



Exposure to boat noise in the field yields minimal stress response in wild reef fish

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ABSTRACT: Aquatic anthropogenic noise is on the rise, with growing concern about its impact on species that are sensitive to low-frequency sounds (e.g. most fish and invertebrates). We investigated whether the reef fish *Halichoeres bivittatus* living in both noisy and quiet areas had differing levels of baseline stress (measured as whole-body cortisol) and whether they would exhibit a physiological stress response when exposed to boat noise playbacks. While the playback experiments significantly increased cortisol levels in fish from our experiment compared to baseline levels, there were minimal pairwise differences across treatments and no difference in baseline stress for fish living in noisy vs. quiet areas. These results may be explained by low overall auditory sensitivity, habituation to a fairly noisy environment (due to biological sounds), or that boat noise simply may not represent an immediate threat to survival in this species. These findings contrast recent studies that have shown elevated stress responses in fishes when exposed to boat noise and highlights that inter-specific differences must be considered when evaluating potential impacts of anthropogenic noise on marine life.

KEY WORDS: Anthropogenic noise · Reef fish · Stress

1. INTRODUCTION

As human activity in the oceans has increased, so have levels of anthropogenic noise. Noise is one of many stressors that marine species are facing in the oceans of today, yet understanding its potential consequences on wildlife is challenging due to the complex interactions of physics, biology, physiology, and animal behavior. Initially, most concerns about anthropogenic noise in marine environments focused on acute impacts (e.g. physical injury and hearing loss) from the most intense sound sources (e.g. explo-

sions, navy sonars, and seismic airguns), with a particular emphasis on marine mammals because of their complex vocalizations and hearing systems (Parsons et al. 2008, Finneran 2015, Williams et al. 2015). As the field has progressed, however, greater attention has been directed toward lower trophic level species, as investigators have realized that they, too, are sensitive to sound and could be impacted by noise (Slabbekoorn et al. 2010, De Soto 2016, Hawkins & Popper 2017, Cox et al. 2018). In addition, there is growing evidence of non-injurious effects, such as changes in behavior, increases in physiological stress,

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or interference with communication systems (Wright et al. 2007, Kight & Swaddle 2011, Erbe et al. 2016). These impacts may be more long-term and more widespread, as lower sound levels are required to elicit such responses.

Noise from vessel traffic is an example of a stressor that is both chronic and widespread. The cavitation of boat propellers creates a continuous, low-frequency sound that is now nearly ubiquitous in the ocean and has steadily increased with the growth of the global economy (Hildebrand 2009, Andrew et al. 2011, Frisk 2012). Automatic identification systems (AIS) on commercial ships have proven useful for quantifying and predicting ocean sound levels (Hatch et al. 2008, Roul et al. 2019) as well as potential impacts on marine mammals (Erbe et al. 2014, 2019, Merchant et al. 2014). Although smaller boats do not have this technology, some studies have demonstrated that noise from small vessels makes a significant contribution to coastal soundscapes and may affect behavior of wildlife in these areas (Codarin et al. 2009, Kaplan & Mooney 2015, Wladichuk et al. 2018). Considering how frequent such boat traffic is in some coastal areas, this is an important area of ongoing research. Furthermore, because small boats produce sounds that overlap in frequency with the hearing range of most fishes and invertebrates (typically < 1 kHz), boat noise may be a significant stressor for these species (Popper & Hastings 2009). Studies on fishes have shown that responses vary depending on engine noise type, field versus laboratory settings, and within and between taxonomic groups (Normandeau Associates 2012, Hawkins & Popper 2017, Jain-Schlaepfer et al. 2018).

Indeed, previous research on the effects of boat noise on fishes has yielded variable results: laboratory studies tend to focus on physiological endpoints, while field studies tend to investigate behavioral responses. Codarin et al. (2009) found that, when exposed to boat noise in the laboratory, hearing thresholds of 3 Mediterranean fishes increased. Scholik & Yan (2002) found a similar result in fathead minnows. A tank study on 3 freshwater fishes resulted in significant increases in glucocorticoid hormones upon exposure to ship noise for just 30 min (Wysocki et al. 2006), and juvenile kelp bass reacted similarly in response to intermittent (but not constant) boat noise (Nichols et al. 2015). In another tank study, potential longer-term impacts of noise exposure were explored: after 10 d of noise exposure, gilthead seabream showed significant increases in several blood parameters including adrenocorticotropic hormone, cortisol, glucose, lactate, and hematocrit (Celi et al. 2016).

Heart rates have also been used as a measure of stress: Graham & Cooke (2008) exposed largemouth bass to real operational engine motors inside a tank and saw that heart rates increased the most in response to motorboat engines compared to quieter trolling engines or non-motorized paddle strokes; a similar result was found in embryonic damselfish (Jain-Schlaepfer et al. 2018).

Several field studies have also shown that fishes change their behavior in response to boat noise. For example, a brooding damselfish, when subjected to playbacks of boat noise in the field, changed its typical brooding behaviors (Nedelec et al. 2017), and a different damselfish species showed a diminished reaction to predators after exposure to motorboat noise (Simpson et al. 2016). In largemouth bass, nest-guarding males slightly altered natural parental care behaviors when exposed to noise, but only when their offspring were a certain age (Maxwell et al. 2018). These studies suggest that the presence of noise either decreased vigilance or masked the ability of these fishes to detect potential threats. Swimming behaviors can also be affected by noise: noise from passenger ferries and small boats disrupts schooling in captive bluefin tuna (Sara et al. 2007), and Norwegian herring seem to flee in response to an approaching vessel (Vabo et al. 2002). Similarly, Schwarz & Greer (1984) showed that Pacific herring exhibit avoidance responses when exposed to sounds meant to simulate approaching vessels. Taken together, these studies indicate that fishes respond to the presence of real boats, as well as the sounds of boats in both laboratory and field environments. And while behavioral responses are important metrics to track, there has been a noticeable lack of focus on stress physiology in field studies; such work is important because primary responses to stressors can underlie whole-body responses that are typically observed as changes in behavior (Barton 2002).

One particular topic that has not been sufficiently investigated is whether fishes habituate to high levels of background noise. Chronic stress, which can result in elevated glucocorticoid levels for extended periods of time, can have significant metabolic and fitness-level consequences (Barton 2002). One study found that a coral reef fish initially hid behind rocks when encountering boat noise, but was less likely to react this way after 1 wk of noise exposure, indicating that its initial response was dampened (Nedelec et al. 2016). When cortisol levels were tested after 1 wk, they were no different from those of controls, providing no evidence of chronic stress due to repeated noise exposure (Nedelec et al. 2016). Simi-

larly, sounds of pile-driving and seismic airguns initially triggered a stress response in European seabass, but after 12 wk of exposure, this response was diminished; the exposed fish showed no evidence of chronic stress (Radford et al. 2016). Given the relatively few studies on this subject, it is unclear whether fish living in high-noise areas become habituated or sensitized to noise. It is also unclear whether populations might diverge in their responses. All of this could depend on background noise conditions of their respective habitats. In other words, do fishes living in areas with chronic noise experience chronic stress, and do they still exhibit a stress response when exposed to acute noise events?

To explore these questions, we collected baseline physiological data from populations of a common Caribbean reef fish, the slippery dick wrasse *Halichoeres bivittatus* that lives in areas with varying degrees of boat activity. We also measured the physiological stress responses in fish from these different populations following a series of boat noise playback experiments. We addressed 3 primary questions: (1) Are there differences in baseline stress biomarkers for populations from noisy vs. quiet areas? (2) Does *H. bivittatus* exhibit a change in stress markers when experimentally exposed to boat noise? (3) Are there variations in the stress response among populations from noisy vs. quiet sites upon exposure to boat noise playbacks? We discuss our findings as they relate to the growing field of research seeking to evaluate the effects of anthropogenic noise on aquatic ecosystems.

2. MATERIALS AND METHODS

2.1. Collection sites, baseline acoustic recordings, and whole-body cortisol methods

Our work was conducted on the Caribbean coast of Panama in the Bocas del Toro region (9.33° N, 82.25° W), where *Halichoeres bivittatus* is highly abundant. We identified 2 sites within the region that had substantially different levels of boat traffic; acoustic recordings and fish collections were conducted at these 2 sites. The 'noisy' site, Hospital Point, is a hardbottom habitat with scattered corals and reef-associated fauna that lies directly underneath a busy channel, where passenger ferries and water taxis frequently pass at high speeds (Fig. 1A). The 'quiet' site, STRI Point, is a protected seagrass bed fringed by mangroves and small coral reefs, near the Smithsonian Tropical Research Institute's field station (STRI; Fig. 1A). Typical boat traffic in this area is lim-

ited to small boats moving at slow speeds as they come and go from the research station. We deployed passive acoustic recorders (DSG, Loggerhead Instruments; 20 kHz sampling rate) at each of these sites for several days in order to quantify boat activity and ambient sound levels. A full analysis of soundscapes around different parts of the Archipelago can be found in Staaterman et al. (2017). Briefly, our recordings confirmed that the acoustic conditions (below 1 kHz, the frequency range that most fish can hear) of these 2 areas differ by up to 10 dB during daytime hours (Fig. 1B). By manually scanning the recordings, we found that an average of approximately 30 boats h⁻¹ (between 07:00 and 19:00 h) pass nearby at Hospital Point, compared to 10 boats h⁻¹ at STRI Point. Sound levels during a representative 24 h window are depicted in Fig. 1B.

To understand whether there were differences in baseline stress biomarkers for *H. bivittatus* individuals living at the noisy and quiet sites, we measured whole-body cortisol and blood glucose (2 common stress biomarkers in fish; Sopinka et al. 2016). Over the course of 5 d (18–22 January 2016), 20–30 animals were collected at ~400 m² areas at each of the 2 sites. Individual fish were captured using a lift-net baited with crushed sea urchins. Upon capture, animals were immediately sacrificed via cerebral percussion, as it was the fastest and most efficient means possible (IACUC permit 2016-0101-2019-A1). An incision was made near the heart to withdraw a fresh blood sample (0.1 ml), which was then analyzed on a test strip for glucose using a portable glucose meter (ACCU-CHEK glucose meter; Roche Diagnostics) previously validated for use in fish (Stoot et al. 2014). The carcasses were measured for total length and frozen immediately for later whole-body cortisol processing. The total time for capture, euthanasia, and sampling prior to being frozen was less than 3 min (Lawrence et al. 2018). The goal of our rapid processing was to characterize the condition of the fish prior to capture without data being unduly influenced by handling.

To obtain whole-body cortisol levels for each fish, we generally followed the procedures outlined in Sopinka et al. (2014), Jeffrey & Gilmour (2016), and Redfern et al. (2017). Individual fish were removed from their respective frozen bags and the proximal portion of each fish (anterior of midway between the operculum and pelvic girdle) was removed using a serrated duct knife. The fish tissue was ground into a very fine homogeneous powder with a mortar and pestle, then transferred into 5 ml bullet tubes. The tubes with the ground fish tissue were returned to

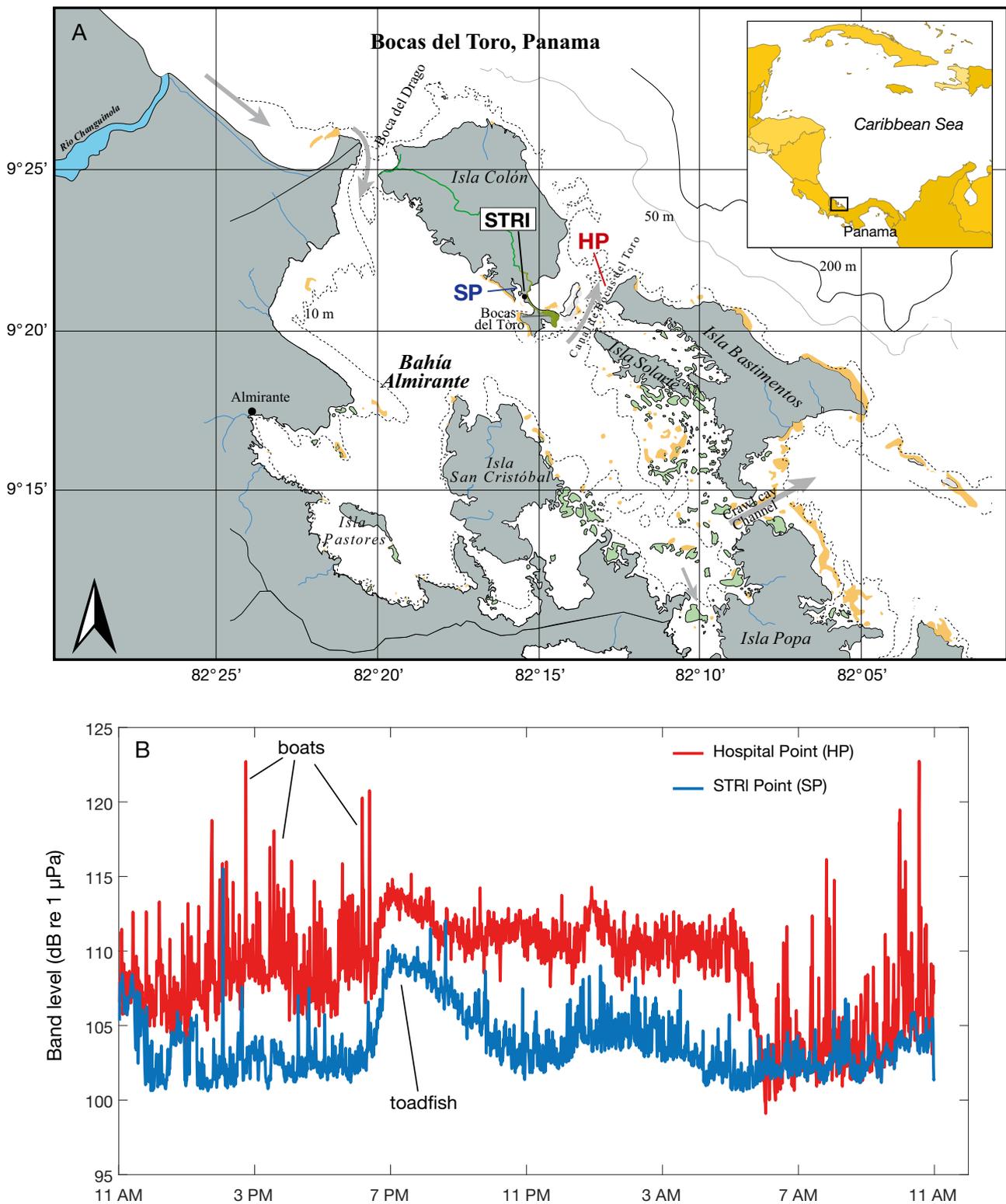


Fig. 1. (A) Study site within the Bocas del Toro Archipelago on the Caribbean coast of Panama; *Halichoeres bivittatus* specimens were collected at Hospital Point (HP), a hardbottom area with frequent vessel traffic, and STRI Point (SP), a quiet, protected bay with seagrass and patchy corals. Playback experiments took place near SP. (B) Representative 24 h acoustic recording at the 2 sites showing different acoustic conditions within the frequency band of 1–1000 Hz. The frequent passage of boats at HP (average: 30 boats h^{-1} during the day) can be seen as sharp peaks. At both sites, low-frequency sound levels increased at night due to chorusing toadfish (Staaterman et al. 2018). STRI: Smithsonian Tropical Research Institute's field station

the bag with the rest of the carcass and stored at -80°C until the extraction process. Throughout the process, the knife, scoopula, mortar, and pestle were cooled with liquid nitrogen and cleaned between samples to prevent cross-contamination. Next, the pulverized frozen tissue was decanted into a small test tube; an extraction buffer (Neogen Cortisol Kits, Neogen) was added ($730\ \mu\text{l}$ of buffer g^{-1} of tissue). Samples were then homogenized on ice using sonication, and diethyl ether was added to the sample ($2.5\ \text{ml g}^{-1}$ of tissue). The sample was vortexed and placed in a refrigerator at 4°C for 60 min. Next, samples were centrifuged at $3000 \times g$ for 5 min at 4°C , then flash frozen at -80°C for 30 min. Supernatant liquid was pipetted into a separate tube, and remnants were left to thaw before 2 further repetitions of vortexing and centrifuging. Remnant supernatant liquid in each tube was evaporated using a gentle stream of air, leaving only the cortisol-containing residue. Samples were reconstituted using Neogen extraction buffer ($2\ \text{ml g}^{-1}$ of tissue; Neogen Cortisol Kits). Samples were used at full strength for enzyme-linked immunosorbent assay (ELISA) quantification. ELISA quantification combines sample cortisol with a cortisol conjugate, both of which compete for the same antibody-dependent binding sites on a single plate. As only the conjugate reflects light, more color corresponds with less analyte. Sample absorbency ($\lambda = 450\ \text{nm}$) is then compared to 8 in-plate standards to quantify cortisol concentration. It was difficult to obtain adequate blood volumes for glucose readings during collection of our baseline samples, so the sample sizes for this component of our work were too low for statistical analyses.

2.2. *In situ* acoustic playback experiment

To test whether *H. bivittatus* exhibited a stress response when exposed to boat noise, we collected fish at each of the 2 sites and conducted an *in situ* playback experiment. We collected a total of 40 fish site^{-1} over a span of 9 d (12–20 February 2016), with a maximum of 10 ind. d^{-1} due to limitations of our experimental arena. Fish were collected using the same

lift-net as described above. Upon capture, fish were immediately transferred to a cooler filled with seawater and transported back to the experimental arena: a shallow seagrass bed (2 m water depth) near STRI Point, where *H. bivittatus* are commonly observed. We crafted cylindrical mesh cages (30 cm length, 10 cm diameter, 5 mm mesh size) that were placed 50 cm apart along horizontal PVC frames (Fig. 2). Each PVC frame held up to 5 cages, and the 2 frames were 4 m apart with the speaker located in the middle. All *H. bivittatus* individuals were placed one each into the 5 mesh cages on one side of the arena. After a minimum of 1 h of acclimation, they were either subjected to the ‘noisy’ or ‘control’ treatment.

For the noisy treatment, playbacks were generated from the 24 h recordings from Hospital Point. Using Raven Pro 1.4 (Cornell University), we created two 60 min playback files consisting of approximately 30 intermittent boat pass-overs (typical for this location), randomly spaced in time. Two boat noise playback files were created to avoid pseudo-

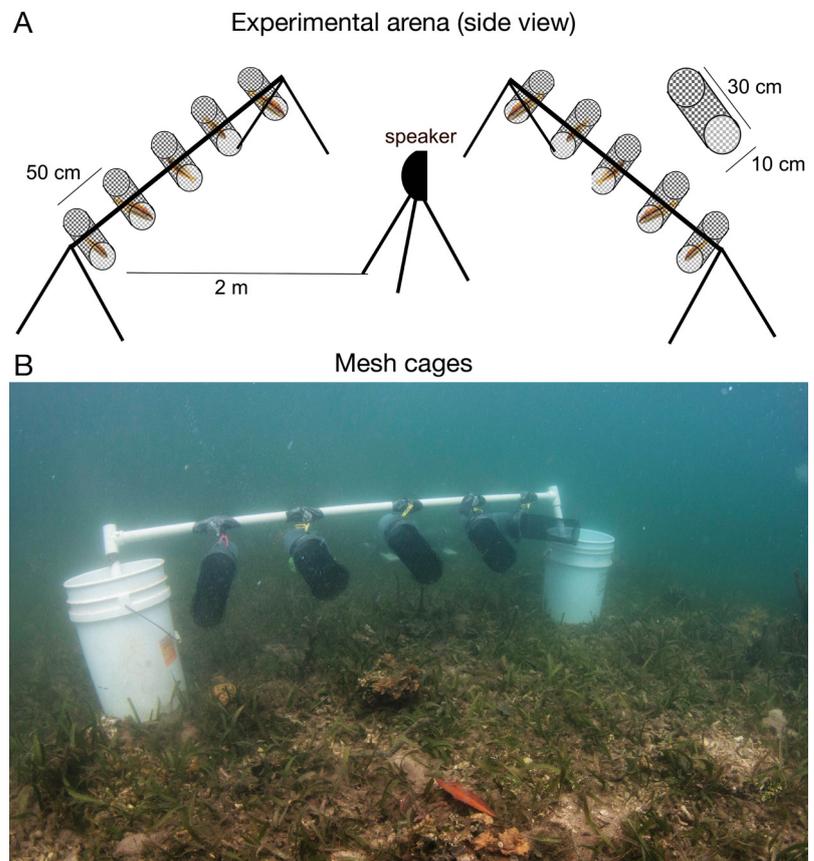


Fig. 2. (A) Diagram of the experimental arena, with the 2 rows of small mesh cages and the speaker in the middle; (B) photo from behind one of the rows of cages. The speaker (not visible) is on the other side of the setup

replication and were randomly chosen throughout all experiments. We recorded the natural habitat sounds in our experimental arena and found that they were equivalent to those measured at the quiet site; thus, there was no need to play back additional sounds from the speaker during the control treatment (Fig. 3).

To match the boat playbacks to real boat sounds recorded *in situ*, we calibrated our underwater speaker system. The underwater speaker (Aquasonic AQ339, Clark Synthesis) was powered by an amplifier (SX220.2, Cerwin-Vega) and 12 V marine battery. The passive acoustic recorders measured the received levels at the location of the fish during the playbacks. Using the RMS sound pressure level of a specific boat pass-over as recorded at Hospital Point (125 dB re 1 μPa) as a target, we adjusted the gain on the amplifier so that received levels at the site of the fish were within 2 dB of this target during the same boat pass-over. Some spectral distortion due to the speakers was unavoidable (Fig. 3), however. Sounds were high-pass filtered at 3 kHz prior to playback to avoid high-frequency artefacts from the speakers.

After the 1 h playback period, the fish were sacrificed and processed using the same protocol as described above for baseline stress markers and then im-

mediately frozen for future cortisol assays. On any given day, we were only able to conduct one noisy and one quiet treatment (i.e. we collected and used a maximum of 10 ind.), and between days we alternated the order of the 2 treatments and the frames used for each.

The effects of experimental handling stress, collection site, and noise treatment on fish cortisol levels were evaluated by comparing the baseline, control playback, and noisy playback treatments between sites using ANCOVA. Prior to analysis, cortisol data were square root transformed to meet assumptions of normality. A 2-way ANCOVA was conducted in MATLAB R2017B (MathWorks) with site and treatment as independent variables and body length as the covariate. Pairwise multiple comparisons were conducted to identify which treatments were significantly different.

To examine potential differences in glucose levels across playback treatments, we first used a linear regression to determine whether there was a significant relationship between body length and glucose. We found no relationship, so we used ANOVA to look for differences in mean glucose levels between the 4 groups described above. Since the glucose data did not meet assumptions of normality, the data were first square root transformed.

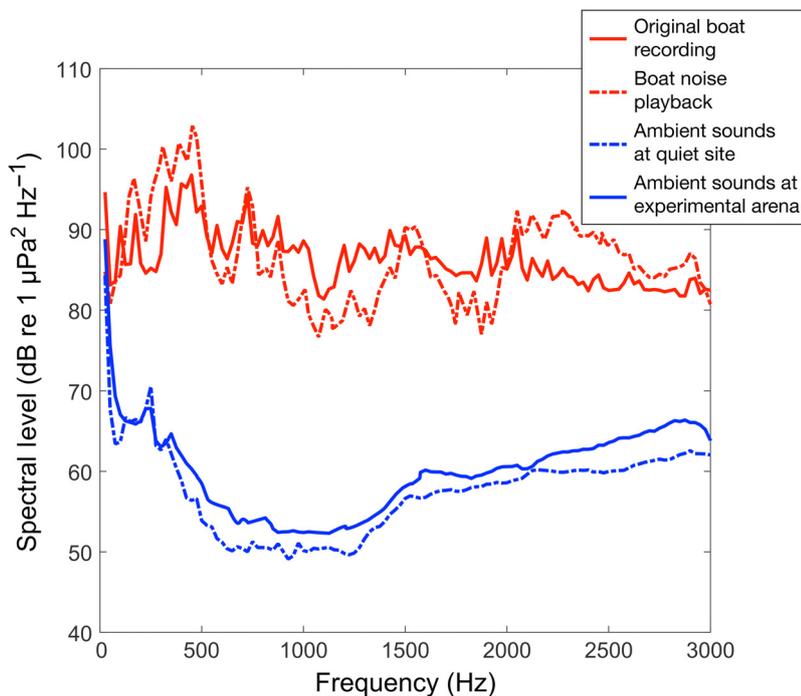


Fig. 3. Underwater speakers broadcast boat sounds (spectrum of representative boat pass shown as dashed red line) from recordings made at the noisy site (same boat pass; solid red line) in the *in situ* experiment. Because ambient sounds at the experimental arena (solid blue line) were similar to the quiet site (dashed blue line), no playbacks occurred during the control treatment

3. RESULTS

Fish included in the playback experiments had higher whole-body cortisol levels than those sacrificed immediately to measure baseline stress levels, but differences among sites and treatments were minimal. The mean (\pm SE) whole-body cortisol level varied from $1.17 \pm 0.22 \text{ ng g}^{-1}$ for baseline fish collected at STRI Point to $4.80 \pm 0.39 \text{ ng g}^{-1}$ for fish collected at Hospital Point and exposed to the noisy treatment (Table 1). There was a negative linear relationship between body length and cortisol levels, and there were significant effects of site and fish body length (Table 2). Post hoc multiple comparisons tests (Table A1 in the Appendix) revealed that fish from STRI Point were smaller than those from Hospital Point, but there were no differences in size among treatments. Baseline cortisol levels did not differ between sites. Fish exposed to experimental treat-

Table 1. Sample sizes, mean body length, whole-body cortisol, and glucose levels for *Halichoeres bivittatus* used in both the baseline study and the *in situ* experimental portion of the study. Values are reported as means \pm SE. We were not able to obtain glucose readings for all fish, so the sample sizes for glucose data are reported separately in the last column

Experiment	Collection site	Noise treatment	Sample size	Mean body length (cm)	Mean whole-body cortisol (ng g ⁻¹)	Mean glucose (mmol l ⁻¹)
Baseline	STRI Point	–	31	6.17 \pm 0.53	1.17 \pm 0.22	3.9 (n = 1)
Baseline	Hospital Point	–	19	8.19 \pm 0.58	1.28 \pm 0.29	4.4 \pm 0.6 (n = 16)
<i>In situ</i> playback	STRI Point	Control	19	6.70 \pm 0.38	4.53 \pm 0.72	4.17 \pm 0.37 (n = 14)
<i>In situ</i> playback	STRI Point	Noisy	21	7.21 \pm 0.39	3.87 \pm 0.59	4.52 \pm 0.55 (n = 20)
<i>In situ</i> playback	Hospital Point	Control	17	7.93 \pm 0.46	3.97 \pm 0.68	3.25 \pm 0.23 (n = 15)
<i>In situ</i> playback	Hospital Point	Noisy	19	9.11 \pm 0.47	4.80 \pm 0.39	3.97 \pm 0.29 (n = 18)

Table 2. *In situ* playback experiment: results of the 2-way ANCOVA for whole-body cortisol levels in *Halichoeres bivittatus*, with site and treatment as independent variables and length as a covariate

Source	df	SS	MS	F	Prob > F
Treatment	1	0.7951	0.79508	5.71	0.0184
Site	2	14.8229	7.41146	53.23	<0.001
Length	1	3.0505	3.05052	21.91	<0.001
Error	124	17.2641	0.13923		
Total	128	35.9212			

ments had significantly higher cortisol levels than baseline fish, with fish in the Hospital Point noisy treatment having slightly, but significantly higher values than fish in the Hospital Point control treatment and both STRI Point treatments.

In contrast, there was no relationship between body length and glucose levels ($F = 0.69$, $df = 69$, $R^2 = 0.01$, $p = 0.41$). There was also no difference in the mean glucose levels across the 4 groups ($F = 1.01$, $df = 3$, $p = 0.39$).

4. DISCUSSION

While boat noise playback experiments significantly increased cortisol levels in *Halichoeres bivittatus* from our experiment compared to baseline levels, there were minimal pairwise differences across treatments and no difference in baseline stress for fish living in noisy vs. quiet areas. The one difference — fish from the noisy site had slightly but significantly higher cortisol levels in response to the noisy vs. control treatments, whereas no difference was observed for fish from the quiet site — was potentially confounded by fish from the noisy site being significantly larger on average than those from the quiet site. This result of minimal differences was somewhat

surprising, considering the recent studies that have shown a heightened stress response in fishes exposed to boat noise (e.g. Wysocki et al. 2006, Nichols et al. 2015, Celi et al. 2016). Thus far, the majority of studies examining physiological responses of fishes to boat noise have been conducted in laboratory settings, rather than the field. Playback work in the laboratory is inherently flawed, as the physical boundaries of tanks can distort acoustic frequencies and present abnormal cues to the study subjects (Akamatsu et al. 2002). Because most fish are sensitive to particle motion, which is the dominant acoustic cue close to a sound source, when very close to the speaker they may have perceived high levels of particle motion and/or abnormal directional cues (Popper & Hawkins 2018, 2019). In addition, 3 laboratory studies (Wysocki et al. 2006, Nichols et al. 2015, Celi et al. 2016) used sound pressure levels that were 10–30 dB above those used in our experiments (within an equivalent frequency range), so it is not surprising that they may have seen a more pronounced response. The playback of a boat at 20 m distance in Nichols et al. (2015) most closely approximated our playback levels and, interestingly, this study showed a negative correlation between playback levels and cortisol concentrations. Therefore, it is likely that both the proximity and amplitude of the sounds received will influence the degree of response observed. Future experiments should further explore this relationship.

We calibrated our overall playback amplitudes to match recordings from the reef, where real boats passed anywhere from 5–50 m from the recorder. During the experiments, fish subjects were at least 2 m from the speaker and, with no reverberant boundaries nearby, the sound field they received during the intermittent boat sounds would have resembled that of a real boat passing several meters away. Therefore, it is reasonable to assume that the received levels in the experiment matched what *H. bivittatus*

would encounter in its natural habitat. However, it should be acknowledged that the underwater speaker was a stationary monopole source, and that the directionality of particle motion created by a real, moving boat would be quite different. Not much is known about hearing capabilities of wrasses in general (Tavolga & Wodinsky 1963, Boyle & Cox 2009) and there is no published audiogram for *H. bivittatus* so it is difficult to know how well our specimens were able to detect the playback sounds. The fact that we failed to observe a strong stress response in animals exposed to boat noise may be explained by poor auditory sensitivity — either the particle motion field that was received at 2 m distance from the speaker was too faint to be detected above ambient levels, or that it simulated a sound that was non-moving so it did not represent a true threat. It also could be that since this species is accustomed to fairly high levels of natural sounds, it was simply unphased by the additional noise from the boats. Future field-based studies of this nature should include a particle motion sensor at the location of the specimens so the actual received acoustic cues can be quantified, and other investigators should consider having real boats drive by the animals, rather than a stationary speaker. Finally, additional work on auditory sensitivity in wrasses would shed further light on these results.

We hypothesized that *H. bivittatus* that lived in noisy habitats would have different levels of baseline stress, and that fish from these areas would exhibit a different response to boat noise compared to animals living under quiet conditions. Our results support neither of these hypotheses. While fish from the noisy site appeared to respond to the noisy playback treatment with significantly higher cortisol values than the control treatment, we can not determine whether their responses were different from fish from the quiet site due to significant differences in fish body length between sites. We found no evidence of 'chronic' stress (i.e. no difference in whole-body cortisol in baseline fish) for fish from the noisy area compared to fish living in quiet areas, although we acknowledge the limit in replication ($n = 1$ for each site type) and the fact that we only measured glucocorticoids as an endpoint. Nonetheless, our results are consistent with previous work in fishes. Weeks of boat noise exposure had no effect on plasma cortisol concentrations in damselfish (Nedelec et al. 2016), long-term exposure to white noise had no effect on cortisol in goldfish (Smith 2004), and long-term exposure to pile-driving noise did not affect ventilation, growth, or mortality rates in European sea bass (Radford et al. 2016). Our findings suggest that popula-

tions of *H. bivittatus* exhibit no evidence of chronic stress, based on our assessment of baseline cortisol levels.

We observed a significant negative correlation between body size and whole-body cortisol (Fig. A1 in the Appendix) but no relationship with plasma glucose. We intended to use plasma cortisol as an indicator of stress, but we quickly discovered that, due to its small size, *H. bivittatus* did not appear to have enough blood to make this method feasible. Instead, we had to use lethal sampling and obtain whole-body cortisol measurements, as in Ramsay et al. (2006). Whole-body cortisol levels have been used to assess the stress response of developing salmonids and sturgeon because blood volumes are insufficient to provide measurements of circulating cortisol (de Jesus & Hirano 1992, Simontacchi et al. 2009). Similarly, whole-body corticosteroids have been measured in smaller adult fishes such as three-spined sticklebacks (Pottinger et al. 2002). In the present study, cortisol measurements were standardized per mass of tissue, so the inverse relationship with size was not an artefact of sampling but instead relates to fish life history. The fact that smaller fish had higher levels of whole-body cortisol suggests that these individuals may be more sensitive to stressors such as noise or handling stress. Our study did not resolve the role of sex in whole-body cortisol levels. *H. bivittatus* are protogynous hermaphrodites, and the exact size at which they transition from female to male may vary across populations (Robertson et al. 1978). However, using a general assumption that they change sex at 80% of maximum total body length (Allsop & West 2003), with the maximum length of 35 cm (Froese & Pauly 2019), this would occur at 28 cm. All of our fish were <15 cm, so it is reasonable to assume that they were either juveniles or females. Future studies could specifically target large males (based on coloration) to further examine the potential relationship between reproductive state and cortisol response.

We recognize that we did not obtain a positive control in this study which would improve the tractability of boat noise as a stressor and the validity of whole-body cortisol as a useful biomarker across treatments. However, cortisol is indeed the universal biomarker for evaluating stress in wild fish (Barton 2002), and conducting numerous treatments, beyond exposing fish to boat noise, was beyond the scope of the study. While there is a general trend in the literature towards observing positive physiological and behavioral effects of boat noise in temperate and tropical fish, those results have largely been obtained

in laboratory settings (Cox et al. 2018). Our findings suggest that null results or subtle effects (e.g. Maxwell et al. 2018) may be more common in field-based studies than previously thought. We also learned that these more subtle effects can be masked by experimental procedures involving handling.

5. CONCLUSIONS

Taken together, our results suggest that boat noise does not represent a significant stressor for *Haliichoeres bivittatus*. Despite living in very different acoustic conditions (Fig. 1B), fish from the 2 sites exhibited no differences in baseline (i.e. chronic) stress. When exposed to boat noise at similar levels that they would experience in these 2 habitats, fish from the quiet site showed no differences and fish from the noisy site showed slightly higher cortisol levels, although direct comparisons between sites were confounded by differences in fish body length. The biggest effects observed related to body size and experimental handling. Smaller fish and all fish that were held in the experimental cages exhibited a heightened stress response. This result suggests that being handled represents a proximal stressor, perhaps simulating a predation event, to which small fish are particularly sensitive. Boat noise — especially if perceived as coming from a distant boat — may represent a more benign threat that does not pose an immediate risk to survival. To our knowledge, this is the first study to demonstrate that wild reef fishes may be somewhat resilient or indifferent to the presence of boat noise. This work stands in contrast to the majority of recent studies on fish and noise, underscoring the need to consider species-specificity when assessing the overall impacts of anthropogenic noise on marine life.

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Appendix.

Table A1. Two-way ANCOVA to test the effects of experimental handling stress, collection site, and noise treatment on fish cortisol levels. Pairwise multiple comparison test results are shown. For each treatment pair, the difference in y-intercepts and corresponding p-values are reported. SPB: collected at STRI Point, baseline; SPC: collected at STRI Point, control treatment; SPN: collected at STRI Point, noisy treatment; HPB: collected at Hospital Point, baseline; HPC: collected at Hospital Point, control treatment; HPN: collected at Hospital Point, noisy treatment

Comparison	Difference	p-value
SPB vs. HPB	-0.1790	0.5228
SPB vs. SPC	-0.6763	0.0000
SPB vs. HPC	-0.6047	0.0000
SPB vs. SPN	-0.6774	0.0000
SPB vs. HPN	-1.0683	0.0000
HPB vs. SPC	-0.4973	0.0004
HPB vs. HPC	-0.4256	0.0045
HPB vs. SPN	-0.4983	0.0003
HPB vs. HPN	-0.8893	0.0000
SPC vs. HPC	0.0716	0.9914
SPC vs. SPN	-0.0011	1.0000
SPC vs. HPN	-0.3920	0.0193
HPC vs. SPN	-0.0727	0.9903
HPC vs. HPN	-0.4636	0.0019
SPN vs. HPN	-0.3909	0.0157

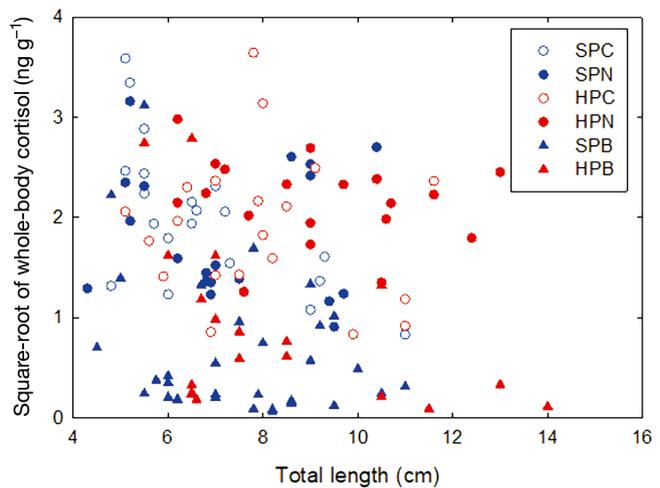


Fig. A1. Whole-body cortisol levels (ng g^{-1}) and body length (cm) for *Halichoeres bivittatus* from STRI Point (blue) and Hospital Point (red) after *in situ* acoustic playback experiments. Treatment groups were STRI Point or Hospital Point 'control' (SPC and HPC), 'noise' (SPN and HPN), and baseline (SPB or HPB)

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