment at baited remote underwater video stations. From these analyses it appears that juvenile black sea bass observations tended to be lower during pile driving, particularly at the near sites. Zero-peak sound pressure levels recorded at the 5 m BRUV were 196.9 dB re 1 μPa (±1.9).

Further, for both juveniles and adults there appears to be a cumulative effect of the pile driving, with later pile driving episodes having statistically significantly lower counts of juveniles as pile driving sequences increased throughout the day, and as both juveniles and adults decreased over the course of the 2022 noise exposure season, but not the 2023 control (no pile driving) year. In particular, these decreases occur despite the renewed availability of food in the bait bags.

However, animals may have been weighing some tradeoffs. Notably, there were not significant decreases in observed animals, even at the near site which reached 206.8 ± 1.6 (SPL_{zpk}; dB re 1 μPa) for the initial IH hammer sequence. Only after the bait was present for some time, so animals were perhaps either satiated or not receiving enough food to stay around the pile driving sound. Further, there was a ca. 10-15 dB (dB re 1 μPa) difference between the near sites and the 10 m sites, which showed generally fewer differences between sound and control trials, implying that there are sound levels where black sea bass are not deterred from the food resource.

Perhaps most striking were the decreases in observed fish over the course of several weeks of sound exposure sessions, which the subsequent control year, without pile driving, did not experience. While it is impossible to measure all extrinsic factors in a field study, water temperatures and weather patterns were similar for both years, indicating that it was not likely the seasonal environment that drove those changes and suggests that the pile driving could have been the event that induced those differences. The premature departure from the area under pile driving conditions may have negative effects on the fitness of black sea bass. Black sea bass are known to have relatively high site fidelity and migrations strongly linked to temperature (Moser and Shepherd, 2008). While the extent of movement away from the sound source was not assessed in this study, and could have been as minor as the next harbor over, OSWs can occupy thousands of acres, thus the spatial extent of real windfarm installation may be costly to black sea bass habitat use.

Finally, it seemed that juveniles generally seemed to be more sensitive to the sound exposures. Juvenile observations decreased at the closer sites, across the timeline of an afternoon of impact hammering, and across the multiweek exposure period. This is interesting given that they tend to have more sensitive hearing (Stanley et al., 2021) compared to adults. Thus the sound may actually be perceived as louder. They also may be more susceptible to noise induced hearing loss, something that should be quantified in follow-up work.

In a larger perspective, some fieldwork indicates that black sea bass populations may not be largely affected by installed offshore windfarm pilings, and perhaps such habitat provides structure for this species to aggregate around. Yet, those observations are largely addressing adults, and examine populations substantially after construction. Our study underscores that life stages may be differentially impacted, and that juveniles may be particularly sound-sensitive to the acoustic or related impacts of pile driving related to construction. Further, in their sound-sensitive responses, animals have to weigh foraging opportunities and stressor exposure. How they respond appears dependent upon multiple factors including received level (or distance from the sound source), age, and exposure history. Such a multi-factorial context certainly makes evaluating impacts in nature more challenging, but not taking these diverse factors into account could result in incorrectly predicting black sea bass responses to noise.

4 Species 2: Longfin squid (*Doryteuthis pealeii*)

4.1. Study 3: PD Sound Induces Transient Gait Disruptions in the Longfin Squid (*Doryteuthis pealeii*)[1]

4.1.1. Background

There is a global investment in offshore wind (OSW) infrastructure as many countries increasingly prioritize renewable energies over fossil fuels (Gielen et al., 2019). The increased human presence in the ocean poses challenges to marine life since the pile driving noise emitted during OSW construction has been shown to cause physical damage (Halvorsen et al., 2012), sensory harm (Kastelein et al., 2016), and behavioral changes (Jones et al., 2020) to a myriad of marine taxa. Consequently, anthropogenic noise is recognized as a global pollutant of paramount concern (Halfwerk et al., 2011; Kunc et al., 2014; Duarte et al., 2021). Noise-induced behavioral changes can have direct fitness consequences, and the spatial extent is likely greater than that of noise-induced physical and physiological harm (Popper et al., 2022). However, movement responses are rarely quantified. Fine behavioral changes are difficult to measure in marine environments where animals are largely inaccessible, leading to key knowledge gaps on the effects of noise on behaviors that can influence individual fitness.

Much of the existing research on noise-induced behavioral changes has focused upon large marine mammals, and to some extent fishes (Miller et al., 2000; Southall et al., 2007; Miller et al., 2012; Popper and Hawkins, 2019). There is scant data on marine invertebrates such as cephalopods. This is a surprising fact considering their central position in many ocean food webs (Clarke, 1996) and their high commercial value exceeding $1 billion USD per year worldwide (Hunsicker et al., 2010). Cephalopods have been shown to detect sounds within the same frequency range (<500 Hz) as pile driving noise, indicating a likely susceptibility to adverse effects of noise (Mooney et al., 2010; Mooney et al., 2020). Indeed, recent laboratory studies showed that solitary longfin squid (*Doryteuthis pealeii*), an important U.S. fishery taxon, exhibit alarm responses to pile driving playbacks (Jones et al., 2020; Jones et al., 2021). However, these studies used solitary squid in tanks, which makes behavioral inferencing challenging since *D. pealeii* is an aggregating species and the acoustic field differed from field conditions (Birkett and Newton-Fisher, 2011; Jones et al., 2019). One field study examined caged squid (*Sepioteuthis australis*) behavioral responses to seismic air-gun surveys (Fewtrell and McCauley, 2012). The authors found that both the proportion of alarm responses (e.g., escape jetting) and swimming speed were positively correlated with received noise levels. Nonetheless, this preliminary study only assessed movement qualitatively, leading to important questions regarding the ecological consequences, energetics, and duration of the observed behavioral changes.

Most bioacoustic studies have not measured the duration of noise-induced behavioral changes (but see Miller et al., 2012) despite being a key consideration for policy makers (Finneran et al., 2017; Southall et al., 2021). Measuring the duration of noise-induced behavioral impacts is critical because it is inherently linked to impact severity and persistence of effect. For

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[1] Cones SF, Jézéquel Y, Ferguson S, Aoki N, Mooney TA.. 2022. Pile driving noise induces transient gait disruptions in the longfin squid (*Doryteuthis pealeii*). Front Mar Sci. 9: 1070290. https://doi.org/10.3389/fmars.2022.1070290. This work was funded by the Bureau of Ocean Energy Management Cooperative Agreement #M20AC10009.

example, the energetic cost incurred from a transient increase in acceleration is less severe than a prolonged heightened acceleration state if an individual does not habituate or desensitize to a noise stimulus (Southall et al., 2007). The few studies measuring disturbance durations in aquatic animals have been restricted to large vertebrates capable of carrying motion sensor tags (Miller et al., 2012). For many marine species, quantifying individual movement is difficult, particularly over time scales comparable to pile driving operations; yet such data are needed to quantify behavioral changes and energetic costs. As a result, most studies on smaller and more abundant animals are conducted in tanks, providing key data but limiting the knowledge that can be applicable to actual noise exposures in field settings. New tools and methods are thus needed to accurately describe and quantify noise-induced behavioral changes, especially in more real-world conditions (Popper et al., 2022).

To date, there has been no field study quantifying the movement behavior of cephalopods, or any invertebrate, during real-time pile driving construction. Given that OSW construction has already commenced and numerous more OSW's are planned, directly overlapping cephalopod fisheries globally(Figure 19), there is an urgent need to experimentally examine whether commercially important cephalopods alter movement behaviors during pile driving noise exposure, and if so, quantify how long those changes persist. In this context, our present aim was to quantitively examine the fine-scale swimming movements and kinematics of *D. pealeii* during field-based pile driving activities to assess potential ecological and energetic consequences of noise exposure. We utilized high-resolution movement sensors to measure individual-level swimming kinematics at sub-second to hourly temporal resolutions and at multiple spatial scales during the two main types of piling installation: continuous vibratory and impulsive impact hammering. Both installation methods are known to produce intense sounds, but the characteristics are vastly different (Amaral et al., 2020; Jézéquel et al., 2022). We then assessed the probability of squid changing their movement behavior associated with specific received noise levels, characterized the observed behavioral changes, and measured the durations of those alarm behaviors. These anthropogenically induced alarm responses were then compared to natural swimming movements and gait disruptions observed throughout the course of quiet, control days to evaluate the potential biological and energetic implications of the noise-induced stress. To address these questions, we developed a new approach to quantify the movement of cephalopods that can be used to address similar questions for other species more broadly.

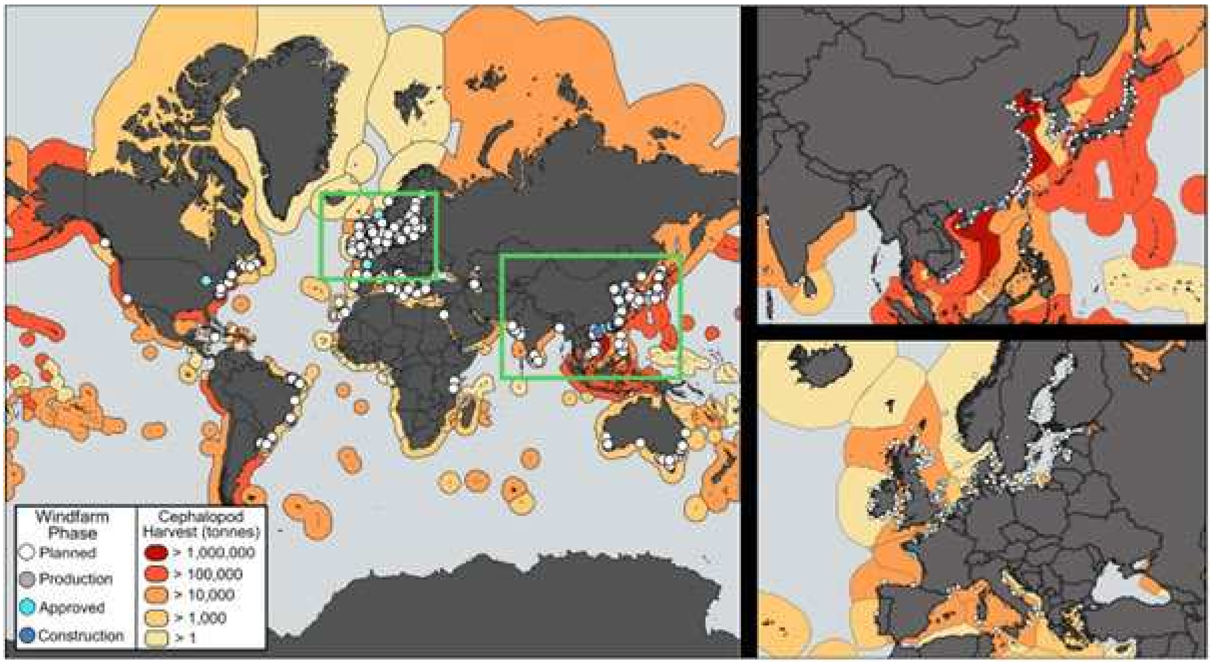

Figure 19. Future offshore windfarm construction largely overlaps with areas of high cephalopods harvest.

The global map depicts individual OSW projects (dots) at four stages of development as well as the extent of cephalopod harvest within a country's ocean governance area (The Wind Power (www.thewindpower.net), Food and Agriculture Organization).

4.1.2 Materials and Methods

4.1.2.1 Study animals

Squid used in the present study were collected from Vineyard Sound, MA (41.22 N; 70.47 W). Animals were hand-selected and only animals without visible lesions and muscular damage were chosen for experimental use. Prior to the experiment, squid were held in multiple 1.2-m diameter cylindrical tanks constantly supplied with ambient, local seawater from the study area. Squid were fed mummichogs (*Fundulus heteroclitus*) and grass shrimps (*Palaemonetes spp.*) daily. Experimental squid were kept in holding tanks for no longer than three days before trials started, and new squid were used each experiment day. This study was carried out in accordance with the principles of the Basel Declaration and recommendations and approval of the Woods Hole Oceanographic Institution's (WHOI's) Institutional Animal Care and Use Committee scientific protocol to TAM.

4.1.2.2 Experimental procedure

Pile driving was conducted for 11 days in September 2021 off the WHOI's dock (Figures 20A, B). At the start of each pile driving day a cylindrical steel pile (length: 10 m, diameter: 0.3 m, wall thickness: 0.02 m) was positioned into the sediment using a vibratory hammer (VH, weight: 212 kg, H&M model 135) at 1150 blows per minute. Squid were then introduced into cages (see below for details) and given 15 minutes to acclimate. Exposures began as (1) a steel impact hammer (IH, weight: 1500 kg) was dropped at 1.2 m height at a rate of 8 -12 strikes per minute until the bottom edge of the steel pile was approximately 5 m into the substrate, taking (mean ± standard deviation) 14.9 ± 0.47 min. (2) The VH was then used to pull the pile out of the substrate and to reposition the pile in an adjacent location for another round of impact hammering. This process was repeated five times per experiment day, which lasted for three to four hours.

To assess potential dose-dependent responses, squid were monitored at two different distances from the pile (near site: within 8 m, far site: 50 m; received levels noted below). The exact distance from the noise source varied slightly because consecutive piles could not be driven in the exact same locations. Squid were placed in 1.5 m 3 cages constructed using a polyvinyl chloride frame covered with 1.5 cm knotless polyester mesh netting (Figures 22C, D). Each cage contained 4–7 squid of mixed sexes to represent wild aggregations (Shashar and Hanlon, 2013). Two underwater cameras (GoPro Hero 7 Black, San Mateo, CA) were placed in the cages for visual observations. Cages were lowered roughly 5 m and hovered 0.5 m above a sandy substrate. The largest squid (male) in each cage was affixed with a modified ITAG, a biologging tag designed for soft-bodied animals (Mooney et al., 2015; Fannjiang et al., 2019; Cones et al., 2022; see Figure 21). The ITAG was used to measure fine-scale swimming kinematics during noise exposure and control periods (see Section 2.3). The analysis focused on the swimming behavior of the tagged squid. Hence, a typical squid group consisted of one large, tagged male (dorsal mantle length (DML): 25.2 ± 2.6 cm) associated with smaller untagged squid (DML: 16.3 ± 2.5 cm).

Control experiments (n=7) were conducted using the same methods, but without pile driving noise exposure. To compare metrics between the two experiment types, noise exposure time periods from experiment days were randomly assigned to control experiments.

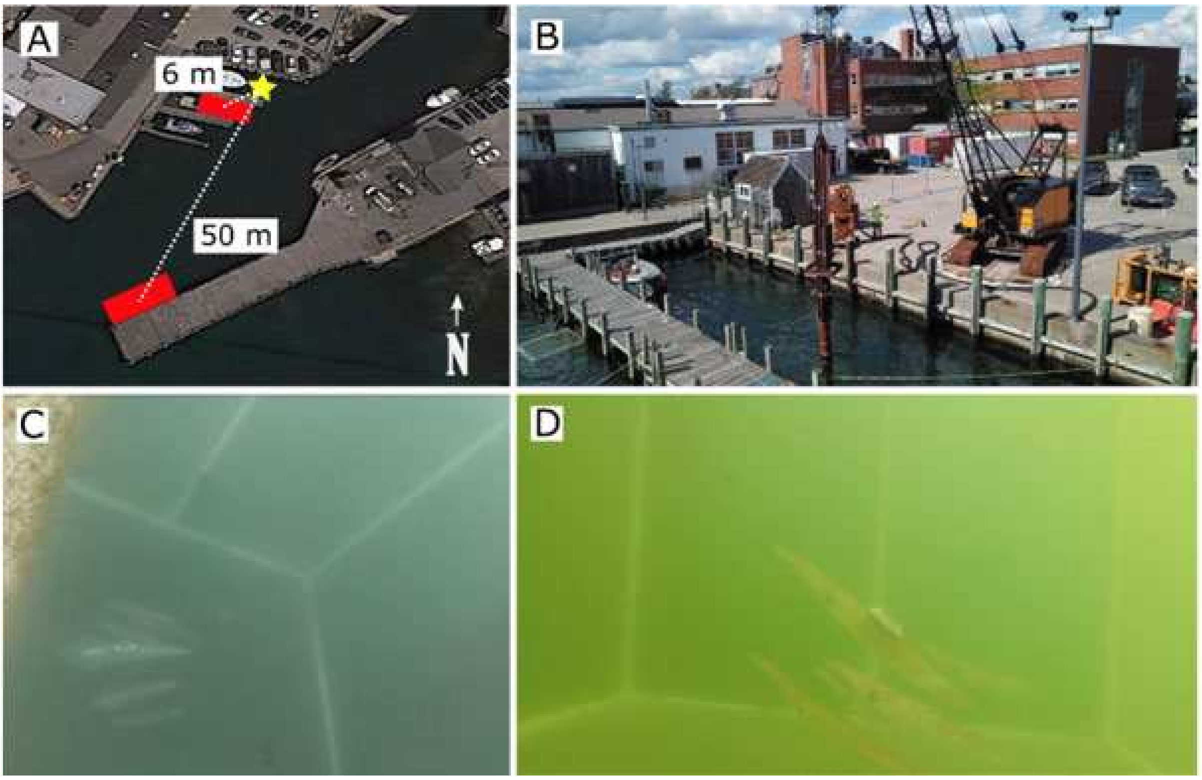

Figure 130. The experimental setup including a (A) map of the two sites: near (2–8 m) and far (50 m).

The yellow star denotes the pile driving location, while the shaded red regions are the position of squid cages. The northern and western boundaries around the pile driving were solid sea walls. There were no physical barriers between the noise source and squid cages apart from a series of 0.3 m diameter piles supporting the dock slips. (B) Drone images during both impact pile driving. (C, D) Video footage from an experiment showing a focal tagged squid schooling with conspecifics.

Figure 14. Example of the tagging procedure of a longfin squid before pile driving sound exposure.

4.1.2.3 Gait classification

ITAGs were used to measure squid movement dynamics. The sensor package was small (length: 7 cm, width: 3 cm, height: 1 cm) and was affixed using surgical sutures (Mooney et al., 2015; Flaspohler et al., 2019; Cones et al., 2022). Additionally, ITAGs were neutrally buoyant, hydrodynamic, and focal tagged squid exhibited normal swimming and schooling behaviors with other conspecifics. ITAGs contain an inertial measurement unit (IMU) which measures acceleration, magnetic field strength, and angular velocity. These high-resolution (100 Hz sampling rate) accelerometers allowed for the estimation of overall dynamic body acceleration (ODBA), a widely used metric to quantify behavior (Zhang et al., 2018) and estimate energetic cost (Wilson et al., 2006; Halsey et al., 2009). The ITAG IMU was used to measure two swimming gaits: jet propulsion and finning.

Jet propulsion is pulsatile and entails the intake of water into the mantle cavity and its expulsion through a flexible funnel (Bartol et al., 2001). Intense jet propulsion events are high acceleration movements employed in response to predators or during conspecific interactions, but is also the common response of squid to recorded pile driving noise (Wells and O'Dor, 1991; Hanlon et al., 2002; Jones et al., 2020). The jetting gait was quantified using similar methods described in detail in previous studies (Flaspohler et al., 2019; Cones et al., 2022). In brevity, a movement was deemed a jetting event if ODBA exceeded 0.3 gravities (g).

Finning is a more continuous movement generated by fin-mediated thrust from waves propagating down the length of the squid mantle-fin. In contrast to intense jet propulsion events, finning is frequently used during low-speed swimming and maneuvering (Stewart et al., 2010; Bartol et al., 2016). To measure finning rates, two small cylindrical magnets (diameter: 3 mm, height: 1 mm) were placed dorso-ventrally on one fin and remained in position without any additional measures. The position of the fin and magnet were coupled, and movements distorted the ambient magnetic field measured by the ITAG magnetometer, resulting in fin position and magnetic field strength to be coupled. Concurrent video and tag data from a subset of six squid in preliminary lab control experiments revealed continuous fin-dominated swimming produced a sinusoidal curve with a frequency equivalent to fin rate. First, a low-pass filter of 20 Hz was applied to the raw signal to smooth the high frequency noise. Then, a MATLAB (Mathworks, Natick, MA, USA) peak detector was used to enumerate crests in the signal which represented individual finning events. The technique was tested on 410 s of movement data from six squid. The algorithm had an average classification accuracy of 97.4%, and its worst segment performance was 95.8% correct detections.

The video data from the cages were used to corroborate and enumerate the number of intense jetting and startle alarm behaviors during noise exposure (defined in detail in Jones et al., 2020). For the impact hammer, only alarm behaviors coinciding with the impact hammer were considered. Alarm behaviors during agonistic encounters with conspecifics were not considered. Using kinematic data from the confirmed alarm behaviors, we created a custom MATLAB algorithm to identify similar movement patterns during the three noise treatment periods using the ITAG (control, vibratory hammer, impact hammer). If focal squid ODBA exceeded 0.3 g and had a concurrent two standard deviation change in finning rate, it was deemed a kinematic disturbance.

To assess if noise exposure impacted the overall swimming patterns, we applied the algorithm to all kinematic data (control and noise exposure sequences) to isolate all sequences, termed kinematic disturbances, during all noise treatments. For this analysis, noise exposure periods were treated as continuous, and all kinematic disturbances during impact and vibratory hammer periods were considered. This differs from the video analysis described above where only alarm behaviors coinciding with the hammer strike were considered.

Lastly, finning rates and ODBA were also used to measure the duration of a gait disruption. The disturbance duration was defined as the time required for the focal squid (1) to return within 25% of the mean finning rate for at least five consecutive finning events and (2) ODBA to decrease below 0.3 g. This method is analogous to Lowe (2002), which used tail-beat frequency as a metric to assess when captured sharks returned to baseline behavior after capture and handling.

4.1.2.4 Acoustic measurements

Given cephalopods sensitivity to low frequency (< 1 kHz) underwater particle motion (Mooney et al., 2010), the sound field was quantified in particle acceleration using a calibrated PCB triaxial accelerometer (model W356B11; sensitivity: x = 10.26 mV m s^{-2}, y = 10.38 mV m s^{-2}, z = 10.62 mV m s^{-2}) with a frequency sampling of 2 kHz. All acoustic measurements were taken during the behavioral experiments. The recording device was wired through a signal conditioner (Model 480B21, Piezotronics), which multiplied the recorded voltage by a factor of 10. The accelerometer signal was input to three analog filters (one per axis; Model FMB300B, Krohn-Hite), which each applied a bandpass filter between 0.06 and 2 kHz. Outputs of the filters were input to a data acquisition board (USB 6251, National Instruments), which was in turn connected to a laptop that ran a custom MATLAB script to record the audio files. Voltage values for each axis (x, y, and z) were calibrated to the sensitivity of the accelerometer and used to calculate the different following acoustic metrics. Recordings were taken at three distances from the pile (1, 8, and 50 m) during both IH and VH pile driving throughout the experimental period. For acoustic measurements, triaxial data were combined as the 3-D vector quantity.

For the IH, the pulse length (in ms) was measured as the time between 5% and 95% cumulative energy, and the rise time as the duration (in ms) from 5% of total energy to the peak acceleration of the signal (ISO standards 2017). The intensity was assessed by computing 0-peak accelerations (PAL_{zpk}; in dB re 1 µm s^{-2}). Next single strike sound exposure levels (SEL_{ss}; in dB re (1 µm $s^{-2})^2$.s) were calculated by integrating PAL_{zpk} over the pulse length containing 90% of the signal energy, and cumulative sound exposure levels (SEL_{cum} ; in dB re (1 µm $s^{-2})^2$.s) were calculated using the following equation:

$$SEL_{cum} = SEL_{ss} + 10 * \log_{10}(N)$$

where N is the number of impulses.

Because VH signals were characterized as continuous (compared to transient IH signals), PAL was described in root mean square (PAL_{rms}; in dB re 1 µm s^{-2}) in the 90% energy window and the 0-1 kHz frequency range, as well as SEL_{ss}.

Finally, PAL_{rms} of the IH signals were calculated with identical methods as for VH signals. Based on PAL_{rms} datasets from both IH and VH, we estimated transmission losses (TL; in dB) by fitting nonlinear least-squared regressions using custom-made scripts in MATLAB (Ainslie, 2010). TL represents the loss of intensity due to the geometrical spreading of sounds in a physical medium (Ainslie, 2010), and was calculated as the slope of the logarithmic regression between PAL_{rms} and the distance from the noise source, which was expressed as:

$$TL = \alpha \times log10(r)$$

where r is the distance between the piling and the accelerometer (in m), and alpha is the geometrical TL term.

4.1.2.5 Statistical analyses

The non-parametric Mann-Whitney U test was used to test for differences in the number of alarm behaviors at the near versus far site and between the IH versus VH. A two-sample t-test was used to test for differences in ODBA during alarm behaviors versus baseline schooling movements. Since our data fit normality assumptions, a one-way ANOVA was used to test for differences in finning rates during noise treatments and to test for differences in the frequency of kinematic disturbances during IH at the near site, far site, and control periods. Lastly, a two-sample Kolmogorov-Smirnov test was used to test if the duration of kinematic disturbances elicited during noise exposure and control periods had similar probability distributions.

4.1.3 Results

4.1.3.1 Acoustic field

A full summary of acoustic data is in Table 5. The IH and VH pile driving produced clear signals above background noise levels at both exposure sites, which allowed for isolation and analysis of all noise sequences (Figure 22A). Both rise time and pulse length increased with distance from the pile, with pulse length ranging from 190–990 ms and rise time increasing from 5.8 to 68 ms. PAL_{zpk} decreased from 122.96 dB re 1 µm s^{-2} at 1 m to 96.45 dB re 1 µm s^{-2} at 50 m. SEL_{ss} for the IH ranged from 81.30 at 1 m to 68.28 dB re (1 µm $s^{-2})^2$.s) at 50 m. In contrast, SEL_{ss} for the continuous VH signals were greater, ranging between 137.76, 134.62, and 126.96 dB re (1 µm $s^{-2})^2$.s)at 1, 8, and 50 m, respectively. SEL_{cum} for the IH was 102.04, 93.24, 88.32 dB re (1 µm $s^{-2})^2$.s) at 1, 8 and 50 m. Interestingly, TL values were similar for both IH and VH signals (a = 12.9 and 11.8, respectively) despite greater PAL_{rms} for the IH (Figure 24B), which was consistent with acoustic propagation in shallow waters.

Table 5. Particle acceleration levels from the IH (black) and VH (red) at three different distances from the pile.

Distance from pile (m)	Pulse Length (ms)	Rise time (ms)	PAL_{zpk} (dB re 1 $\mu m\ s^{-2}$)	PAL_{rms} (dB re 1 $\mu m\ s^{-2}$)	SEL_{ss} (dB re (1 $\mu m\ s^{-2}$)*s)	SEL_{cum} (dB re (1 $\mu m\ s^{-2}$)*s)
1	190 ± 100	5.8 ± 9	122.96 ± 7.98	105.22 ± 1.7	81.30 ± 9.1	102.04 ± 9.8
				95.21 ± 1.6	137.76 ± 0.8	
8	270 ± 200	9.5 ± 20	112.32 ± 3.2	95.79 ± 2.4	72.95 ± 4.0	93.24 ± 2.6
				82.88 ± 4.52	134.62 ± 4.0	
50	990 ± 40	68 ± 30	96.45 ± 3.3	83.22 ± 1.9	68.28 ± 2.6	88.32 ± 1.6
				75.26 ± 1.7	126.93 ± 1.6	

Single strike sound exposure levels (SELss) for the impact hammer were measured for individual hammer strikes, and a single strike for the VH was considered one pile driving sequence. Cumulative sound exposure levels (SEL cum) at 1, 8, 50 meters for the impact hammer were calculated from, on average, 126, 118, 94 strikes respectively.

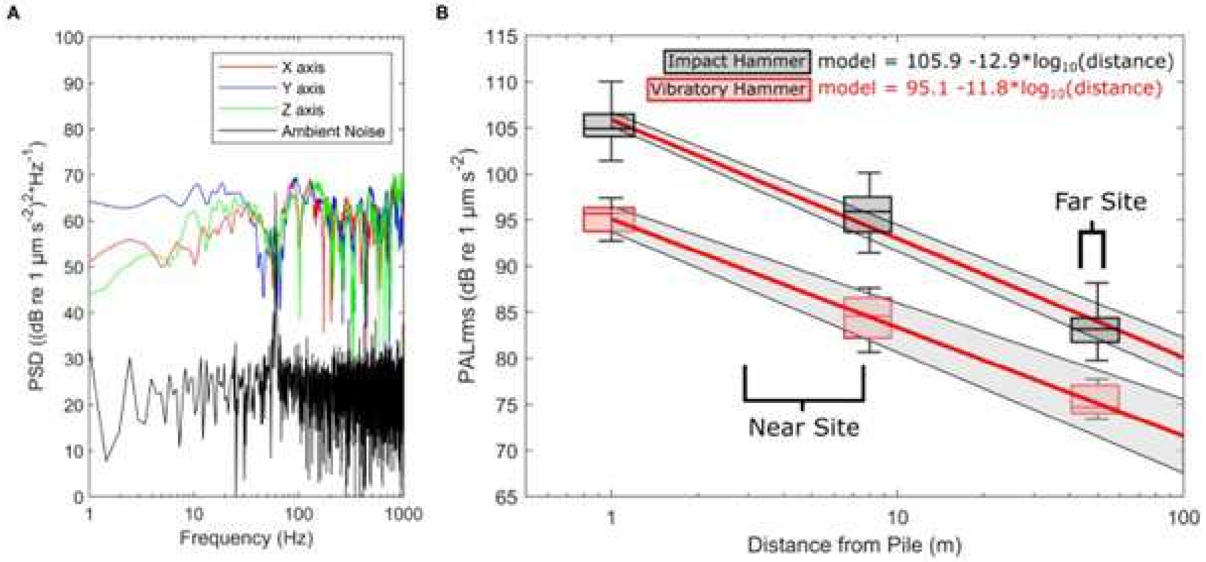

Figure 22. (A) Power spectral density curves of the impact hammer and ambient noise measured at 1 m. The PSD curves were generated from a 1 min segment during both noise treatments, and the x (red), y (blue), and z (green) represent the three accelerometer axes during the impact hammer. (B) PALrms propagation model labeled with the brackets denoting the distances of the experimental cages at the near and far sites. Particle acceleration was measured at multiple distances: 1, 8, and 50 m from the pile driving. The red line represents the empirically-based model fit, and the shaded region denotes the 95% confidence interval.

4.1.3.2 Kinematic disturbances

Over 11 experiment days, we tagged 20 squid and each animal was considered an individual noise exposure experiment. In total, 1101 and 416 minutes of kinematic and video data were collected during IH and VH pile driving, respectively. Thirteen of the 20 experiments were located at the near site, while seven experiments were conducted at the far site. Additionally, we conducted seven control experiments (409 minutes of kinematic data) with identical methods but with no pile driving noise exposure. There were significantly more noise-induced alarm behaviors at the near site [compared to the far site (near site = 17 alarm behaviors, far site = 0 alarm behaviors, Mann-Whitney U test, z = 2.19, p = 0.0284)]. Alarm behaviors were high acceleration jet propulsion events coinciding with the impact hammer or at the onset of the vibratory hammer (Figure 23). Kinematic data from the ITAG revealed that alarm responses resulted in a significant increase in ODBA (two-sample t test, t = 2.11, p = 0.0438; Figure 24). At the near site, nine of the 13 focal squid exhibited one or multiple alarm behaviors in response to the impact and vibratory hammer. Five squid elicited more than one alarm behavior. Of the squid eliciting an alarm response at noise onset, there were more alarm behaviors in response to the IH (16 alarm behaviors) compared to the onset of VH (1 alarm behavior). Eighty-two percent of the alarm responses occurred during the first or second impact or vibratory hammer sequences within a given exposure day, and a separate 82% of the alarm responses occurred within the first three impact hammer strikes or at the onset of vibratory hammer. No focal squid at the far site reacted to either pile driving noise type.

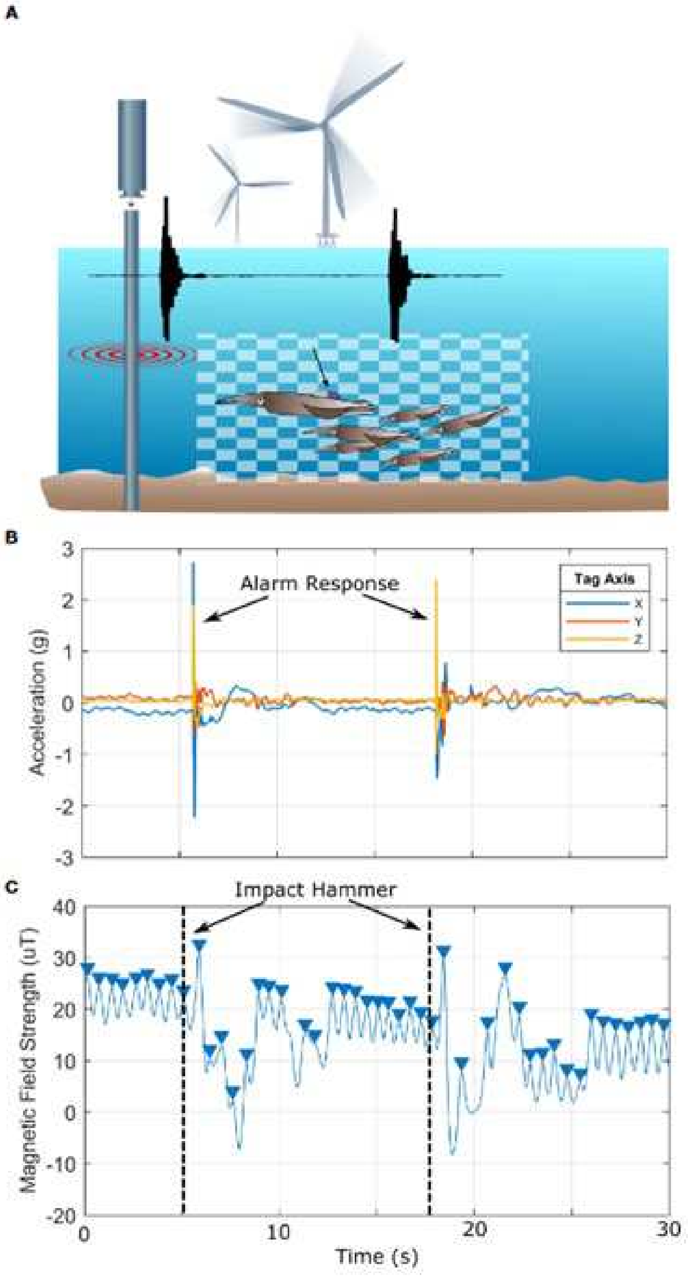

Figure 23. Squid elicit alarm behaviors in response to pile driving sound.

(A) A schematic of the experimental setup with an overlaid example impact hammer signal. Black arrow highlights tagged large squid. (B) Focal tagged squid acceleration during a typical kinematic disturbance. Heightened acceleration occurs at the moment of the impact hammer strike. (C) Concurrent magnetic field strength data used to calculate finning rate. Magnetic field strength is a consistent sinusoidal signal before impact hammer, but becomes erratic as the focal squid transitions to jet propulsion swimming.

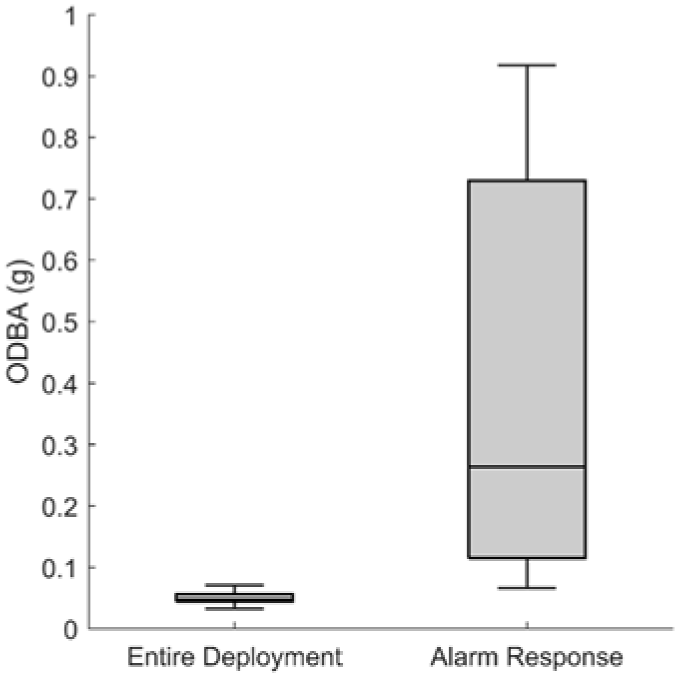

Figure 24. ODBA averaged over the entire experiment periods and (left) across all 17 alarm behaviors in response to pile driving noise (right).

4.1.3.3 Kinematic disturbance probability

Although alarm behaviors occurred in response to the IH, there was no significant change in the number of kinematic disturbances over the course of an experiment vs. control day. Indeed, focal squid at the near (0.037 ± 0.034 kinematic disturbance min -1) and far (0.062 ± 0.048 kinematic disturbance min -1) sites had statistically similar kinematic disturbance frequencies compared to the quiet control periods (0.058 ± 0.058 min -1 ; One-way ANOVA, F2,26 = 0.88, p = 0.43, Figure 25).

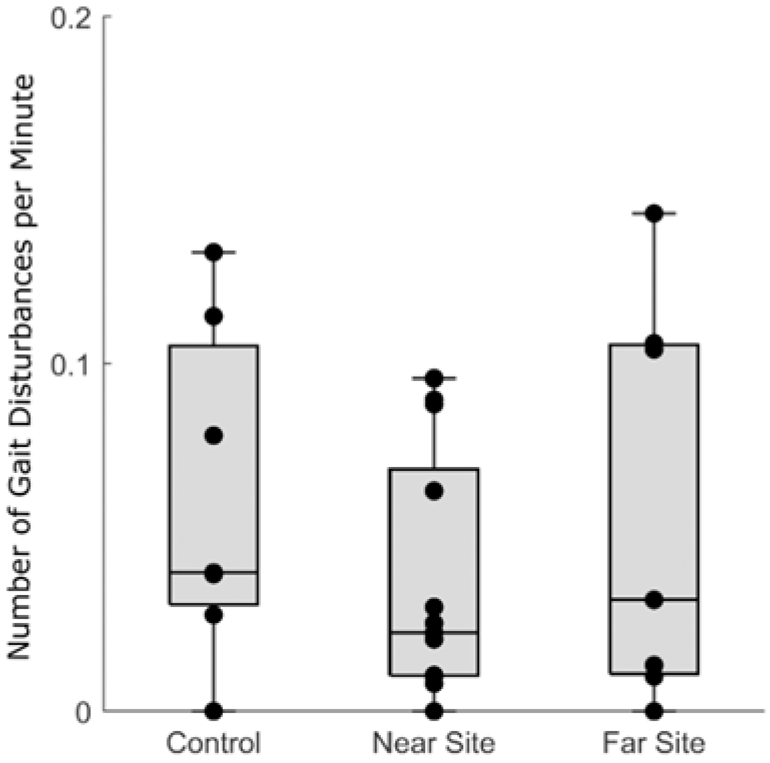

Figure 155. The number of daily gait disturbances calculated from kinematic algorithms trained by confirmed reactions.

Although squid reacted to pile driving noise, it did not significantly increase the number of total gait disturbances over an experimental day.

4.1.3.4 Duration of disturbances

Alarm behaviors during IH sequences persisted for 4.2 ± 4.7 s. This was significantly shorter than kinematic disturbances measured during ‘quiet’ control periods 6.1 ± 4.2 s (two-sample Kolmogorov-Smirnov test, $p < 0.001$, Figure 26A). For each noise-induced disturbance, focal squid accelerated rapidly (i.e., high ODBA), but ODBA for each disturbance returned to similar baseline levels within ca. 4 seconds (Figure 28B). However, for some individuals, the finning gait continued to deviate from baseline or individuals reacted to consecutive hammer strikes, resulting in longer recover times, with a maximum recovery time of 14.7 s.

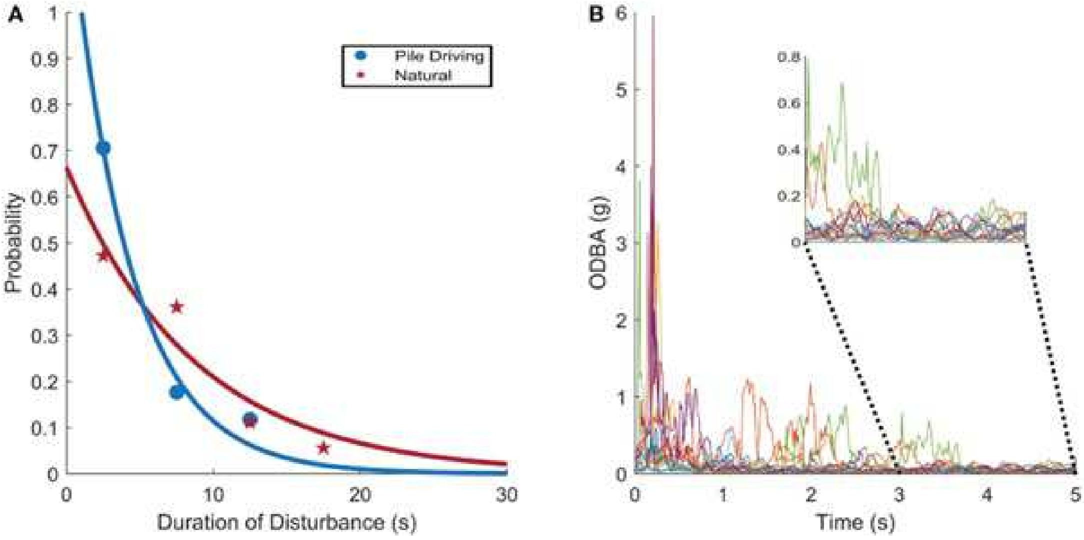

Figure 166. (A) Squid exhibited brief kinematic disturbances in response to pile driving noise, and these disturbances are similar in duration to natural kinematic changes during inter-individual interactions. (B) ODBA during all 17 confirmed alarm responses to pile driving noise.
The models compare the recovery time from both pile driving noise and naturally-induced kinematic changes.

Although finning behavior changed at short time scales during kinematic disturbances, average finning rates during IH periods were not significantly different at the near site (1.563 ± 0.13 fin s -1), far site (1.624 ± 0.063 fin s-1), and during silent control periods (1.587 ± 0.11 fin s -1 , One-way ANOVA, F 2,39 = 0.63, p = 0.54, Figure 27A). Additionally, after combining all finning data across the two sites, there was no difference in average finning rates during noise exposure (IH: 1.584 ± 0.11 fin s -1; VH: 1.583 ± 0.11 fin s-1) and silent periods (1.587 ± 0.11 fin s -1; One-way ANOVA, F2,59 = 0.01, p = 0.99, Figure 27B).

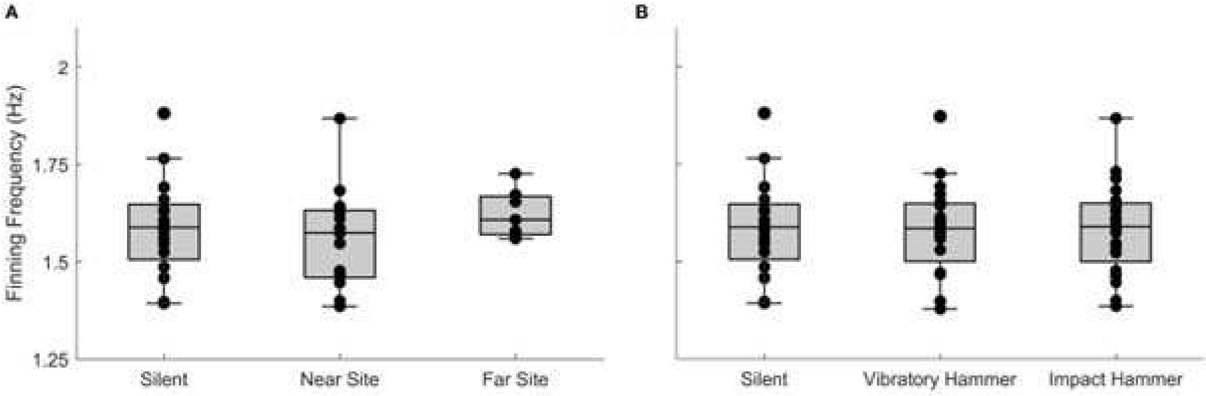

Figure 17. Focal squid finning rates averaged over the impact pile driving periods, separated by (A) near and far site and the control quiet periods. (B) Finning rates for both near and far site separated by noise treatment.

4.1.4 Discussion

We present the first study quantifying the fine-scale movement behaviors of a marine invertebrate in response to an actual field-based anthropogenic noise source. We used high-resolution movement sensors to quantitively measure changes in swimming kinematics and measure how long gait disruptions persisted. Our results demonstrate that while field-conducted pile driving noise elicited clear alarm responses at high received levels, these were short-term evasions that persisted for only 4 s on average. Further, these escape behaviors were found only at a site of relatively high received sound levels, although the measured noise levels corresponded to roughly 1 km from actual windfarm construction pile driving (Table 1, Sigray et al., 2022). Interestingly, alarm behaviors were shorter in duration than similar high acceleration movements during natural, intraspecific agonistic encounters observed during quiet control periods indicating that the animals quickly returned to sensory vigilance. Additionally, when considering overall jetting and finning gait behaviors throughout an exposure or control day, there was no detectable impact of pile driving noise on swimming behavior. Although, the experimental cage may have constrained certain swimming behaviors, particularly horizontal dispersion from the sound source.

This study used novel accelerometer-based particle acceleration measurements at multiple distances to create an acoustic propagation model and identify probabilities of movement behavior changes at specific received noise levels. Nine of 13 *D. pealeii* at the near site elicited at least one or more alarm movements in response to the IH between 122.96 and 112.32 PAL zpk dB re 1 µm s^{-2}, which are noise levels greater than 880 m from a one OSW construction site (Sigray et al., 2022). We know of no other sites in which we there are comparable, published, particle acceleration data. This suggests that behavioral disruption will likely occur at the kilometer scale and at a relatively substantial range, especially if we consider wind turbine pile spaces to be roughly 1 km apart and noise levels to stay consistent. More intense or persistent responses may occur within that 880 m range especially if larger pilings are used or if multiple platforms are constructed concurrently. Hence, the alarm responses described here may impact a significant majority of animals within the entire OSW development area, leading to potential regional impacts on squid populations. However, more information on noise-induced disruptions to group-level behaviors is needed to better assess impacts on populations.

Although there were clear alarm behaviors in response to pile driving noise, we found no significant difference in the number of kinematic disturbances measured from the ITAG between control and noise exposure periods (Figure 23). To be more representative of wild conditions, we used squid groups of mixed sexes in our experiments. *D. pealeii* are still reproductively active into September when our experiments took place (Stevenson, 1934), and squid are known to swim dynamically in breeding aggregations, and these movements were likely classified as kinematic disturbances in the present study (Shashar and Hanlon, 2013). This result provides more evidence that pile driving did not change long term swimming behaviors and it demonstrates the importance of considering the biology and group-level behaviors when quantifying noise-induced behavioral impacts. Future studies should avoid studying aggregating species in isolation because it may constrain individual behavior and limit interpretations.

Most alarm behaviors were associated with one or multiple rapid jet propulsion events; these jets resulted in elevated ODBA and a change in finning rate (Figure 23). An increase in ODBA and a transition to primarily jet propulsion indicates a higher energetic cost (Webber and O’Dor 1986, Halsey et al., 2009). Squid are thought to operate at or near their metabolic limit (O’Dor and Webber 1991), which suggests that an anthropogenically-induced high energy alarm behaviors may be detrimental to squid energy budgets. However, because the disruptions were transient and only elicited a maximum of three times per individual over 3-4 hours of pile driving, we suspect the impact to be non-substantial, especially considering squid frequently elicited similar dynamic kinematics during non-noise exposure periods. Additionally, free-ranging muscular squid naturally display high acceleration jet propulsion at rates, > 9 jets min-1 (Cones et al., 2022). Thus, the additional 0-3 jetting propulsion alarm responses over multiple hours of noise exposure are not likely detrimental to energetic expenditure.

No squid at the far site (with lower received levels) elicited alarm behaviors in response to either IH or VH pile driving noise despite noise levels occurring within *D. pealeii* sound detection abilities (Mooney et al., 2010). This result suggests there was either a dose-dependent response or there exists a minimum threshold that induces alarm behaviors, where animals detecting amplitudes 112-123 and 96 dB re 1 μm s^{-2} have a 69% and <1% probability of eliciting at least one alarm response, respectively. In fact, dose dependence behavioral responses were found in *S. australis* exposed to air gun noise (Fewtrell and McCauley, 2012). Squid elicited a higher proportion of alarm behaviors with increasing noise levels, implying the severity of noise impact on squid is related to the distance from the noise source.

Interestingly, 16 of the 17 alarm behaviors were observed during IH (7 alarm behaviors at the first hammer strike) pile driving, with only one instance of reaction to the onset of VH pile driving. This finding suggests that high amplitude and transient signals are more detrimental to squid swimming kinematics compared to low amplitude and continuous signals. Previous noise studies have largely focused upon IH noise impacts on marine life (Herbert-Read et al., 2017; Jones et al., 2020; van der Knaap et al., 2022), while fewer have directly compared noise impact with temporal variation (Neo et al., 2014; Shafiei Sabet et al., 2015). These studies also demonstrated that intermittent noises, rather than continuous, induced more severe behavioral changes including more alarm behaviors. Further research should seek impact severity comparisons between IH and VH techniques for a broader range of species. Considering some OSW farms have been successfully installed with only the VH, it could serve as an important mitigation technique in areas with suitable substrate type (OSPAR, 2014).

The duration of a behavioral disturbance is a key metric to address impacts to individual fitness, and it can inform models and evaluations of impacts by managers as they develop policy recommendations (Southall et al., 2007; Tyack et al., 2011; Ranaweerage et al., 2015; Finneran et al., 2017). Observed *D. pealeii* alarm responses were transient and had similar movements as anti-predator behaviors observed in other squid species (Mather, 2010). By resuming baseline swimming within only a few seconds, squid may be selecting to maximize other sensory systems or detection needs, particularly audition, to enable vigilance for predators. In late summer, coastal Massachusetts waters and the habitat of this squid are turbid. Such conditions likely renders auditory cues more useful than vision for long-term sensory perception. Low acceleration swimming could serve to decrease chaotic flow around sensory hair cells, which aid in predator detection (Mooney et al., 2010; York and Bartol, 2014; Higham et al., 2015). Another explanation for the short-term alarm responses was that *D. pealeii* experienced temporary or permanent shifts in hearing thresholds as seen in other species (Smith et al., 2004; Mooney et al., 2009). If so, squid may lack perception of the noise stimulus, explaining the rapid decline in alarm behaviors throughout exposure. However, given that no hearing threshold shifts were observed in *D. pealeii* exposed to very similar impulsive sound levels (Jezequel & Mooney, in review), this is unlikely.

There was no significant difference in finning rates over noise treatment periods, which is more evidence suggesting pile driving noise does not alter longer term natural swimming patterns. To our knowledge, these are the first data on squid finning rates in semi-wild conditions. Most research on squid locomotion, especially in the field, has focused upon jet propulsion despite finning being integral to squid energetics and ecology (Anderson and DeMont, 2005; Bartol et al., 2016; Cones et al., 2022). Fin-dominated movements increase propulsive swimming efficiency at certain speeds and allow for versatile maneuvers which are thought to aid in squids' ability to compete with fishes (Hoar et al., 1994; Bartol et al., 2016). Although we did not measure specific energetic costs throughout noise exposure, the finning detection method described here could be used in combination with other metrics (i.e., speed) in the future to estimate free-ranging squid energetics in response to real OSW constructions and more broadly (Anderson and DeMont, 2005; Bartol et al., 2008).

4.1.5 Conclusion

This work revealed that pile-driving noise induced clear but transient disruptions to squid swimming behavior. However, the scale of our experimental pile driving was much smaller than planned future pile driving associated with OSW development within the D. pealeii range in the U.S. eastern coast. The diameter of our steel pile was 0.3 m, while OSW turbines are using piles exceeding 8 m in diameter, perhaps approaching or exceeding 10 m diameter (Steelwind Nordenham, FHL Corporation). As a result, noise propagating from OSW constructions will likely be higher in amplitude and farther reaching, which would expand the volume of ocean where behavioral impacts may be elicited.

Consequently, this study represents a significant step toward understanding how an abundant and commercially important species will be impacted by current and planned offshore constructions. Our novel high-resolution movement and particle acceleration data allowed us to be the first study to document both the probability of behavioral change and its duration in multiple spatial scales and noise exposure contexts. Future studies should aim to assess if pile-driving causes horizontal displacement, which is of particular concern to the management of commercial fisheries.

4.2 Study 4: Short-term Habituation of the Longfin Squid (*Doryteuthis pealeii*) to Pile Driving Sound[2]

4.2.1 Background

Anthropogenic sound is increasingly considered a major underwater pollutant of international concern that can affect sound-sensitive animals (Duarte et al., 2021). Pile driving (PD) is associated with offshore windfarm (OSW) construction and generates repeated, high intensity impulsive sound that can propagate over tens of kilometres underwater, underscoring concerns for wide-ranging impacts (Bailey et al., 2014; Dahl et al., 2015; Mooney et al., 2020). Several studies have described the various impacts of PD sound on marine mammals and fish, ranging from temporary changes in behaviour to mortality (Madsen et al., 2006; Slabbekoorn et al., 2010). However, little is known about the impact of PD activity on marine invertebrates despite their oft-central role in ecosystems and fisheries (Williams et al., 2015; Popper et al., 2022).

Cephalopods are sensitive to low-frequency sounds in the same bandwidth as PD sound (Packard et al., 1990; Mooney et al., 2010, 2020). Previous studies in tanks showed both artificial and PD sounds elicited short-term alarm responses in squid and cuttlefish, but these responses attenuated over time, suggesting a potential habituation to sound exposure (Samson et al., 2014; Mooney et al., 2016; Jones et al., 2020). However, for many taxa, including marine invertebrates such as cephalopods, the translation of these laboratory results into the field and actual PD is not straightforward, especially when considering the different sound intensities as well as the spatial and temporal scales of potential impacts by anthropogenic activities (Popper et al., 2022). An initial field-based study showed dose-dependent responses in individual squid (*Sepioteuthis australis*) to airgun sounds from seismic surveys, suggesting the severity of sound impact was related to the sound level and (often corresponding) distance from the sound source (Fewtrell and McCauley, 2012). Another field-based study used biologging tags and showed PD events disrupted individual squid fine-scale movements, but these impacts were transient, suggesting minimal energetic impacts over the entire exposure period (Cones et al., 2022). However, all studies mentioned above have only examined individual-level responses. PD sound impacts on squid shoals are not yet known, a knowledge gap that is particularly striking because they live in groups (Hanlon and Messenger, 2018).

Intraspecific aggregations and collective movements are a widely conserved phenomenon across many distinct evolutionary trajectories (Allee, 1927). In many marine species, shoaling can decrease predation risk (Ioannou et al., 2008), enable more efficient navigation through collective learning (Berdahl et al., 2018), and decrease metabolic demands by leveraging beneficial flows from conspecifics (Marras et al., 2015; Burford et al., 2019). One laboratory study showed that PD sound disrupt the structure and dynamics of fish shoals (Herbert-Read et al., 2017). In the field, free-ranging sprat and mackerel shoals exposed to PD sound increased dispersion (i.e. greater shoal area) and shoals moved to deeper water (Hawkins et

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[2] Published as: Jézéquel Y, Jandial P, Cones SF, Ferguson S., Aoki N, Girdhar Y, Mooney TA. 2023. Short-term habituation of the longfin squid (*Doryteuthis pealeii*) to pile driving sound. ICES J Mar Sci. p.fsad157. https://doi.org/10.1093/icesjms/fsad157. This work was funded by the Bureau of Ocean Energy Management Cooperative Agreement #M20AC10009.

al., 2014). To date, there are no data of PD sound effects on shoal-level behaviours in marine invertebrates, leaving questions on how OSW development could impact the ecology of commercially important squid.

Recent calls for future OSW constructions have been planned in nine US states (Musial et al., 2019), encompassing the distribution area of many marine invertebrates, such as the longfin squid (*Doryteuthis pealeii*; Hanlon et al., 2013). The occurrence rate and spatial range of PD exposure events are expected to affect this taxon and its vital habitat. Considering the economic importance of squid, which have contributed mean annual landings and value of 13000 mt and $26 million since 2000 (NMFS, 2019), conflicts between fishermen, policymakers, and the offshore wind industry are expected to increase dramatically (Lacroix and Pioch, 2011). New studies are now needed to develop effective management strategies and to design suitable mitigation methods (Popper et al., 2022).

In this field-based study, we assessed real-time behavioural responses of squid (D. pealeii) shoals exposed to real PD. Squid were placed in enclosures installed at different received sound levels and distances from the PD activity and subsequent responses were recorded using underwater cameras. We first characterized the effects of PD sound on individual behaviours and sought to assess potential habituation rates to repeated PD events. Next, we used video data to measure squid group cohesiveness by calculating the collective area of the shoals both prior to and while exposed to PD sound. Because different construction techniques are used in OSW constructions, we also examined the influences of the two main types of piling installation tools with different sound characteristics: the “impulsive” impact hammer, and “continuous” vibratory hammer (termed IH and VH, respectively). While IH is the most prevalent method, some OSWs have installed pile structures VH (OSPAR, 2014). Given that there is some interest in expanding this technique, we sought to compare sound effects from these multiple construction techniques in squid.

4.2.2 Materials and methods

4.2.2.1 Animal collection and holding conditions

A total of 189 adult squid (dorsal mantle length = 17.9 ± 3.3 cm, mean ± standard deviation) were used for this study. Squid were collected from Vineyard Sound (41.22 N, 70.47 W) via trawling by the R/V *Gemma* of the Marine Biological Laboratory (Woods Hole, MA, USA). Squid were held in groups of four to seven individuals in cylindrical tanks (1.2 m diameter) constantly supplied with ambient seawater (temperature range: 21.1 to 22.5°C). Tanks rested on rubber gaskets and concrete blocks, both of which served to further isolate the squid from surrounding vibrations during their respective holding periods. The top of each tank was covered with plastic sheeting to create shaded zones. Squid were fed daily with mummichogs (*Fundulus heterclitus*) and grass shrimp (*Palaemonetes spp*.). Given that squid can be relatively fragile and healthy animals are vital to behavioural experiments, individuals were held <72 h before being used for experiments, and all animals incorporated had no visible skin damage and exhibited normal swimming behaviour (e.g. Jones et al., 2023). All procedures regarding the use of animals in research followed local guidelines and were approved by the Woods Hole Oceanographic Institution’s Institutional Animal Care and Use Committee (approval to TAM).

4.2.2.2 Location and PD characteristics

Ten days of PD experiments were conducted between the 14th and 29th of September 2021. Procedures took place at near-shore experimental testbed area off the Woods Hole

Oceanographic Institution pier (41.52◦N, 70.67◦W; Figure 28a and b). It is a shallow water habitat with depth varying between 3 and 5 m depending on tide, the bottom is flat and consists of homogeneous sand and silt. PD incorporated a single 10 m long, 0.3 m diameter cylindrical steel monopile (wall thickness: 0.02 m). At the start of an experimental day, a crane (American 595) with a 20-m long boom moved a VH (weight: 212 kg, H&M model 135) into place to first secure the pile into the seabed. This VH, similar to a jack-hammer, would hit the pile at a rapid rate of 1150 blows per min. Once the piling was in position, squid were introduced into their respective cages (see details below). The VH was then removed and replaced with a steel IH (weight 1500 kg), the head of which was manually dropped onto the top of the pile from a height of 1.2 m at a rate of 10 strikes per min (~16 kJ per strike) for a duration of 15 min (constituting the IH exposure). After 15 min of IH, the piling was typically driven 5 m below the water sediment surface. The VH was then used to pull the pile out and reposition it in an adjacent location for another round of IH. This procedure started at 1330 each day to control for any potential circadian rhythm effects and was repeated five times within an afternoon. Hence, in total, each squid shoal was exposed to five IH and four VH sequences, always starting with the IH exposure. Control days were essentially mimics of this procedure (i.e. adding squid to cages shortly before 1330).

4.2.2.3 Experimental design

Prior to the first IH sound exposure, squid were quickly transferred from holding tanks to submerged 3.4 m3 cubic cages (1.5 × 1.5 × 1.5 m) built with polyvinyl chloride (PVC) pipes and covered with 1.5-cm knotless polyester mesh netting (Figure 28c). Note that the shortest dimension of the cage (1.5 m) was always >5 body lengths even for the largest squid used, which allowed animals to naturally exhibit shoal behaviours throughout the experiments (see Supplementary materials). A door on the top of the cages permitted the squid transfer into the undersea cages; once all squid were introduced, the cage was lowered to 0.5 m from the seabed. Two cages were placed within 2–8 m and one cage was positioned at 50 m from the pile (Figure 28b). Squid were allowed 15 min of acclimatization in the cages to recover from handling before the first IH sound exposure started.

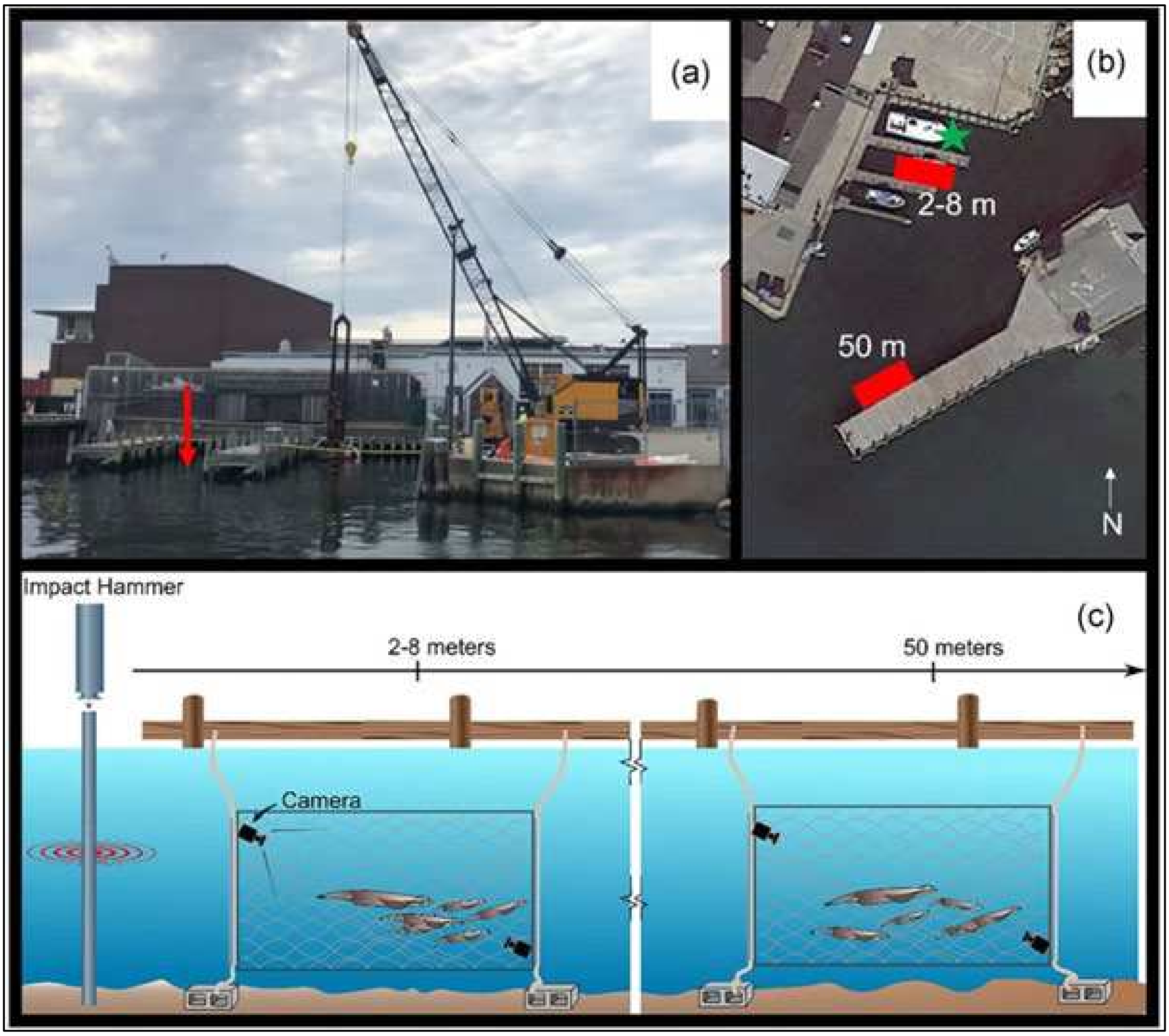

Figure 28. Experimental set-up used to investigate the behavioural responses of squid to repeated pile driving sound exposure.

(a) Picture of the crane driving a pile into the seabed off a dock with the impact hammer. Red arrow shows the location of the cages placed within 2–8 m from the pile. (b) Map of the two near (2–8 m) and far (50 m) sites. The green star denotes the pile driving location, while the red regions are the positions of the squid cages. (c) Shoals of four to seven squid were placed in large cages and behavioural responses to repeated pile driving sound were monitored using two different cameras. Controls were performed using the same experimental design but without pile driving sound.

Within the cages, we sought to quantify squid shoaling behaviour, which was defined as three or more individuals swimming within one body length from each other (Oshima et al., 2016). Each cage contained four to seven haphazardly chosen individual squid of mixed sexes. Hence, each day, two new squid shoals were studied at the near site (i.e. two replicants), while one new squid shoal was studied at the far site (Figure 28b). It is notable that this group size was certainly lower than that of many wild squid aggregations (often easily upwards of 100 individuals; see Shashar and Hanlon, 2013). Yet, this quantity provided a reasonable number of animals to coherently track and quantify shoaling behaviour (see below).

Squid responses to PD sound were recorded using GoPro Hero 7 Black cameras. In each cage, one camera was positioned horizontally near the bottom against the net, while the second was mounted in the top corner and angled towards the cage center (Figure 28c). The bottom camera was used to monitor individual squid responses, while the top camera was used for shoaling behaviour (see details in the section "Data analyses").

Control experiments (n = 7 squid shoals) were conducted at both sites using the same experimental procedures, but without PD noise exposure. To compare metrics between the two experiment types, sound exposure time periods from experiment days were randomly assigned to control experiments.

Individual squid were used only once and released back into the marine environment at the end of each experiment.

4.2.2.4 Sound exposure

Squid detect low frequency (<1 kHz) underwater acoustic particle motion (Mooney et al., 2010). Correspondingly, the sound field was measured and quantified in presence and absence of PD using a calibrated PCB triaxial accelerometer (model W356B11), and details of the particle acceleration levels (PALs) generated by the PD were presented in Cones et al. (2022). Briefly, the cages placed at the near site were exposed to "high" PALs (in 0-peak; PAL_{0p}) from the impulsive IH that ranged between 112 and 123 dB re 1 µm s^{-2} at 8 and 1 m, respectively, while the cage at 50 m had lower PAL_{0p} (83 dB re 1 µm s^{-2}). The VH generated PALs (in rms; PAL_{rms}) between 83 and 95 dB re 1 µm s^{-2} within 8 m, while PAL_{rms} decreased at 75 dB re 1 µm s^{-2} at 50 m. The PALs recorded at the near site were roughly equivalent to those measured at 1 km from OSW constructions (Table 1, Sigray et al., 2022). An example of underwater particle acceleration recordings from an entire PD experiment (including five IH and four VH sequences) is shown in Figure 31. Note that given the in situ and realistic nature of this actual PD, pulse amplitudes and inter-pulse time intervals did vary slightly within and across IH sequences as the pile was driven into the sediment (Figure 31). The variability in these metrics across impulses reflected that which a wild squid may experience near an offshore PD site (Amaral et al., 2020). The near site was always characterized by higher PALs compared to the far site (~30 dB difference). Here, we were interested in studying behavioural effects of PD sound at two distances from the pile, rather than studying responses dependent on these specific metrics.

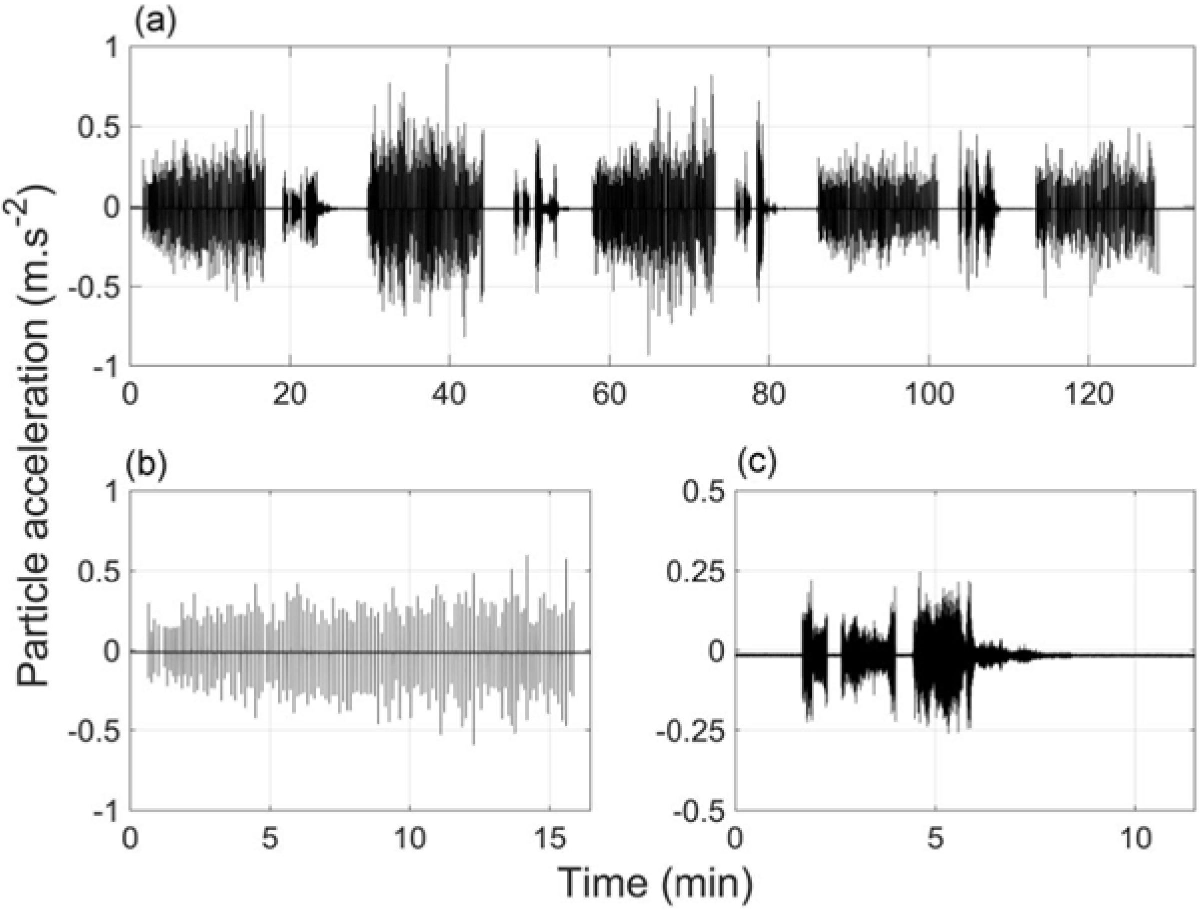

Figure 29. Example of underwater particle acceleration (x-axis) from a field-based in situ pile driving experiment recorded at 8 m from the pile (a). Sound exposures consisted of five 15-min long IH sequences (transient pulses, b) that were separated by VH sequences (continuous vibrations, c).

There were 5 min "silent" sequences between each IH and VH sequence due to the crane switching hammers. Each experiment started daily at 1330 when squid shoals were first exposed to the IH pile driving.

The PALs of ambient sound in the holding tanks and underwater in absence of PD sound were below the self-noise floor of the accelerometer, which was evidenced by flat power spectral densities of these recordings at 30 dB re 1 (µm s−2)2 Hz−1 (Figure 29; Cones et al., 2022). These relatively quiet environments enabled us to isolate and assess potential effects of PD sound on squid while minimizing extraneous stimuli that are typically found in natural field environments.

4.2.2.5 Data analyses

4.2.2.5.1 Individual behaviour

Behavioural responses of individual squid were assessed using bottom-mounted camera videos. Manual annotations started 5 min after squid were transferred inside the cages. All visible squid were observed and behaviours were annotated using tools in BORIS (v7.12.2; Friard and Gamba, 2016). Videos were first viewed by a trained observer at half-speed without sound (i.e. blind to the sound treatment) to quantify occurrences of four alarm responses (inking, jetting, startle, and body pattern change) following descriptions from previous studies (Hanlon et al., 1999; Mooney et al., 2016; Jones et al., 2020). Because the GoPro cameras also recorded sounds from PD at both sites, a second annotation was then performed by listening to the audio, which permitted us to synchronize particular behaviours to either IH or VH sequences. Annotations were then compared with another independent observer (see Cones et al., 2022) who manually annotated one third of the video footages, and comparisons showed 100% agreement. Body pattern changes were defined as alterations in chromatic components of at least half of the squid bodies (Hanlon et al., 1999). Squid raising their arms as postural component was termed startle. Jetting was characterized by a rapid jet-propelled escape, which was sometimes followed by inking. Alarm responses related to agonistic encounters were not taken into account in this study. Because squid were not tagged with a specific mark, it was not possible to assess behavioural responses by specific squid throughout an entire experiment. However, most squid were present in the video recordings majority of the time. Hence, we reported the proportion of individual squid responding to PD sound per number of squid visible on the video. Using the sound recordings on the camera to synchronize both video and acoustic data, we were able to associate observed responses to each strike from the IH events.

4.2.2.5.2 Shoaling behaviour

We used top camera videos to assess the potential impacts of PD sound exposure on the cohesion of squid, quantified as the variance in the area covered by the shoal using a trained neural network. Analyses were conducted for the near site only (within 8 m from the pile) because there were no behavioural reactions of squid found at the far site (50 m form the pile). To compute the shoal area, we first tracked each squid individually in the video by anchoring and tracking three virtual point markers along the length of their body (arms, centre, and mantle tip). Then, we computed the convex hull of the set of all points from all squid visible in a frame to find the smallest bounding convex polygon, and then computed its 2D area in pixel space. To train the squid tracker, we used 260 randomly selected frames and annotated them by marking the arms, centre, and mantle tip of each squid. Then, we used the DeepLabCut (Lauer et al., 2022) algorithm to train the squid tracker (Figure 30), and then apply it to rest of the data to track the squid in all frames of all the videos. We analysed 6 out of 18 squid shoals when the water turbidity was low enough for all squid within a shoal to be detectable by our algorithm throughout the entire video recordings.

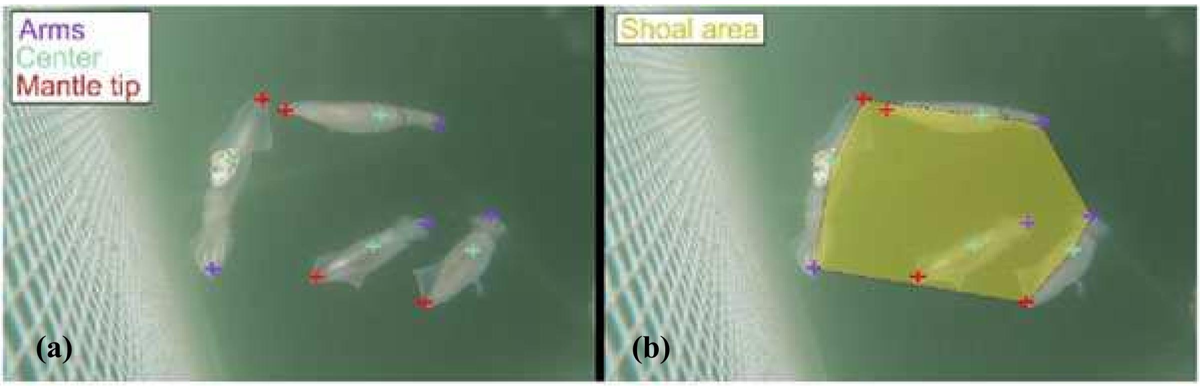

Figure 30. Example of a manually annotated frame (a) and associated polygon (b) created by the machine learning model using DeepLabCut software (Lauer et al., 2022) to estimate the area of the squid shoal during pile driving sound exposure.

False positives and negatives in the squid detections can result in extremely noisy estimates of the shoal area. Instead of using the standard outlier elimination approach based on running averages, we found the Hampel Filter to be far more robust to the noisy detection (Davies and

Gather, 1993). Any value that was considered as an outlier by the filter was replaced by the median of the filter's running window. We set the window size to be the same as the number of frames per second for that particular video.

4.2.2.5.3 Statistical analyses

Statistical analyses were performed using R v4.1.3 (http://www.R-project.org/). Behaviours from individual squid (n = 103) were measured as the proportion of visible squid that responded to PD exposure at the near site. No statistics were conducted for the far site samples since squid did not respond to either IH or VH sequences at 50 m (see the section "Results"). We focused our analyses on IH events because most alarm responses occurred during IH compared to VH events (see the section "Results"). We also focused on the first 30 strikes of each IH event to compare our results with previous laboratory and bioenergetic studies (Samson et al., 2014; Mooney et al., 2016; Jones et al., 2020; Cones et al., 2022). Generalized linear models (GLMs) were used for regression analysis to describe the occurrence of each behaviour over the first 30 strikes. The strike numbers and IH sequences were used as explanatory variables, and ordinary Poisson (log-link) GLMs were performed. Since two different squid shoals were exposed daily at the near site, making them non-independent samples, we included "day" as a nested factor in the model.

Finally, we used one-way repeated measures analyses of variance (ANOVAs) to determine the effects of PD treatment on the squid shoaling area at 8 m, as no alarm responses were found at 50 m (see the section "Results").

4.2.3 Results

4.2.3.1 Individual behaviour

A total of 30 different squid shoals (near site: n = 18; far site: n = 12) were experimented upon during 10 days of PD sound exposure. Another seven shoals were monitored as controls without PD. Individual alarm responses only occurred at the near site. No alarm responses besides natural conspecific interactions were found at the far site (50 m form the pile) during PD exposure and during controls.

At the near site, a total of 305 alarm responses were detected from individual squid in response to PD sound (Figure 31, Table 6). Among these responses, 89% occurred during the IH exposure, while only 11% occurred during VH exposure. While all types of alarm responses were exhibited by squid exposed to the IH (i.e. inking, jetting, colour change, and startle), only colour change patterns and startle responses occurred during VH exposure (Table 6). During IH exposure, the most common alarm response was jetting (65%) and startling (21%); inking only occurred four times and was associated with jetting (Table 6).

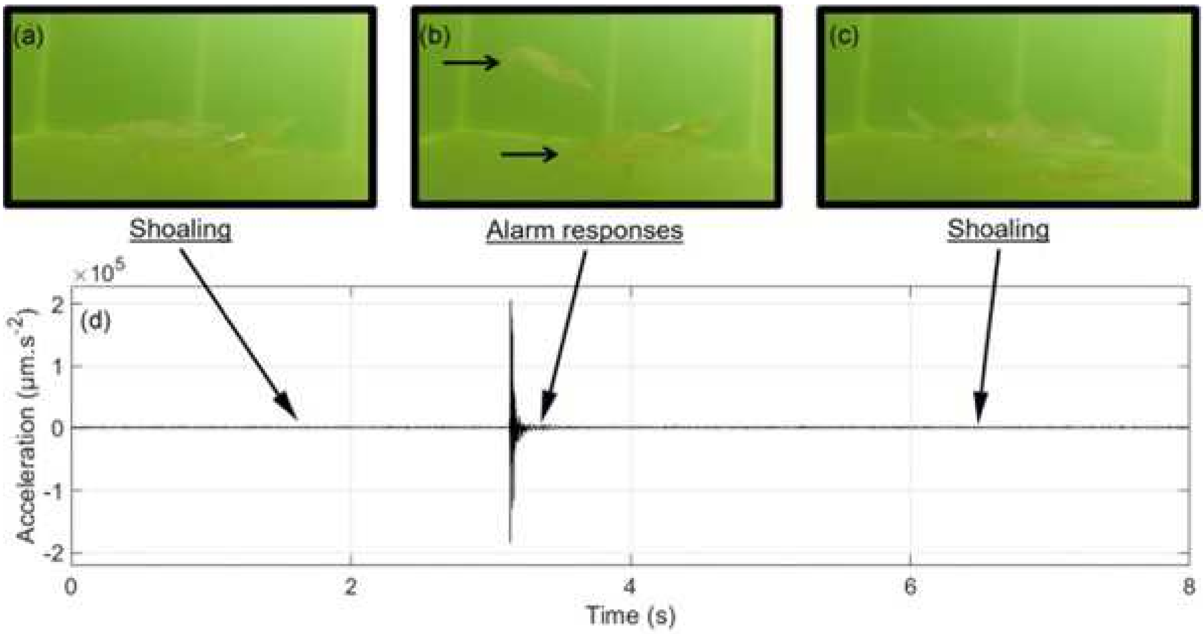

Figure 31. Examples of squid alarm responses observed before (a), during (b), and after (c) exposure to the first strike of an IH sequence (d). Arrows indicate the time when the captions occurred during sound recordings in (d).

Among the five squid present in the shoal, two individuals reacted by jetting and startling in response to the strike (b, horizontal black arrows), yet quickly returned to the shoaling behaviour (c).

Table 6. Summary of individual alarm responses annotated during pile driving sound exposure at the near (n = 103) and far (n = 56) sites and during control (n = 30).

Note that squid exhibited alarm response only at the near site.

Behaviour	**Near site**		**Far site**		**Control (No sound)**	
	IH	VH	IH	VH	IH	VH
Startle	57	20	0	0	0	0
Body pattern change	34	14	0	0	0	0
Jet	176	0	0	0	0	0
Ink	4	0	0	0	0	0

During IH sequences at the near site, 80% of alarm responses were seen during the first two IH sequences of the day and squid showed significantly more responses to the first IH sequence compared to subsequent IH sequences (LMM: X2 = 764, df = 2095, $p < 0.001$; Figure 32). In addition, 84% of alarm responses were seen within the first five strikes, which corresponded to the first 30 s of each IH sequence (Figure 32). Hammer strike number was a significant predictor of the alarm response rate over the first 30 strikes for all IH sequences (Poisson GLM, $p < 0.001$; Figure 34). There were also individual variations in alarm responses among squid shoals, with <40% of individual squid within a shoal reacting to PD sound (Figures 31 and 32). Finally, for two squid shoals, no alarm responses were exhibited by any squid over the entire PD sound exposure experiment at the near site.

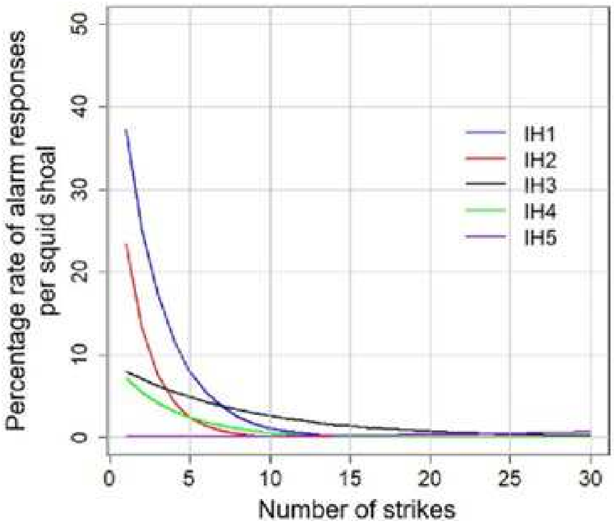

Figure 182. Proportion of alarm responses (percentage) across daily consecutive IH sequences (from one to five) for squid located at the near site within 8 m from the pile driving (n = 103). IH1 to IH5 represent the five sequences within a single day of pile driving exposure.

Lines represent Poisson GLMs performed on the first 30 strikes of each IH sequence.

4.2.3.2 Shoaling behaviour

We studied the area change of squid shoals during PD sound exposure at the near site and during the first strikes of each PD sequences (i.e. where most individual alarm responses occurred). Our computer vision-based model successfully computed the areas of six different squid shoals. While alarm responses occurred in response to IH strikes, squid quickly (within few seconds) returned to the shoal (Figure 31). This was further confirmed by comparing shoal areas at different time scales prior to sound exposure, after the first IH strike (5 s) and after 10 IH strikes (60 s). Indeed, despite these short behavioural disruptions, the squid shoal areas were not significantly affected by PD sound after the first IH strike (one-way ANOVA, p = 0.299) nor after the last 10 IH strikes of a sequence (one-way ANOVA, p = 0.345; Figure 33).

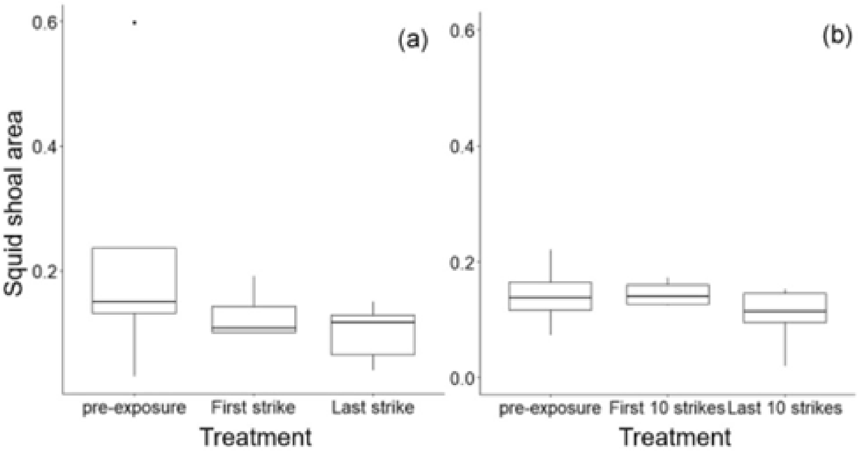

Figure 19. Areas computed on six squid shoals exposed to PD sound at the near site.

Two different temporal resolutions are shown: during the first strike and the last strike (i.e. 5 s scale; **a)** and the first 10 first strikes and last 10 strikes (i.e. 60 s scale; **b)** of IH sequences. No significant differences were found in both treatments.

4.2.4 Discussion

This field-based study is the first to quantify both shoaling behaviour and individual alarm responses within squid shoal exposed to actual PD. Behavioural changes were transient and occurred mostly at the onset (<1 min) of the PD sound exposure, and response rates decreased after consecutive PD sequences. Despite these short-term responses, PD sound did not disrupt overall squid shoaling behaviour. Given that *D. pealeii* do not experience hearing threshold shifts at these sound levels (Jezequel & Mooney, in review), our results suggest potential quick habituation of wild squid to PD sound during OSW constructions.

The alarm responses observed during PD sound exposure were typical squid behaviours to perceived predatory threats (Figure 31; Hanlon and Messenger, 2018). These results are consistent with previous tank studies on the same species as well as cuttlefish in response to both artificial and PD sounds (Samson et al., 2014; Mooney et al., 2016; Jones et al., 2020). This striking similarity highlights the valuable asset of sound exposure experiments in tanks when the sound field is accurately calibrated (Popper and Hawkins, 2018). Using the same field set-up, our previous study used movement sensors to demonstrate that while alarm responses were mostly high acceleration jetting responses, these behavioural changes were transient and persisted for <15 s (Cones et al., 2022). Additionally, there was no evidence of activity changes on longer time scales, suggesting minimal effects on squid energetics (Cones et al., 2022). However, our results are different from another study where PD exposure repeatedly affected scallop behaviours (Jézéquel et al., 2022), showing the importance of studying anthropogenic sound impacts across different taxa.

The logarithmic decrease in alarm responses within and across PD events is similar to previous tank studies that reported rapid habituation of squid and cuttlefish to sound (Figure 34; Samson et al., 2014; Mooney et al., 2016; Jones et al., 2020). This reduction in alarm responses over several sound impulses indicates increased tolerance over time to PD sound, and suggests these squid may have behaviourally habituated. Interestingly, habituation to repeated sound exposures has also been noted in some fish (e.g. Nedelec et al., 2016; Neo et al., 2018; Currie et al., 2020). However, using the same experimental set-up as in this study, Jézéquel et al. (2022) found that scallops did not habituate to repeated PD exposure, suggesting inter-specific differences for marine invertebrates in response to PD activity. Behavioural habituation can be defined as a learned, persistent reduction of an individual's response to a stimulus repeated over time, as individuals learn the stimulus has neither adverse nor beneficial consequences (Bejder et al., 2009). Another hypothesis to the decrease of alarm responses over time could be the occurrence of temporary thresholds shifts due to sound exposure in squid. Some studies highlighted that artificial sound can induce anatomical damages on statocysts (André et al., 2011), the sensory organ responsible for sound detection in squid (Mooney et al., 2010). While we did not analyze potential squid statocyst damages after sound exposure, acoustic trauma could have been responsible for the decrease of squid sensitivity to sound.

We found that only a portion of squid in each shoal responded to PD sound, with overall <40% of squid showing alarm responses (Figures 29 and 30). These results suggest inter-individual differences in squid sensitivity, which has been highlighted in fish (Harding et al., 2019). This result is vastly different from previous tank studies where >90% of squid showed alarm responses to PD sound (Jones et al., 2020). This difference can be attributed to the fact that the previous studies tested solitary squid, which may be more sensitive to predator cues than squid in a shoal (Hanlon and Messenger, 2018). This also highlights the importance of studying anthropogenic sound impacts on shoals and realistic groupings, rather than individuals for social species (Popper et al., 2022). Another hypothesis is that sound exposure levels were not high enough to induce alarm responses in all squid. Our measured sound levels corresponded to roughly 1 km from actual windfarm constructions (Sigray et al., 2022). Hence, it is possible that more squid would react at closer ranges where sound levels are much higher.

Despite the occurrence of short-term alarm responses, PD exposure did not disrupt squid shoaling areas (Figures 31 and 33). This result is consistent with the findings of Ginnaw et al. (2020) where fish shoal collective motion was not affected by pure low-frequency tones in tanks, but differs from Herbert-Read et al. (2017) who found that PD sound exposure (5 min duration) in tanks disrupts collective dynamics of fish shoals. First, it is possible that the cages used in our study, while relatively large and comparable to other studies (e.g. Dahl et al., 2020), could have influenced squid shoal movements, preventing any horizontal dispersion. Despite these physical constraints, squid exhibited normal shoaling behaviours without any collisions with the cage netting. For example, wild shoals of fish can disperse and even change depth when exposed to PD sound (Hawkins et al., 2014). Second, shoaling is a vital behaviour for squid to reduce predatory threats and for reproduction, thus there is a likely high motivation for individuals to return quickly to shoaling after behavioural disruption (Figure 31; Hanlon and Messenger, 2018). While our study was performed using four to seven squid per shoal, wild *Doryteuthis spp*. shoals can be composed of 100 s of individuals (Shashar and Hanlon, 2013), and dynamics could be different for larger groups. Thus, while our data reflect behavioural responses and interactions within small (but more manageable) shoals, there is still a need to address how PD sound may influence larger, wild mating squid. Finally, while the PD sound levels at our near site were roughly equivalent to 1 km from OSW constructions (Sigray et al., 2022), they might not have been high enough to induce any shoal disruption (Cones et al., 2022). Further studies should assess potential horizontal dispersion of wild squid shoals in the vicinity of OSW constructions, which have much higher intensity PD activity (Sigray et al., 2022).

The present study focused on behavioural responses of both squid individuals and shoals to repeated PD sound exposure. Here, we did not study dose-dependent responses that could have occurred in individuals located at the near site where PAL 0p varied between 112 and 123 dB re 1 μm s−2 , depending upon the distance pile-cages (Cones et al., 2022). Dose-dependence behavioural responses were previously described in *D. pealeii* to pure tones in tanks (Mooney et al., 2016), as well as in S. australis exposed to seismic air gun sound (Fewtrell and McCauley, 2012). Squid elicited a higher proportion of alarm behaviours with increasing sound levels, implying the severity of sound impact on squid is related to the distance from the source. Considering the higher sound levels and propagation distances arising from OSW constructions (Sigray et al., 2022), further studies are now needed to assess dose-dependent responses, as well as minimum acoustic thresholds that induce alarm behaviours in *D. pealeii* in the marine environment.

4.2.5 Conclusion

To conclude, our results highlight two main potential mitigation procedures that could be used by OSW developers to reduce behavioural impacts on squid. First, far fewer behavioural reactions were found in squid exposed to the VH continuous and low-amplitude signals, which is consistent with our previous study in scallops (Jézéquel et al., 2022). This suggests that the VH can be used as a mitigation technique to drastically reduce behavioural impacts on squid during OSW constructions. Second, the fact that squid mostly responded at the onset of the IH events highlights that the first IH blows have the most impacts on squid. Here, ramp-up could also be used as a mitigation technique. This soft-start procedure with slowly increasing sound levels could provide animals with a warning signal before sound exposure levels rise to the full power (Robinson et al., 2007; Bailey et al., 2014). The gradual increase in sound level of the ramp-up procedure may allow the squid to habituate to the sound exposure faster thus reduce the occurrences (and consequent energetic costs) of avoidance behaviour.

4.3 Study 5: Pile Driving Sound Does Not Induce Hearing Loss in the Longfin Squid[3]

4.3.1 Background

International interest in renewable energy production is rapidly growing due to climate change concerns. Offshore wind is a key renewable resource. Pile driving (PD) activity during the installation of offshore windfarms (OSW) generates repeated, high sound levels that can propagate over kilometers underwater (Amaral et al. 2020, Sigray et al. 2022). This major pollutant underscores clear concerns for wide-ranging impacts on sound-sensitive animals, including marine invertebrates (Mooney et al., 2020). However, examining impacts to invertebrate taxa has received relatively little attention and research despite their ecological and economic importance (Williams et al. 2015).

Cephalopods are sensitive to the particle motion component of acoustic signals in the same low-frequency bandwidth as PD sound (Packard et al. 1990, Mooney et al. 2010a). Studies in tanks initially revealed that repeated PD sound playbacks elicit short term alarm behavioral responses in longfin squid (*Doryteuthis pealeii*), but these responses attenuated over time (Jones et al. 2020). Field-based experiments demonstrated comparable results; where the rate of individual alarm responses quickly (< 1 min) decreased both within and across repeated actual PD sound sequences (Cones et al. 2022, Jézéquel et al. 2023). This reduction in behavioral responses over repeated sound exposures may indicate increased tolerance over time, suggesting squid may have behaviorally habituated to this acoustic stimulus. However, high sound level exposure could also induce temporary threshold shifts (TTS), reducing squid sound sensitivity by an increase in auditory thresholds.

Temporary threshold shifts (TTS) following sound exposure have been measured across a variety of taxa, including marine mammals and fishes (Nachtigall et al. 2003, Popper et al. 2005), but such phenomena remain poorly studied in marine invertebrates. The one study that has examined TTS in this broad taxonomic group demonstrated TTS in three age classes of the hummingbird bobtail squid (*Euprymna berryi*). Animals were exposed to 15-min continuous duration vessel sound playbacks in tanks, with sound sensitivity recovering within two hours (Putland et al. 2023). Recovery in sound sensitivity may occur after sensory cells regeneration following sound exposure as shown in fish (Smith et al. 2006). An explanation for TTS following sound exposure in cephalopods could be related to anatomical and physiological damages on statocysts (André et al. 2011), the sensory organ responsible for sound detection in squid (Mooney et al. 2010a). It is important to note that these aforementioned studies have been performed in tanks, rending the translation of these laboratory results into the field and actual sound source exposure difficult. This is especially true when considering the different sound levels and the varying spatial and temporal scales of potential impacts by anthropogenic activities (Popper et al. 2022).

Future OSW construction has been planned in nine U.S. states (Musial et al., 2019), encompassing the habitat of many marine invertebrates including longfin squid (Hanlon et al., 2013). The occurrence rate and spatial range of PD exposure events are expected to affect this taxon and its vital habitat. Considering the economic importance of squid, which have

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[3] Jézéquel Y Mooney TA. 2024. Impulsive pile driving sound does not induce hearing loss in the longfin squid (*Doryteuthis pealeii*). J Acoust Soc Am. Oct 1;156(4):2200-2210. doi: 10.1121/10.0030404. This work was funded by the Bureau of Ocean Energy Management Cooperative Agreement #M20AC10009.

contributed mean annual landings and value of 13000 mt and ca. $26 million per year since 2000 (NMFS, 2019), conflicts between fishermen, policymakers, and the offshore wind industry are expected to increase (Lacroix and Pioch, 2011). New studies are now needed to support the development of effective management strategies and to design suitable mitigation methods.

In this context, we quantified potential TTS in longfin squid exposed to repeated, real-time PD sound following the same field-based design used in our previous studies (Cones et al. 2022, Jézéquel et al. 2023). Hearing thresholds were measured using auditory evoked potentials (AEPs), a technique that was previously applied to measure the audiogram of the studied species (Mooney et al. 2010a). Given that TTS studies are new for invertebrates, we sought to compare thresholds from PD exposed squid to both squid and goldfish exposed to continuous white sound. Overall, the study aimed to address the following questions: 1) Does PD sound induce TTS in the longfin squid? 2) Does PD sound duration increase potential TTS? 3) Finally, is (potential) TTS affected by the duration after PD sound exposure?

4.3.2 Materials and methods

4.3.2.1 Animal collection and holding conditions

A total of 89 adult squid (dorsal mantle length = 15.1 ± 1.9 cm, mean ± standard deviation) were used for this study. Squid were collected from Vineyard Sound (41.22 N, 70.47 W) via trawling by the R/V Gemma of the Marine Biological Laboratory (Woods Hole, MA) using a long-established, taxon-specific, soft net and catch process. Squid were initially maintained in groups of 4-7 individuals in cylindrical outdoor tanks (1.2 m diameter) constantly supplied with ambient seawater (temperature range: 18 to 22 °C) for 1-3 days before the start of the experiment. Tanks rested on rubber gaskets and concrete blocks, both of which served to further isolate the squid from surrounding vibrations during holding. The top of each tank was covered with plastic sheeting to create shaded zones. Squid were fed daily with mummichogs (*Fundulus heterclitus*) and grass shrimp (*Palaemonetes* spp.). Only visibly healthy squid exhibiting normal patterning and swimming, and with no discernable skin damage were tested. To ensure we only used the healthiest animals, animals were only held no longer than 72 h before the start of an experiment. The study was carried out in accordance with the principles of the Basel Declaration and recommendations and approval of the WHOI Institutional Animal Care and Use Committee scientific protocol to TAM.

4.3.2.2 Pile driving set up

Pile driving was conducted for 11 days between September 20th and October 12th, 2022, off a dock at the Woods Hole Oceanographic Institution (Woods Hole, Massachusetts, US; figure 34A). A cylindrical steel pile (length: 10 m, diameter: 0.3 m, wall thickness: 0.02 m) was used as the impact pile and hammering methods were identical to previous experiments (figure 34B; see Cones et al. 2022, Jézéquel et al. 2023 for additional details). Exposures began as (1) a steel impact hammer (weight: 1500 kg) was dropped onto the pile from a height of 1.2 m at a rate of 10 strikes/min for 15 minutes, which resulted in the steel pile penetrating a minimum of 3 m into the seabed. (2) Next, a vibratory hammer (weight: 212 kg, H&M model 135, 1150 blows per minute) was used to pull the pile out of the substrate and to reposition it in an adjacent location for another round of the impact hammer pile driving. This process was repeated five times per experiment day, with pile driving activity commencing at 13:30 and lasting for ~3 h (Figure 34C).

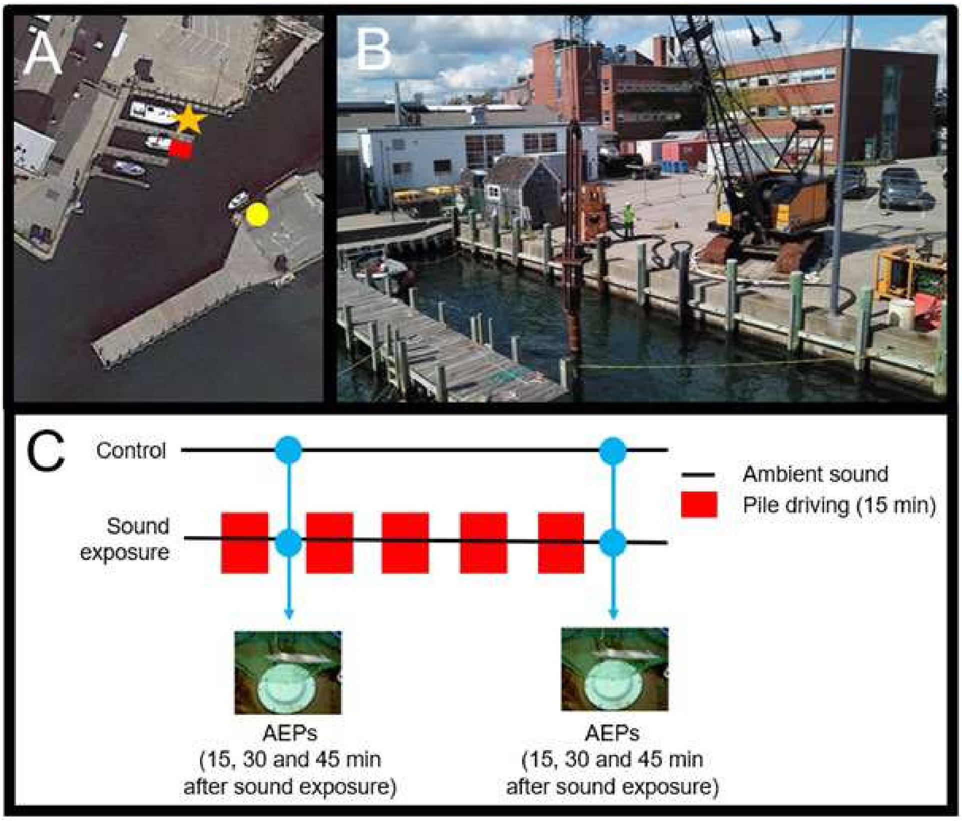

Figure 20. Experimental set-up used to investigate the potential temporary threshold shifts of squid to repeated pile driving sound exposure.

A: Map of the pile driving site. The orange star denotes the pile driving location, the red rectangle shows the position of the squid cage and the yellow circle the shed inside which were measured squid auditory evoked potentials after pile driving sound exposure. B: Picture of the crane driving a pile into the seabed off a dock with the impact hammer. The squid cage was placed on the other side of the dock, within 10 m from the pile. C: Experimental procedure during pile driving exposure. Groups of three to four squid were used after each sound treatment and AEPs were obtained at three different time scales. Sound treatments corresponded to 15-min long impulsive pile driving.

Prior to the first impact hammer sound exposure, groups of 6 to 8 squid were quickly transferred from holding tanks to one submerged 3.4 m^3 cubic cages (1.5 × 1.5 × 1.5 m) built with polyvinyl chloride pipes and covered with 1.5 cm knotless polyester mesh netting. A door on the top permitted the squid transfer into the undersea cages; once all squid were introduced, the cage was lowered to 0.5 m from the seabed. Squid were allowed 15 min of acclimatization in the cages to recover from handling before the first PD sound exposure started.

Because our previous studies showed squid were only affected at short distances (see Cones et al. 2022, Jézéquel et al. 2023), the cage was placed within 10 m from the same pile driving set-up. The pile could not be driven in the same exact location every time because each PD effort and subsequent extraction left a 0.3 m diameter hole in the seabed, thus there was a small difference in PD distances (mean = 6.1 m ± 1.1, min = 4.3 m, max = 9.1 m) to the experiment animals. The received level (RL) variations across this distance were minimal (ca. 9 dB re 1 $\mu m.s^{-2}$) and quantifiable (more information in the 'Sound exposure' section below), and this variation includes the fact that RLs also vary with pile strike. Such variation, while potentially different from highly controlled laboratory studies, is to be expected for field-based sound exposure studies.

Control experiments (n = 17 squid) were conducted at the same site using the same experimental procedures, but without PD sound exposure. To compare metrics between the two experiment types, sound exposure time periods from experiment days were randomly assigned to control experiments.

4.3.2.3 Sound exposure

Given squid detect low frequency (< 1 kHz) underwater particle motion (Mooney et al. 2010a), the sound field was recorded in presence and absence of pile driving using a calibrated PCB triaxial accelerometer (model W356B11 sensitivity: x = 10.31 mV m s^{-2}, y = 10.56 mV m s^{-2}, z = 10.58 mV m s^{-2}) at a sampling frequency of 2 kHz, with the same setup and methods as in Cones et al. (2022). Recordings were done with the accelerometer suspended in the water column (1 m below the water surface) and placed between the pile (2.0 - 3.6 m) and the experimental cage (1.6 - 3.6 m). We focused our analyses on impact hammer PD only since squid showed minor disturbances to vibratory hammer signals (Jézéquel et al. 2023).

We calculated the rise time (in ms) of each hammer strike by measuring the duration (in ms) from 5% of total energy to the peak acceleration of the signal (ISO standards 2017). The intensity was assessed by computing 0-peak accelerations (PAL_{zpk}; in dB re 1 µm s^{-2}). Next single strike sound exposure levels (SEL_{ss}; in dB re (1 µm $s^{-2})^2$.s) were calculated by integrating PAL_{zpk} over the pulse length containing 90% of the signal energy, and cumulative sound exposure levels (SEL_{cum} ; in dB re (1 µm $s^{-2})^2$.s) by taking into account the number of impulses with and across pile driving sequences. Note that pulse amplitudes and inter-pulse time intervals did vary slightly within and across PD sound sequences as the pile was driven into the sediment (see Jézéquel et al. 2023 for details), an occurrence that also occurs in OSW constructions. Thus, the variability in these metrics across impulses reflected that which a wild squid may experience near an offshore pile driving site (Amaral et al. 2020).

4.3.2.4 AEP recordings

We assessed whether TTS in squid occurred after one PD sound exposure (15 min) and after 5 PD sound exposures (~3 h) (figure 34C) using the same materials and methods as in Mooney et al. (2010a). After each treatment, three to four squid were randomly collected from the experimental cage and immediately transferred to a 10-litter plastic bin (0.30 x 0.18 x 0.12 m) filled with fresh ambient seawater (~19°C). The bin was covered to reduce stress for the animal. Squid were then individually sedated prior to each AEP recording in a bath of $MgCl_2$ solution (0.15 mol.l^{-1}) following Mooney et al. (2010b). $MgCl_2$ sedation does not have an effect on cephalopod evoked responses (Mooney et al., 2010b). An animal was considered anaesthetized when turning it upside down would not exhibit any resistance, but breathing through siphon was still clearly visible. This procedure lasted 5 to 10 min (depending on size of the individual).

Anesthetized squid were moved from the $MgCl_2$ bath to a rectangular fiber-glass tank (0.80 x 0.50 x 0.50 m, 0.16 m^3) for AEP measurements (see figure 35 for details). The tank rested inside a larger plywood box lined with acoustically dampening open-cell foam which served to reduce sound and dampen vibrations from the surroundings. This box sat on two rubber gaskets and concrete blocks that further isolated the tank from the substrate. The tank was previously filled with same ambient seawater as the sedation bath. The squid rested below the surface (~5 cm depth) on acoustically transparent plastic mesh. An underwater speaker (UW-30; Lubell Labs Inc., Columbus, OH, USA) rested on the bottom of the tank on rubber gaskets. The speaker was located directly below the squid eye region and its statocyst organs (35 cm depth). The squid was placed dorsal side up (ventral side towards the speaker) in the tank. The tank was grounded using a wire connected to the outgoing seawater flow of the experimental site.

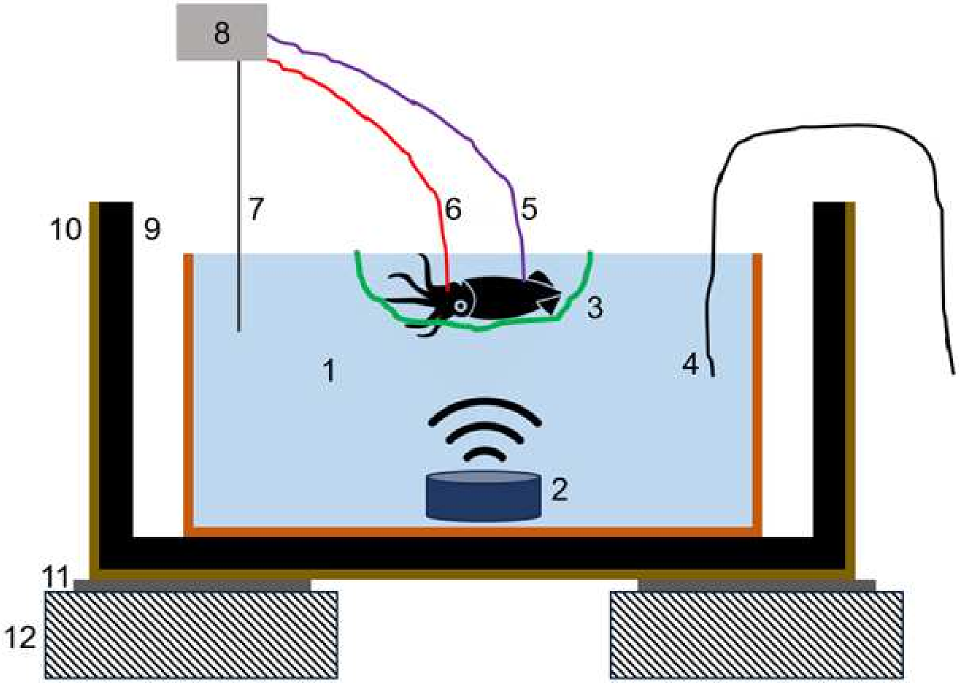

Figure 21. Schematic of experimental tank set-up during squid AEP measurements (not to scale).

1: rectangular plastic tank; 2: Underwater speaker (Lubell Labs UW-30); 3: acoustically transparent mesh sling with animal suspended 5 cm below water surface; 4: ground wire; 5: reference electrode; 6: recording electrode; 7: ground electrode; 8: filters connected to a laptop; 9-10: larger plywood box lined with acoustically dampening open-cell foam; 11: rubber gaskets; 12: concrete blocks; for further details see the text.

The recording electrode (diameter, 27 ga.; length, 13 mm; Rochester subdermal needle electrode, LifeSync Neuro, New York was inserted medially, from the squid's dorsal side, posterior to the eye but on the anterior side of the statocyst and into the surrounding cartilage. This was the standard location of all recordings. A reference electrode was inserted into the muscle of the squid body, 5–10 cm from the head. A ground electrode was suspended in the water column of the tank. Both electrodes were modified by coating the entire stainless-steel portion, except the very tip (0.5–1 mm), with a thin layer of Por-15 (Morristown, NJ, USA), which reduced extraneous electrical sound. The connection of the stainless steel to the electrode cable was also lightly coated in epoxy resin to prevent saltwater from penetrating the connection. The electrodes were connected to a battery-powered Grass CP-511 biological amplifier and filter (Astro-Med Inc., West Warwick, RI, USA) that amplified the signal with a gain of 40 dB and bandpass-filtered responses from 100 to 3000 Hz. The received signal was then transferred to a BNC connector box and converted from analog to digital via a National Instruments 6062E data acquisition card (DAQ; Austin, Texas) and custom AEP program (using National Instruments LabView software) on a laptop computer. The AEP data were sampled at 16 kHz, with a modulation rate of 1 kHz. A total of 250 sweeps were collected and averaged for each record over a 100 ms window. These alternating stimuli were presented at 0 and 180 deg phases to remove any stimulus artefacts. The same laptop, custom program and data acquisition card were used to generate acoustic stimuli and collect AEP responses for each tested animal.

Auditory evoked potential responses were recorded using an acoustic stimulus of 125 Hz. This frequency was chosen as it was in the best frequency hearing range (i.e., lowest thresholds) reported in this species (Mooney et al. 2020a). The acoustic stimulus was presented at a rate of 8 s^{-1} with a start SPL_{rms} of 153.9 dB re 1 µPa. The SPLs were then gradually decreased with the attenuator, and the corresponding AEP responses were visually monitored. The SPLs were first decreased in 5- or 10-dB increments depending on the amplitude of the AEP response, and in 5 dB increments when close to the thresholds until the stereotypical AEP response was no longer detectable. Then, one to two additional recordings at 5-10 dB below the visually determined thresholds were made to ensure that low amplitude responses were not missed. The ambient sound level in the experimental tank was typically below that of the acoustic stimuli used during AEP experiments (~50–80 dB re. 1 $\mu Pa^2\ Hz^{-1}$ in the frequency range from 50 to 1000 Hz; see Figure 36). We chose to characterize sound levels in pressure units instead of particle acceleration because the aim here was to not quantify absolute thresholds, but rather shifts in auditory thresholds between squid exposed to ambient vs. PD sound. Indeed, as empirically illustrated by Olivier et al (2023), when sound level increases or decreases by N dB in a tank, both pressure and particle acceleration will increase or decrease by N dB.

The theoretical minimum resonant frequency of the experimental tank was 2.6 kHz (Akamatsu et al., 2002), which was far above the frequency of the acoustic stimulus used during AEP experiments (125 Hz). Hence, the spectral shape of the acoustic stimulus was not distorted (figure 38). However, this low frequency was highly attenuated because its wavelength was larger than the tank size (e.g. a 100 Hz sound as a ~15 m wavelength; Rogers et al., 2016). In this context, after the acoustic calibration, we carefully positioned each squid at the same location and distance (35 cm) from the speaker for each animal's threshold measurements to enable comparisons between individuals.

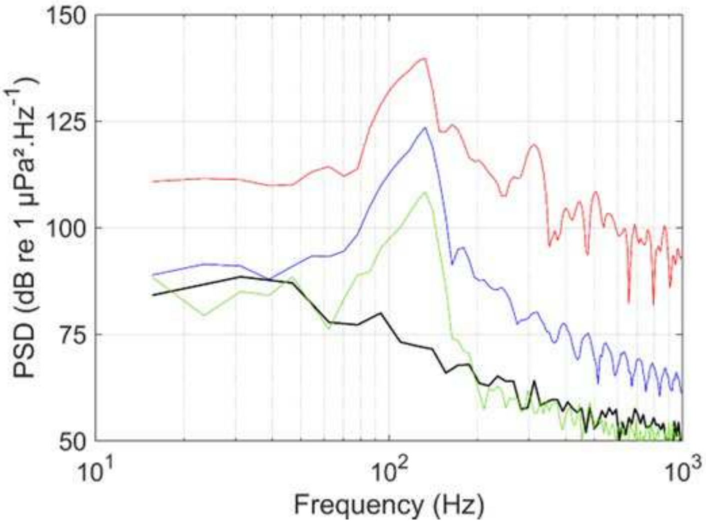

Figure 22. Power spectral densities (PSD) of the ambient sound recorded in the tank (black), and the amplitude modulate tone-pip at 125 Hz (duration = 30 ms) used for AEP experiments at different sound levels (SPL_{rms}, in dB re 1 µPa).

Red = 153.9, blue = 131.9, green = 122.1. Note that all PSDs from acoustic stimuli were above the ambient sound level in the 50-1000 Hz frequency band.

Thresholds were measured from different animals at 15, 30 and 45 min after either one or five PD sequences (Table 7). These post-exposure durations corresponded to the halfway time necessary to measure thresholds in each individual squid. The animals were released into the waters from which they came from after the experiments. Each animal was used only once during the study. After each AEP experiment, the tank was drained completely, thoroughly rinsed and refilled with fresh seawater for the next experiment.

Table 7. Summary of sample sizes used for measuring auditory thresholds at 125 Hz in squid exposed to pile driving sound.

Evoked responses were obtained in squid exposed to one 15-min pile driving sequence, and after a whole afternoon of pile driving sound exposure (~3h). For each treatment, different squid individuals were measured 15, 30 and 45 min after sound exposure.

Treatment	15-min	30-min	45-min
Control	5	7	5
After one PD sequence	11	9	9
After five PD sequences	14	13	9

4.3.2.5 Control experiments

Several sets of additional exposure experiments were conducted to serve as comparison controls. First, we recorded AEP responses from 7 squid exposed to continuous 3 h long white sound in the same experimental tank used for AEP recordings using the maximum sound level of the underwater speaker, corresponding to $\mathrm{SEL_{cum}}$ of 190.3 dB re 1 µPa^2.s and 151.7 dB re (1 µm s^{-2})^2.s for sound pressure and particle acceleration, respectively. This dataset helped us to validate the potential auditory threshold shifts observed in squid exposed to pile driving *in situ*, and compare our results with a previous study showing TTS in bobtail squid following 15 min of continuous boat sound exposure (Putland et al. 2023). Evoked potential responses were obtained using the same materials and methods as described above.

We also measured AEP responses from four goldfish (*Carassius auratus*) exposed to the same white sound following the methods described in Stanley et al. (2020). This dataset was used as a model control and basis for comparison of our squid data because goldfish have been widely studied in the bioacoustic literature and have been shown to present TTS after white sound exposure (e.g., Smith et al. 2004a, 2004b, 2006). Prior to AEP recordings, the experimental tank was filled with freshwater (25°C). Fish were initially anesthetized with a dilute solution of 100% clove oil (0.1–0.5 ml l^{-1}, dependent on fish size) before the recordings to permit placement in the fish holder and to reduce large movement during experiments. Previous studies showed no effects on auditory thresholds and enabled identification of the lowest concentration possible (Stanley et al. 2020). The anesthetized fish were positioned laterally upon the holding net. The operculum was left free to allow respiration to occur normally. No muscle relaxants were used for these experiments. The fish were placed ~10 cm below the water surface and 35 cm from the speaker. The responses of each fish were recorded using the same equipment as for squid. The recording electrode was placed dorsally, just posterior to the operculum, the reference electrode was placed dorsally in the nasal region. Fish were periodically checked during experiments, mostly by means of operculum and mouth movement. All *C. auratus* fully recovered from these procedures.

Several additional controls were done to ensure recorded squid AEP responses were of neuronal origin and not an artifact of our set up, including AEP recordings without animals (i.e., electrodes were suspended in the tank water column), using deceased animals and recording from various body locations (e.g., arm, mantle) distant from the animals' statocyst (following Mooney et al. 2010a). None of these recordings showed the typical AEP responses notable from live animals with recording electrode placed adjacent to the statocysts. In addition, AEP responses showed different shapes between squid and goldfish (see Results).

4.3.2.6 Data analysis

4.3.2.6.1 Threshold determination

We assessed auditory thresholds using fast Fourier transform power spectrum analysis (FFT; Hamming window: 640 points for squid, 1600 points for goldfish) of the averaged waveforms using custom-written MATLAB scripts. As with fish and squid AEPs, the FFT spectra revealed peaks at approximately twice the stimulus frequency (Egner and Mann, 2005; Mooney et al., 2010a). The amplitudes of the FFT peaks also decreased with sound levels. These values were then plotted relative to the corresponding attenuation levels and a linear regression was calculated using this dataset. The points with the highest r^2 value were used to calculate the regression (Mooney et al., 2010a). The point at which the linear regression crossed the y-axis corresponded to the theoretical attenuation level at which no AEP response would occur and coincided with the threshold at a given frequency (Nachtigall et al., 2007).

4.3.2.6.2 Statistical analysis

Differences in sound detection thresholds across the different PD treatments were assessed using linear mixed models (LMM) using the “lmer” function in the “lme4” package available in R v3.6.3 (http://www.R-project.org/). Treatment (control, after 1 PD sequence, 5 PD sequences) and time after PD sound exposure (15, 30 and 45 min) were included as categorical explanatory variables, while the effects of individuals and dates were modelled as random effects on the intercept. Finally, we tested whether 3-h of white sound exposure in the experimental tank significantly induced TTS in squid and goldfish using non-parametric Mann-Whitney (MW) tests.

4.3.3 Results

4.3.3.1 Acoustic field

The impact hammer pile driving generated clear signals above background sound levels which allowed for isolation and analysis of all sound sequences (Figure 37). Power spectral densities for pile driving sound were ~30 dB higher compared to ambient sound over the squid hearing range (< 1 kHz). A full summary of acoustic data is shown in Table 8.

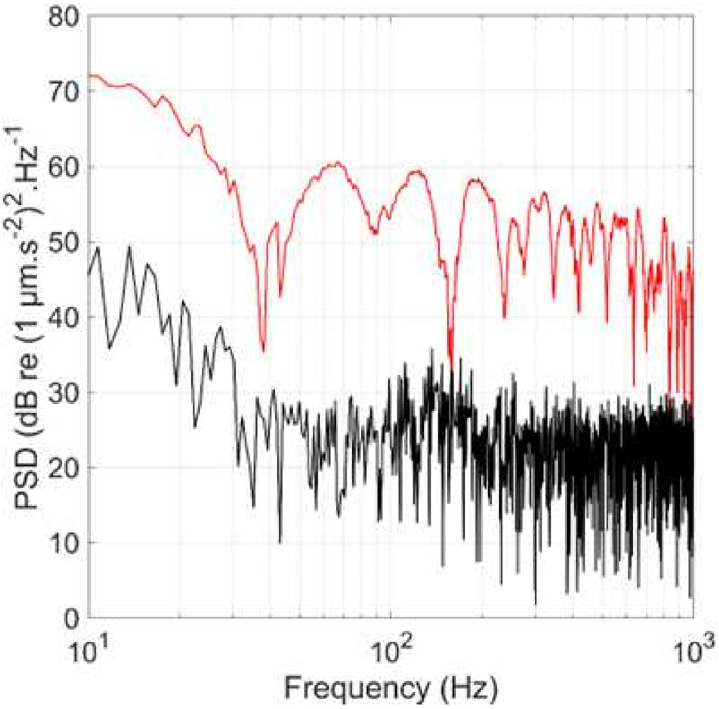

Figure 237. Power spectral densities (PSD) of the ambient (black) and 15-min pile driving sounds recorded by the accelerometer during a field-exposure experiment.

Table 8. Received sound levels measured during one entire pile driving experiment.

Rise Time (ms)	**PAL$_{zpk}$ dB re 1 (µ ms^{-2})**	**SSE dB re 1 (µ ms^{-2})$^{-2}$.s**	**SEL$_{cum}$ / IH seq dB re 1 (µ ms^{-2})$^{-2}$.s**	**SEL$_{cum}$ / day dB re 1 (µ ms^{-2})$^{-2}$.s**
18.0 ± 15.9	122.6 ± 5.1	83.2 ± 4.2	103.6 ± 3.9	110.5

Rise time is the time, in ms, for pile driving impulses to increase from 5% to peak amplitude. Sound exposure levels were measured for one hammer strike (SSE), one pile driving sequence (SELcum, ~110 strikes), and five consecutive pile driving sequences (~550 strikes).

4.3.3.2 Auditory thresholds after PD sound exposure

The results showed no effects of PD sound exposure on auditory thresholds in squid after one or five PD sequences (LMM: $F_{2, 18.01} = 1.21$, $p = 0.32$) (Figure 38). In addition, the time after PD sound exposure (15, 30 and 45 min) did not have any effect on auditory thresholds (LMM: $F_{2, 53.49} = 2.49$, $p = 0.09$). Finally, there was no significance from the combined effects on auditory thresholds (LMM: $F_{4, 51.56} = 0.51$, $p = 0.73$). Overall, our results highlight that PD sound exposure did not induce any TTS in squid exposed over two different time scales.

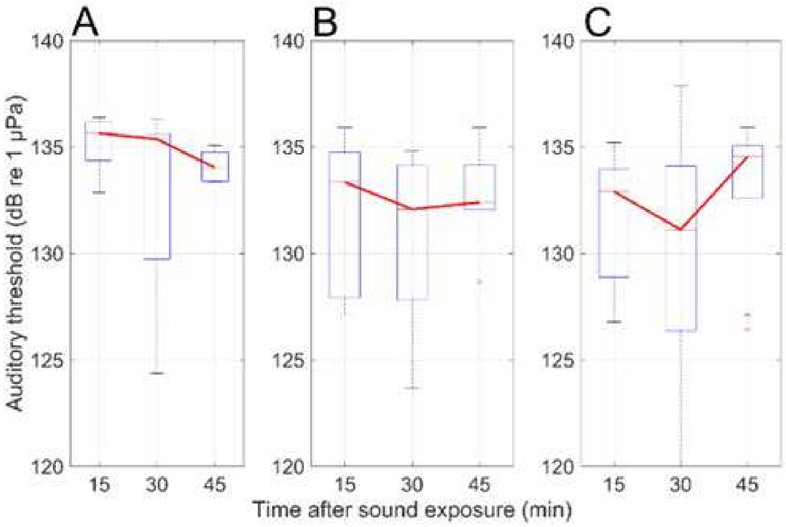

Figure 38. Auditory thresholds measured in squid in absence of PD sound.

(A), after one PD sound sequence (B) and after five PD sound sequences (C). AEP responses were measured at 125 Hz. No significant differences were found among the different treatments. See Table 1 for sample sizes.

4.3.3.3 Auditory thresholds after white sound exposure

White sound exposure in tank did not induce TTS in squid (n = 7; MW, p = 0.16) (figure 37). However, goldfish auditory thresholds significantly increased by 7 dB after white sound exposure (n = 4; MW, p < 0.05), showing the presence of TTS in this species (figure 41).

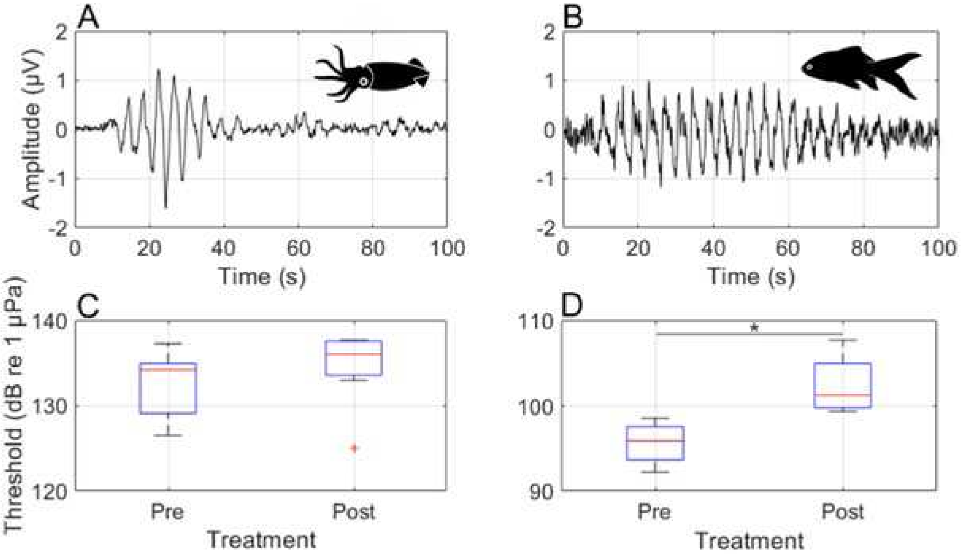

Figure 39. Auditory evoked potential responses from squid (A) and goldfish (B) exposed to 125 Hz pip-tone at 153.9 dB re 1 µPa.

While white sound exposure did not induce any TTS in squid (C), post-exposed goldfish showed a significant increase in auditory thresholds of 7 dB (D).

4.3.4 Discussion

To the authors' knowledge, this is the first field-based study quantifying physiological effects on sound sensitivity in a marine invertebrate following man-made sound exposure. We demonstrated repeated PD sound over different time scales did not induce temporary threshold shifts in squid. These results support previous studies suggesting the decrease in behavioral responses to repeated PD sound exposures are likely related to short-term habituation in this species, and not an increase in auditory thresholds related to anatomical damages on hearing organs.

One study found TTS in bobtail squid exposed to 15-min long vessel sound playbacks in a laboratory tank, and recovery occurred within 2 h post-exposure (Putland et al. 2023). Here, we did not find evidence of TTS from longfin squid exposed to either one or five repeated 15-min long PD sound sequences (figure 38), nor after 3 h of white sound exposure in tank (figure 39A). One explanation could be related to species-specific differences where bobtail squid may be more prone to sound induced hearing loss. The bobtail and longfin squid have very different ecological niches: the bobtail squid is a burrowing species inhabiting reef environments whereas the longfin squid is a relatively robust, schooling, pelagic animal. However, such comparisons of sound sensitivity are challenging given that we know little regarding how these cephalopods actually use sound cues. Further, there is a scarcity of studies quantifying TTS in marine invertebrates to which we may compare with. More broadly, we do see behavioral, loudness-based responses, to sound between the longfin squid and common cuttlefish (*Sepia officinalis*) (Samson et al. 2014, Mooney et al. 2016), implying that at least in other contexts, differences in sound-sensitivity exist.

Acoustic trauma, including lesions, hair cell loss and neuron swelling, have been reported in several cephalopod species (including squid) following exposure to both artificial (50-400 Hz sweeps, 157 ± 5 dB re 1 µPa, André et al. 2011) and PD sound playbacks (173 dB re 1 µPa, Solé et al. 2022). Note that these studies were done in tanks without reporting particle acceleration levels, hence the comparison of these results with our data is not applicable. These ultra-structural damages on statocysts could affect cephalopod's hearing. It was not possible to address potential auditory damage in this study. Yet, none of the animals tested presented evidence of TTS, thus if PD sound exposure did damage longfin squid statocysts or their neurons, it did not affect sound sensitivity.

To further confirm the absence of TTS in longfin squid exposed to PD sound, we performed additional control experiments. This included exposing goldfish and longfin squid to white sound. Goldfish showed clear and significant TTS. Fish exposed to 3 h of white sound demonstrated 7 dB of TTS following sound exposure (figure 38). In the exact same experimental setup, longfin squid auditory thresholds remained similar, thus it appears that white sound exposure also did not induce TTS in this species (figure 38). These comparisons to goldfish and white sound are important because small tanks and AEP methodologies can vary and potentially impact interpretations of hearing thresholds (Popper and Hawkins 2021). It was critical to establish that TTS can be induced here. Further, because they are relatively well-studied in the bioacoustic literature, goldfish can represent a "standard" or baseline from which we can compare (Smith and Popper 2023). The sound induced TTS in goldfish noted here were similar to those measured elsewhere (Smith et al. 2004a, 2004b, Smith et al. 2006) and the methods can induce TTS. This indicates that lack of TTS noted in the longfin squid underscore these animals have resilient hearing for these sound exposure conditions. This includes both intermittent and continuous sound exposures with different SEL_{cum} values, 110.5 dB re 1 $(\mu\ ms^{-2})^{-2}$.s for pile driving and 151.7 dB re 1 $(\mu\ ms^{-2})^{-2}$.s for white sound. In other aquatic taxa, the latter often demonstrates TTS at lower SEL_{cum} values (Halvorsen et al. 2011).

Our previous studies in tanks and *in situ* showed fewer behavioral responses as squid were exposed to successive PD sound exposure (Jones et al. 2020, Cones et al. 2022, Jézéquel et al. 2023). Two hypotheses could have explained these trends. First, squid might have habituated quickly to PD sound exposure. Second, high PD sound level may have led to auditory damages or sound-induced hearing loss as successive PD increased. The present results support the first hypothesis and highlight PD sound may not represent a direct threat for these short-lived animals. For example, squid display important reproductive behaviors that are not affected by PD sound playbacks in tank (Jones et al. 2023). However, it is notable that our PD set-up generated sound levels corresponding to roughly 1 km from actual OSW constructions (Sigray et al., 2022). Hence, future work could quantify both the behavioral and physiological responses of squid exposed to the higher sound levels generated during OSW constructions.

4.3.5 Conclusion

This study expands knowledge on cephalopod hearing highlighting that exposure to both short and long duration, high anthropogenic sound level does not cause significant shifts in the sound sensitivity of the economically and ecologically important longfin squid, *D. pealeii*. Understanding baseline sensitivity and potential physiological effects of sound on cephalopods is an important step towards establishing guidelines for management and policy to protect cephalopods from sound pollution. Auditory thresholds for marine mammals and fishes have been established and are regularly used in environmental impact assessments globally (Popper et al., 2014; NMFS, 2018). Despite the absence of physiological effects in squid, we caution more studies are now needed to confirm (or not) these results at the scale of an OSW construction.

5 General Discussion and Perspectives

5.4 General summary

Here we show that both black sea bass and squid behavior may be altered during exposure to actual, in situ pile driving sound. While these disturbances can influence or occur during a range of behavioral contexts (social interactions, communication, feeding, sexual displays, etc...), yet the duration of the impact is varied and somewhat species dependent. And overall, squid generally appeared resilient to impulsive pile driving across behaviors and hearing, but black sea bass responses are more nuanced. While the in-situ results are both critical for natural behaviors and reduce the complications of acoustics in small tanks, the results are largely consistent with previous tank-based studies for both taxa.

This study conducted five major studies summarized here.

The Study 1 addressed the behavioral responses of sub-adult black sea bass at known locations and received levels. Black seabass in cages showed significant changes in the behavioral patterns exhibited by animals reducing swimming in the water column and moving to a more cryptic, anti-predator vigilance on the benthos. The most common observation of sound exposed fish was decreased activity and increased association with the bottom of the cages. Reponses were particularly evident for animals that received higher sound levels and for Day 1 exposures. Although animals exposed a second day did not exhibit statically significant changes in swimming patterns, swimming responses were still altered in a manner similar to the previous day, suggesting impacts were not fully extinguished. Observation of decreased activity is consistent with our previous studies done in tanks (Stanley et al. 2023), as well as responses reported in other studies on fish and anthropogenic sounds (e.g., Knudsen et al. 1992, Neo et al. 2016).

Study 2 examined how free-swimming black sea bass responded to pile driving, measuring behaviors and time at baited remote underwater video systems. The BRUV experiments highlighted decrease in juvenile black seabass abundances when exposed to pile driving sound at 1 and 5 m distances from the pile. Additionally, we observed a decrease in both juvenile and adult sea bass presence throughout several weeks of pile driving, compared to the same time of year with no pile driving.

In three separate studies, squid showed clear behaviors associated with predator evasion in initial responses to pile driving sound (Cones et al. 2022a), and these responses decreased both within and across repeated pile driving sound sequences (Jézéquel et al. 2023a), similar to Jones et al. (2020) in tanks. Despite these dramatic short term behavioral responses, schooling behavior was not altered (Jézéquel et al. 2023b), nor were the responses energetically consequential in the context of normal swimming of these muscular squid (Cones et al. 2022a). Additionally, squid exposed to high intensity pile driving sound did not present hearing losses (Jézéquel et al., under review; Jones et al. 2021; Jones et al. 2023). Overall, these results demonstrate rapid habituation of squid to pile driving sound, and an apparent resilience to this intense sound stimuli, ultimately showing minimal effects on this ecologically and commercially key taxon.

The rapid habituation seen in both tank-bound and field squid is notable here (Jones et al., 2020; Jones et al., 2021; Jézéquel et al., 2023c; Jones et al., 2023). Similarly, for black sea bass, both tank and field studies observed animals moving to the bottom and exhibiting crypsis and vigilance (in response to an apparent threat). This suggests that, at least for some taxa, tank-based studies can provide valuable information addressing how animals may be adversely affected by anthropogenic sound. Care should be taken to address key details. This includes proper calibrations (of pressure and particle motion, in three dimensions) and to allow animals to acclimate to their enclosures as needed. The set up is often specific to the focal species their behaviors and the specific research questions to be addressed. For black sea bass, acclimatization to the tank environment was nearly a year (Stanley, et al. 2023); for squid, this can be considerably shorter. These tank-based studies, while not replacing field experiments, can inform research foci and methods for field studies addressing *in-situ* and population-level impacts.

Our results also highlight two main potential mitigation procedures that could be used by OSW developers to reduce behavioral impacts on squid and black seabass. First, far fewer behavioral reactions were found in squid exposed to the VH continuous and low-amplitude signals (Jézéquel et al., 2023b), which is consistent with our previous study in scallops (Jézéquel et al., 2022b). This suggests that the VH can be used as a mitigation technique to drastically reduce behavioral impacts on squid during OSW constructions. Second, the fact that squid mostly responded at the onset of the IH events highlights that the first IH blows have the most impact on squid. Here, ramp-up could also be used as a mitigation technique. This soft-start procedure with slowly increasing sound levels could provide animals with a warning signal before sound exposure levels rise to full power (Robinson et al., 2007; Bailey et al., 2014). The gradual increase in sound level of the ramp-up procedure may allow the squid to habituate to the sound exposure faster and stay within the exposure area without avoidance behavior.

5.2 Future directions

This series of studies examined animals exposed to water-borne sounds in the water column. However, many ecological and commercially important fauna are benthic. Thus, they likely receive, and may be affected by, substrate-borne vibrations associated with sound exposure. Pile driving generates intense substrate-borne vibrations that can be detected kilometers away from OSW constructions [e.g., (Bruns et al., 2014)]. This acoustic stimulus, despite being largely overlooked in sound impact studies [see (Popper et al., 2022)], may be of high concern for marine animals living in the bottom of the ocean, especially species have limited or no mobility, and thus may not be able to leave the impacted area. This includes corals, bivalves such as scallops, decapods such as lobsters, but also fishes including flounders.

A common criticism of previous sound exposure experiments (including pile driving for marine invertebrates and fishes) is that studies mostly occurred in the laboratory where it is challenging to recreate realistic sound exposure levels, rending their findings difficult to translate to real-life scenarios. To circumvent this issue and more accurately recreate the sound field associated with OSW constructions, we exposed the focal fauna here to actual PD in a coastal environment. However, it is notable that wind turbine foundations are many times larger (often 10 m diameter and 150 m long monopiles) than the 0.3 m diameter, 10 m height steel monopile used here. For example, the IH used in our study generated underwater sound at 8 m from the pile that were similar in particle acceleration and sound pressure amplitude to roughly 800-1000 m from OSW construction (Sigray et al., 2022). Hence, we caution that the short spatial scale behavioral responses observed for squid in our study are likely to occur over much higher distances during OSW constructions. This suggests that behavioral disruption will likely occur at the kilometer scale and at a relatively substantial range, especially if we consider wind turbine pile spaces to be roughly 1 km apart and noise levels to stay consistent. More intense or persistent responses may occur within that 880 m range especially if larger pilings are used or if multiple platforms are constructed concurrently. Hence, the alarm responses described here may impact a significant majority of animals within the entire OSW development area. However, more information on noise-induced disruptions to group-level behaviors is needed to better assess impacts on populations. Tagging animals at proximity from OSW construction [e.g., (Cones et al., 2022a; Cones et al., 2022b)] may allow us to answer these questions (e.g., horizontal dispersion). Because of their soft bodies and fragile skin, squid are notoriously challenging to tag. Yet new methods of attachment including bioadhesives are being developed for more rapid, longer-term, and less-invasive means of biologger attachment (Londono et al., 2024). Overall, the results from larger field studies could potentially highlight dose-dependent responses depending on the distance and received levels of the animals from the turbines, and help define sound impact areas for each studied species.

One key aspect yet to be addressed is impacts to early life stages. Marine fishes and invertebrates often have complex pelago-benthic cycle, and larval stages and juveniles tend to be particularly sensitive to environmental cues [e.g., (Kaplan et al., 2013; Jézéquel et al., 2023a)]. Thus, there is a need to address sound and other influences of renewable energy for these younger taxa, such work could be conducted in well-controlled aquaria conditions as shown in Olivier et al. (Olivier et al., 2023).

Finally, as noted above, the field-based pile driving methodology provided the unique ability to address multiple species, at multiple locations that were increasing distances from the pile driving. It allowed for a Before-During-and-After Gradient experimental design [e.g., (Methratta, 2020)], all in a field-based, semi-naturalistic setting, replicating (albeit at a smaller scale) offshore wind construction. Such a setup was generally recommended by a recent offshore wind study group (Popper et al., 2022). And it provided a setup that was flexible, logistically manageable and much more cost-effective than studies at offshore windfarm sites. The experimental structure allowed for transferability and flexibility between animal species, comparability between years, the monitoring with, and leveraging of, multiple acoustic sensors to quantify pressure, particle motion and ground vibration. Its replicability and accessibility allowed us to use standard biological methods such as cages and baited remote cameras, but also innovate, via novel squid tags. In effect, this study site has become a key testbed for innovation and quantifying the pile driving impacts on key marine taxa. Building from this framework, there is the opportunity to address new species, more complex behavioral and physiological considerations, and overall better understand how offshore wind development may or may not impact key marine fauna of interest.

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