



Boat noise impacts Lusitanian toadfish breeding males and reproductive outcome



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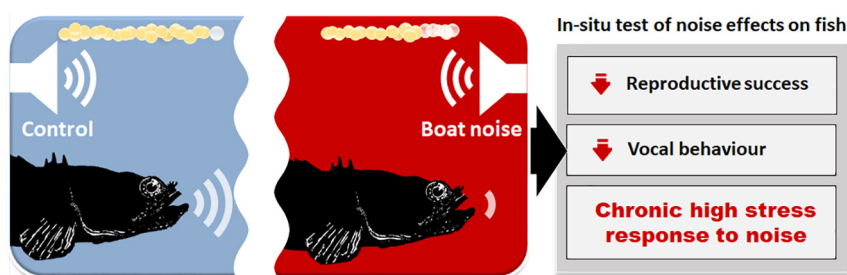
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HIGHLIGHTS

- The effect of boat noise on toadfish fitness was assessed *in situ*.
- Chronic noise exposure impacted calling behaviour and reproductive success.
- Biomarkers and cortisol levels point to stress induced freezing behaviour.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 20 January 2022

Received in revised form 14 March 2022

Accepted 17 March 2022

Available online 23 March 2022

Editor: Daniel Wunderlin

Keywords:

Chronic boat noise

Vocal fish

Stress response

Biomarkers

Field experiments

Fitness

ABSTRACT

Anthropogenic noise is a growing threat to marine organisms, including fish. Yet very few studies have addressed the impact of anthropogenic noise on fish reproduction, especially *in situ*. In this study, we investigated the impacts of boat noise exposure in the reproductive success of wild Lusitanian toadfish (*Halobatrachus didactylus*), a species that relies on advertisement calls for mate attraction, using behavioural, physiological and reproductive endpoints. Two sets of artificial nests were deployed in the Tagus estuary and exposed to either ambient sound or boat noise during their breeding season. Toadfish males spontaneously used these nests to breed. We inspected nests for occupation and the presence of eggs in six spring low tides (in two years) and assessed male vocal activity and stress responses. Boat noise did not affect nest occupation by males but impacted reproductive success by decreasing the likelihood of receiving eggs, decreasing the number of live eggs and increasing the number of dead eggs, compared to control males. Treatment males also showed depressed vocal activity and slightly higher cortisol levels. The assessment of oxidative stress and energy metabolism-related biomarkers revealed no oxidative damage in noise exposed males despite having lower antioxidant responses and pointed towards a decrease in the activity levels of energy metabolism-related biomarkers. These results suggest that males exposed to boat noise depressed their metabolism and their activity (such as parental care and mate attraction) to cope with an acoustic stressor, consistent with a freezing defensive response/behaviour. Together, our study demonstrates that boat noise has severe impacts on reproductive fitness in Lusitanian toadfish. We argue that, at least fishes that cannot easily avoid noise sources due to their dependence on specific spawning sites, may incur in significant direct fitness costs due to chronic noise exposure.

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1. Introduction

Fishes are the most abundant extant group of vertebrates accounting for a large proportion of Earth's biomass. They represent, for example, 7-fold and 100-fold more biomass than livestock and the ensemble of marine and terrestrial wild mammals, respectively (Bar-On et al., 2018). Fishes have adapted to multiple aquatic environments showing a remarkable diversity in behaviour, anatomy, physiology and ecological adaptations (Helfman et al., 2009). Indeed, their notable abundance and biodiversity coupled with ubiquitous distribution make fish pivotal in aquatic ecosystem services, namely by taking major roles in controlling trophic networks, in regulating nutrient cycles and protein supply, and in cultural services such as recreational activities (Villéger et al., 2017). However, anthropogenic pressures are modifying aquatic ecosystems at unprecedented rates, with over-exploitation, introduction of non-native species, habitat loss, climate warming and different forms of pollution, all impacting fish biodiversity and reducing the provision of ecosystem services regulated by fish communities (Villéger et al., 2017).

One important form of aquatic pollution is noise resulting from human activities such as marine traffic, seismic exploration, pile driving, and sonar, which is changing the natural underwater soundscapes globally (Hildebrand, 2009; Popper and Hawkins, 2019). Natural aquatic soundscapes (sound that emanates from biotic and abiotic sources) can deliver crucial information to fish and other animals as sound travels faster and usually further in water than other sensory modalities, providing an invaluable mean to obtain rapid, directional, and long-distance information (Hawkins and Popper, 2018). Aquatic noise pollution has become the focus of many recent studies providing evidence that it can affect many fitness-related traits in fish and other animals (Kunc et al., 2016; Cox et al., 2018; de Jong et al., 2020; Di Franco et al., 2020). In fish, aquatic man-made noise can mask acoustic information, change behaviour, affect physiology such as by increasing stress levels, and cause physical injuries or death; the level of impact depends on noise levels, its frequency and temporal patterns, and distance from the source (Hawkins and Popper, 2018; Duarte et al., 2021; Pine et al., 2021). For example, relatively low levels of underwater noise can affect foraging behaviour or the ability to escape predators due to attention shifts and/or elevated stress levels (Purser and Radford, 2011; Simpson et al., 2016), and increase hearing thresholds due to masking (Alves et al., 2021). In turn, high noise levels are associated, for example, with hearing impairment through damage to the sensory hair cells in the inner ear (e.g., Breitzler et al., 2020).

Of particular concern is the impact due to low-frequency continuous noise generated by boat traffic, which overlaps fishes' hearing ranges, and exhibits an ever-increasing trend in aquatic ambient sound levels (Širović et al., 2016). In a recent study, de Jong et al. (2020) showed that underwater noise, particularly boat noise, has a high likelihood to impact different stages of reproduction such as sexual maturation, establishment of spawning grounds, spawning success and hatching success. Yet, very few studies have addressed the impact of anthropogenic noise on fish reproduction especially in the natural environment, either freshwater or marine (courtship behaviour - Krahforst, 2017; Brown et al., 2021; clutch number and size - Krahforst, 2017; parental care - Nedelec et al., 2017; Maxwell et al., 2018; offspring survival - Nedelec et al., 2017; Faria et al., 2022). To date, only two studies have assessed the impact of boat noise on reproductive outcome in marine fish *in situ*, and both found deleterious effects (Krahforst, 2017; Nedelec et al., 2017).

To investigate the impacts of boat noise exposure in a wild fish population we assessed the effects of boat noise playback in the reproductive success of the Lusitanian toadfish, *Halobatrachus didactylus* (Batrachoididae), in a natural breeding site located near an important ferryboat lane in the Tagus estuary, using behavioural, physiological and direct fitness endpoints. Males of this species nest under rocks or other hard substrates in the intertidal/subtidal areas and produce advertisement calls to attract ripe females (Amorim and Vasconcelos, 2008). Females lay adhesive eggs in the nest and leave the male to provide parental care until the fry are free-swimming (MCP Amorim, personal observation). As nests can be

accessible during spring low tides this species is an excellent model to test the impact of anthropogenic noise *in situ*. We predicted that exposure to boat noise would impact reproductive outcome in this species by affecting one or a combination of possible factors: the occupation of spawning grounds, reproductive behaviour (e.g., male calling behaviour), male stress responses, and reproductive outcome assessed by clutch size.

2. Methods

2.1. Experimental design

The experimental setup consisted of two sets of 12 concrete shelters (50 cm long, 30 cm wide and 20 cm max height) spaced evenly every 2 m in 2 rows (6 nests per row) parallel to the shoreline (Fig. S1a), deployed in an intertidal area of the Tagus estuary (Air Force Base no. 6, Montijo, Portugal; 38°42'N, 8°58'W). Male toadfish (Fig. S1b) spontaneously occupied these shelters during the breeding season (May to July 2016 and 2017) and used them as nests. The shelters were internally lined with a removable plastic sheet where the females laid their eggs. The setup was usually submersed (up to c. 2.8 m water depth) and only exposed to air (or partially exposed) at spring low tides approximately every fortnight, when nests were inspected for occupation and the presence of eggs. Water temperature, measured by a temperature and pressure datalogger (HOBO-U20-001-01, Onset Computer Corp., MA, USA), ranged 17–30 °C in both years.

Twelve custom-made hydrophones were placed next to each male shelter in a mid-lateral position about 10 cm above the substrate (Fig. S1a) and connected to a 16 channel stand-alone data logger (Measurement Computing Corporation LGR-5325, Norton, Virginia, USA, 16 bits resolution, 4 kHz sampling rate). Round-the-clock simultaneous recordings were made to monitor male vocal activity.

Three UW30 underwater loudspeakers (frequency response 0.1–10 kHz, Electro-Voice, Columbus, USA) per nest-set were evenly spaced between the two nest rows and fixed to the substrate facing upwards (Fig. S1a). Each was connected to an amplifier (Sony XM-N1004, Tokyo, Japan) and a mp4 device (A730 Music Player, HOTT, Shenzhen, China). During a fortnight the loudspeakers exposed each nest-set to either ambient sound (control) or boat noise (treatment). Noise treatment mimicked the passages of 10 ferries (Tanstejo ferry boats) plus 4 small outboard engine boats per hour during 18 h (from 6 h00 to 24 h00), similar to what fish experience on average in Tagus estuary (Faria et al., 2022). Playback was set at a level 30 dB (20–40 dB RMS of 5 s centred in the noise of each boat passage) above background corresponding to the increase caused by a ferryboat recorded c. 50–100 m away from the pier near our study site and comparable to the noise level elevation due to boating reported in other studies (Magnhagen et al., 2017; Nedelec et al., 2017; de Jong et al., 2018a; Blom et al., 2019). The ambient sound treatment (control) replaced boat noise by background environmental sound recorded in this area, played back above the background noise level with the same amplification used in the boat noise treatment intending to control for any electromagnetic effects caused by a working loudspeaker. Playback treatment was randomly assigned to a nest-set and swapped in the subsequent fortnight. Three trials (fortnights) were carried out in each year. Nest-sets were sufficiently spaced to prevent noise played treatment being received in the other nest-set.

Sound playbacks were calibrated for pressure with a hydrophone (Bruel & Kjaer 8104, Naerum, Denmark; sensitivity -205 dB re. 1 V μPa^{-1} ; frequency response from 0.1 Hz to 180 kHz) connected to a sound level meter (Bruel & Kjaer 2238 Mediator, Naerum, Denmark) and for particle motion with a 3-axis accelerometer (M20-040, sensitivity 1–3 kHz, GeoSpectrum Technologies, Dartmouth, Canada). Both sensors were located side by side c. 20 cm from the bottom, with c. 1 m water depth and were connected to a 4-channel digital recorder (Edirol R4, Roland Corporation, Tokyo, Japan); the input voltage of each channel used to record the 3 axis accelerometer outputs (xx, yy or zz) and the sound level meter were calibrated by recording sinusoidal waves at pre-set amplitude voltage.

Boat noises used in the playback were recorded nearby at Air Force Base no. 6 pier and comprised noise produced by two small private open deck boats with an outboard engine at 7–20 m from the hydrophone (rms 120–140 dB re. 1 μ Pa, calculated in the 0–20 kHz bandwidth or rms 104–133 dB re. 1 μ Pa, calculated in the 0–2 kHz bandwidth), and two passages of ferryboats that regularly cross the Tagus estuary (50–100 m; rms 122–131 dB re. 1 μ Pa or rms 117–127 dB re. 1 μ Pa calculated in the 0–2 kHz bandwidth) (Fig. S2). Note that playback cannot fully recreate boat noise both in sound pressure and particle motion, particularly at frequencies lower than 100 Hz (Fig. S2). Also, playback levels varied with tide level due to changes in the output of the speakers resulting from variations in water pressure.

To assess stress and immunological response to playback treatment in parental males in each sampling time point, males with eggs were removed from the nests in each nest-set, anaesthetised in a salt water bath with tricaine methane sulphonate (MS222; PHARMAQ, Norway) buffered 1:1 with sodium bicarbonate, sampled for blood (collected from the caudal vein with heparinized syringes within 4 min from first handling the fish), and immediately sacrificed with an excessive dosage of MS222 and dissected. Upon collection, plasma from blood samples was separated by centrifugation (2000 g for 5 min) on site, placed in microtubes in dry ice and taken to the lab, where they were stored at -20°C until cortisol quantification or at -80°C until evaluation of biochemical biomarkers. Samples of body muscle (epaxial muscle) and liver were collected and immediately placed in dry ice until stored in the lab at -80°C . Plastic sheets with eggs were photographed and replaced by new ones. The number of obtained eggs, both live and apparently dead (empty or white eggs, Fig. S1b), was tallied as a proxy for male reproductive success. All fish occupying the nests, including non-parental males, were measured to the nearest mm for total length (TL) and to the nearest g for body mass (M). Non-parental males were released back to the river and swam away from the study site.

This study was authorized by the Portuguese National Authority for Animal Health (Direção Geral de Alimentação e Veterinária), performed in strict accordance with the EU Directive 2010/63/EU for animal experiments and followed the recommendations of the Animal Care and Use Committee of the Faculty of Sciences, University of Lisbon.

2.2. Male acoustic activity analysis

Boatwhistles recorded in two fortnights (during one breeding season, from June to July 2016) of round-the-clock simultaneous recordings were labelled with the help of the matched filter function of the software Ishmael 1.0 (Mellinger, 2002) for 16 males that colonized the nests (8 from control and 8 from boat noise treatment). Recorded sounds could unequivocally be attributed to a particular male as sounds produced by males occupying adjacent nests arrived considerably attenuated (over 6 dB). These files were then screened with Adobe Audition 3.0 (Adobe Systems, San José, CA, USA) to verify the correctness of boatwhistle (BW) identification by the automated procedures of Ishmael. Only the recordings corresponding to a water level above 1.5 m were considered as Lusitanian toadfish males present a very low vocal activity during low tide at this site (Amorim et al., 2011; Vieira et al., 2021a). We quantified mean BW calling rate per hour (Mean CR), maximum BW calling rate (Max CR), mean active BW calling rate (Mean Active CR; i.e. only considering the hours where BWs were registered), and number of hours calling (number of hours when males produced more than 15 BWs) in each fortnight. These calling parameters were tallied for 11 days in each fortnight (ending on the day before sampling, see Fig. 3), as new males took some time to occupy shelters after sampling.

2.3. Cortisol response

Cortisol levels (CORT) were quantified by radioimmunoassay (RIA). From each sample, 100 μ l of plasma were extracted for free and conjugated steroids using the techniques described in Scott and Canário (1992) and

Damasceno-Oliveira et al. (2012). RIA methodology has been published elsewhere (Scott et al., 1984) and cross reactions were previously described in Vasconcelos et al. (2012). Intra-assay and inter-assay precision (coefficient of variation) for RIA were 3.2 and 9.8%, respectively. For logistic reasons we only analysed 2016 data ($N = 16$ males).

2.4. Biochemical responses

2.4.1. Tissue preparation

Approximately 200 mg of liver tissue from each organism ($N = 51$ males) were homogenized using an Ystral d-79,282 homogenizer. The samples were homogenized in a 1:10 (w:v) proportion using K-phosphate buffer (0.1 M, pH 7.4). To assess lipid peroxidation (LPO), part of the homogenate was transferred to a microtube containing an antioxidant (2,6-di-*tert*-butyl-4-methylphenol 4% in methanol). A second portion was separated for the quantification of DNA strand breaks. A third portion of the homogenate was centrifuged at 10,000 g for 20 min (4°C). The obtained post mitochondrial supernatant (PMS) was then aliquoted into different microtubes for posterior protein quantification, as well as for the activity measurements of catalase (CAT) and superoxide dismutase (SOD). Approximately 200 mg of muscle tissue from each animal ($N = 51$ males) were homogenized, in a 1:5 (w:v) proportion, in K-phosphate buffer (0.1 M, pH 7.4) by ultrasonication (4 cycles of 5 s; A: 40%). The homogenate volume was then separated into 2 fractions, one for the evaluation of the electron transport system (ETS) activity and another that followed a centrifugation of 3,000 g for 5 min (4°C). The obtained supernatant was aliquoted into different microtubes for posterior protein quantification, as well as for the activity measurements of isocitrate dehydrogenase (IDH) and lactate dehydrogenase (LDH). Plasma samples ($N = 41$ males) were also separated for later evaluation of humoral defence enzymes (i.e. myeloperoxidase and lysozyme) and cholesterol concentration. All microtubes were stored at -80°C until the day of the respective assay. Every spectrophotometric measurement was performed at 25°C using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Vermont, USA).

2.4.2. Protein quantification

The protein concentration of the supernatants, needed for normalization of measured parameters, was quantified as described by Bradford (1976), adapted from BioRad's Bradford microassay set up in 96-well flat bottom plate, and using the bovine γ -globulin (BGG, Sigma-Aldrich, USA) as standard. Absorbance was read spectrophotometrically at 600 nm and the results were expressed in mg of protein/mL.

2.4.3. Oxidative damage and oxidative stress parameters

The DNA strand breaks were determined according to the DNA alkaline precipitation assay by Olive (1988) adapted from LaFontaine et al. (2000). The damaged DNA present in the supernatant links to Hoesch dye. The fluorescence was measured with an excitation/emission wavelength of 360/460 nm. Calf thymus DNA was used as standard, and the results were expressed as $\mu\text{g DNA/g}$ of wet weight (ww). The LPO levels were measured using the method described by Ohkawa et al. (1979) and Bird and Draper (1984), adapted by Wilhelm Filho et al. (2001) and Torres et al. (2002). The LPO products were quantified colorimetrically at 535 nm using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol of TBARS/g of ww. CAT activity was determined based on the decrease in absorbance resulting from the consumption of substrate (hydrogen peroxide) following the protocol of Clairborne (1985). The activity was monitored at 240 nm for 1 min, using a molar extinction coefficient of $40 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed in $\mu\text{mol/min/mg protein}$. SOD activity was determined according to McCord and Fridovich (1969), adapted to microplate (Lima et al., 2007). This method is based on the reaction of generated superoxide radicals from xanthine/xanthine oxidase with consequent reduction of cytochrome C, measured at 550 nm for 10 min. SOD activity was expressed in U/mg of protein, being 1 U the amount of enzyme in the sample that causes 50% inhibition of cytochrome C reduction.

2.4.4. Energy metabolism biomarkers

ETS activity was measured according to De Coen and Janssen (1997), following formazan generation at 490 nm for 3 min. The cellular oxygen consumption rate was determined using a stoichiometric relationship (2 μmol INT-formazan formed to 1 μmol oxygen consumed) and the activity was expressed in $\text{nmol O}_2/\text{h/g}$ of ww. IDH activity was determined following Ellis and Goldberg (1971) with the adaptations of Lima et al. (2007). This method is based in the measurement of NADPH increase, resulting from decarboxylation of isocitrate by IDH, at 340 nm during 3 min. The results were expressed as $\text{nmol}/\text{min}/\text{mg}$ of protein, using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. LDH activity was assessed according to Vassault (1983) and adapted by Diamantino et al. (2001), following the oxidation of NADH when pyruvate is converted to lactate at 340 nm for 5 min. The results were expressed as $\text{nmol}/\text{min}/\text{mg}$ of protein, using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The cholesterol levels in plasma were determined using the Accutrend Plus instrument (Roche) according to the manufacturer's instructions. The measurement was performed using the Accutrend test strips by photometric light reflection and the results were expressed in mg/dL .

2.4.5. Humoral defence enzymes

Myeloperoxidase (MPO) was determined according to Quade and Roth (1997) with adaptations of Lazado et al. (2015). This method is based on the oxidation of a substrate (3,3',5,5'-tetramethylbenzidine) by hydrogen peroxide (H_2O_2), measured at 450 nm. Lysozyme (LZ) was quantified according to Costas et al. (2011). This method is a turbidimetric assay based on the lysis of *Micrococcus lysodeikticus* at 450 nm. Lyophilized hen egg white lysozyme was used to obtain a standard curve and the amount of lysozyme present in plasma was calculated using this curve and given as $\mu\text{g}/\text{ml}$.

2.5. Statistical analysis

We tested differences in male TL and condition ($K = M/\text{TL}^3$) between treatment groups with linear mixed models (LMM) using sampling date as random factor and treatment as a fixed factor.

The effect of playback treatment on whether males occupied nests (yes/no) and received eggs (after occupying nests, yes/no) was modelled with generalized linear models (GLM, binomial family, link function: logit). Sampling date was included in the models as a random factor. As sampling date did not show a significant effect on the chances of receiving eggs it was removed from the model.

We first assessed the effect of playback treatment on the number of obtained live eggs (a proxy for reproductive success) and the number of dead eggs with LMMs including sampling date as a random factor, treatment as a fixed factor and male TL and K as covariates. Egg number data were log-transformed to meet the model assumptions. As sampling date had no effect on the number of live and dead eggs it was removed from the model.

We tested the effect of treatment on acoustic variables with LMMs including date as a random factor. As we did not find a significant effect of sampling date we used *t*-tests instead. We explored the relation between stress / immune response variables and acoustic parameters with non-parametric Spearman rank correlations as the sample size was small (these responses were only measured in 10 of the fish for which we tallied acoustic activity).

Similarly, we tested the effect of treatment on stress and humoral responses (CORT and all other tested biochemical markers) with LMMs including sampling date as a random factor. The covariates male TL and K were only included in the LMM for plasma lysozyme as it was the only case with a significant effect. When the effect of sampling date was also not significant, we performed *t*-tests instead. LDH-muscle was square root-transformed to meet the model assumptions. The relation between the number of live and dead eggs, and between the number of dead eggs and cortisol was investigated with Pearson correlations. The relation among biomarkers was also explored with Pearson correlations. In addition, we performed a principal component analysis (PCA) to explore

associations between different endpoints (number of live and dead eggs and biomarkers) and noise exposure; serum biomarkers were not considered as the sample size was smaller and were not correlated with egg number or other biomarkers. All variables were log-transformed. Only Principal Components (PC) with Eigenvalues >1 were accepted. We performed *t*-tests on PC scores to investigate the effect of treatment on these new composite variables; sampling date did not have a significant effect.

3. Results

3.1. Nest occupation and breeding success

Males occupying the nests in each sampling period (fortnight) were smaller but with a higher condition factor towards the end of the breeding season (Fig. S3). There was no difference in male TL (LMM, treatment $F_{1,73} = 0.00, P = 0.99$, sampling date $F_{5,73} = 3.19, P = 0.01$) and K (treatment $F_{1,73} = 0.00, P = 0.90$, sampling date $F_{5,73} = 4.73, P < 0.001$) between treatment groups. On average males from environment playback group ($N = 37$) had 38.9 cm TL (\pm SD, range: $\pm 4.1, 30.1\text{--}45.6$ cm) and a condition factor of 1.84 ($\pm 0.19, 1.49\text{--}2.29$) and males from the boat noise playback group ($N = 43$) had on average 38.8 cm TL ($\pm 4.1, 31.2\text{--}48.4$ cm) and a condition factor of 1.82 ($\pm 0.20, 1.46\text{--}2.41$).

We found no effect of treatment in nest occupation (Wald $\chi^2 = 0.51, \text{df} = 1, P = 0.47$). Sampling date had an effect on the likelihood of males occupying a nest, and so it was included as a random effect (95% profile CI for variance due to the sampling date = $[0.075, 2.93]$). In six sampling dates we observed 44 nest occupations by breeding males in the boat treatment vs. 40 in the control. In contrast, treatment had an effect on the likelihood of males receiving eggs in their nest (Wald $\chi^2 = 4.70, \text{df} = 1, P = 0.03$). Nest-holders exposed to environmental-noise playback were more likely to mate successfully than the ones exposed to boat-noise playback. 82.5% of the males under environmental-noise received eggs ($N = 40$) in contrast with 61.4% of the males under boat-noise ($N = 44$). Sampling date did not have a significant effect on the likelihood of receiving eggs and was not included in the model.

Playback treatment had a significant effect on the number of live eggs ($P = 0.03$) and on the number of dead eggs ($P = 0.01$) found in the male's nest (Table 1, Fig. 1). Males exposed to environmental-noise got more live eggs (mean \pm SD, range: $714.9 \pm 751.9, 0\text{--}3230, N = 40$) than males exposed to boat-noise ($513.2 \pm 660.7, 0\text{--}2358, N = 44$). The opposite trend was found for dead eggs. Males exposed to environmental-noise had on average 60.8 dead eggs ($\pm 134.5, 0\text{--}690, N = 32$) in contrast with the 148.7 ($\pm 196.5, 0\text{--}901, N = 27$) dead eggs found in the nests of males exposed to boat-noise. There was no relation between the number of live and dead eggs (Pearson correlation, $N = 59, r = 0.18, P = 0.16$).

3.2. Calling behaviour

Males from the boat noise treatment showed a lower calling activity than control males. Males exposed to boat noise showed a lower Mean CR (2016 data, *t*-test, $t = 2.79, \text{df} = 14, P = 0.01$), Max CR ($t = 2.81, \text{df} =$

Table 1

Effect of playback treatment on the number of live and dead eggs. Results are from linear model testing the effect of treatment and include male total length (TL) and condition factor (K) as covariates.

Dependent variable	Effect	df	F	P
Log no. live eggs	Intercept	1,76	0.34	0.56
	Treatment	1,76	4.63	0.03
	TL	1,76	4.81	0.03
	K	1,76	4.01	0.049
Log no. dead eggs	Intercept	1,52	10.67	0.002
	Treatment	1,52	6.96	0.01
	TL	1,52	4.04	0.049
	K	1,52	5.30	0.03

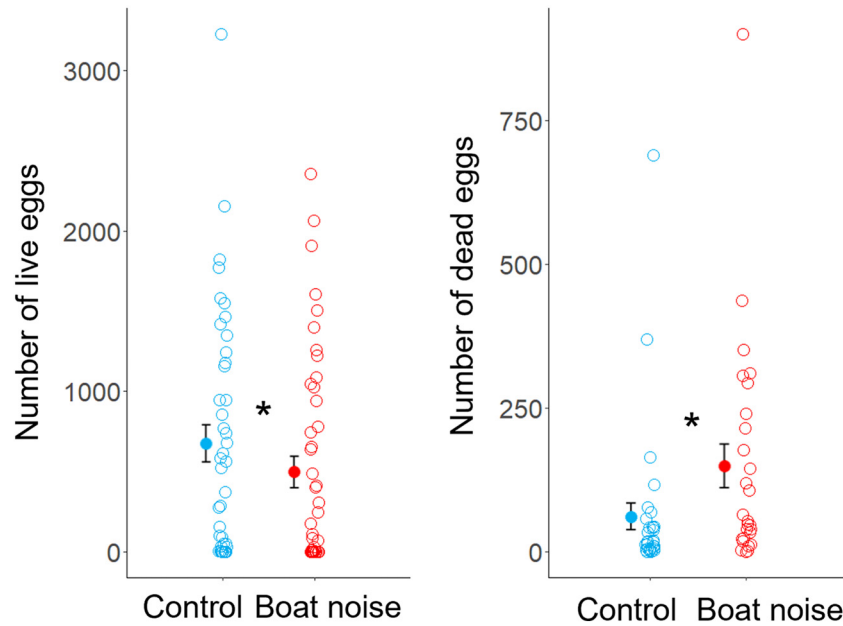


Fig. 1. Effects of treatment on the number of live and dead eggs in males' nests after up to two weeks of noise exposure. Data are means and S.E. * = $P < 0.05$.

14, $P = 0.01$), Active Mean CR ($t = 2.78$, $df = 14$, $P = 0.01$), and called for fewer hours ($t = 2.77$, $df = 14$, $P = 0.02$) than males exposed to ambient noise (Table S1, Figs. 2 and 3). Fig. 3 shows that for both treatment groups calling activity was initially low and only built up some days after spring low tides (when males occupy the nest-sets), denoting the time that took for nest occupation and for the start of chorusing behaviour. In the first fortnight the onset of calling activity was similar between groups but in the second fortnight the males from the control group started to call before the males from the noise exposed group. After the onset of chorusing activity, the calling activity of control males was higher than in noise treatment males.

The number of dead eggs was negatively correlated with Mean CR ($R = -0.66$, $P = 0.04$, $N = 10$), Max CR ($R = -0.73$, $P = 0.02$), mean Active CR ($R = -0.69$, $P = 0.03$) but not with the number of hours calling ($R = -0.44$, $P > 0.05$), indicating that the males that were less acoustically active had a higher number of dead eggs. The number of live eggs was not

correlated with any of the acoustic variables ($R = 0.02-0.16$, $P > 0.05$, $N = 14$).

3.3. Stress and humoral innate immune responses

Boat noise males presented slightly higher values of CORT than the control males (2016 data, t -test, $t = -1.89$, $df = 15$, $P = 0.08$, Table S2, Fig. 4a). In addition, CORT levels were significantly correlated with the number of dead eggs ($N = 16$, $R = 0.62$, $P = 0.01$).

Playback treatment had a significant effect on the oxidative stress biomarker CAT ($P = 0.002$, Tables 2, S2, Fig. 4a), with males exposed to boat noise presenting on average lower values. We did not find a significant effect on DNA damage or SOD activity ($P > 0.05$), but LPO levels were slightly lower in the boat noise group males ($P = 0.08$).

Likewise, we did not find a significant effect for noise exposure in respect to energy metabolism related biomarkers, but LDH was slightly

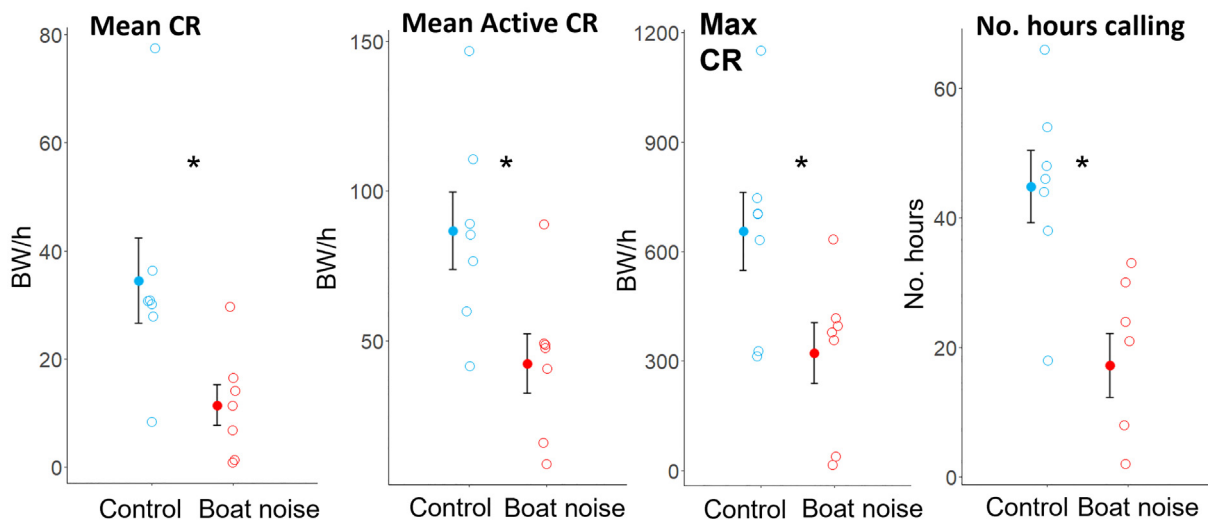


Fig. 2. Effect of treatment on calling activity of 16 nesting males (data for 2016). Mean BW calling rate per hour (Mean CR), mean active BW calling rate (Mean Active CR; only considering the hours where BWs were registered), maximum BW calling rate (Max CR) and number of hours calling are presented. Data are means and S.E. * = $P < 0.05$.

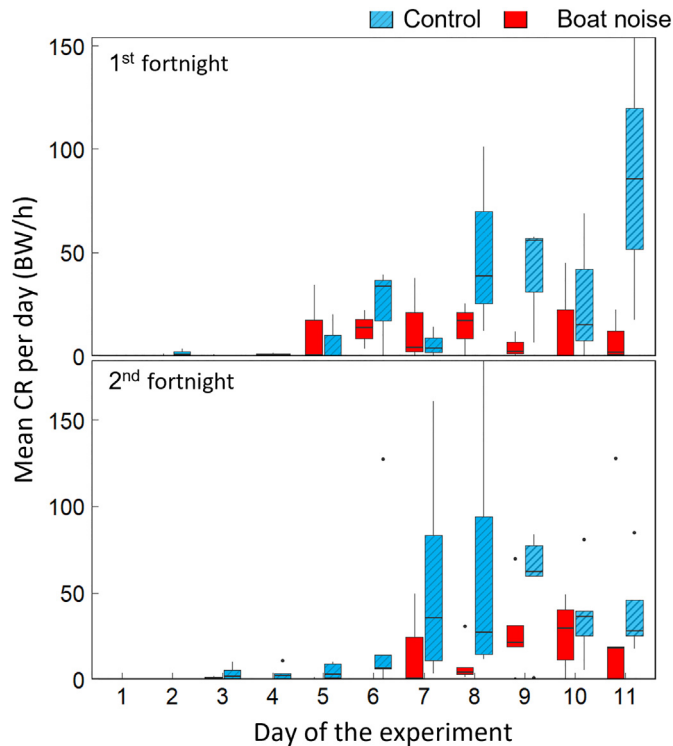


Fig. 3. Daily mean calling rate of 16 nesting males exposed to boat noise ($N = 8$) and control ($N = 8$) in two consecutive fortnights (data for 2016). Boxplots represent medians, 25th and 75th percentiles, range and outliers.

lower ($P = 0.08$) in males exposed to boat noise. The energy metabolism enzymes LDH, IDH and ETS were positively correlated (Table 3), consistent with their high loadings on the first component of the PCA considering oxidative stress and energy metabolism related biomarkers, and the number of live and dead eggs (Fig. S4). The PCA considered the first three components and explained 62% of data variance (PC1–25.0%, PC2–19.5%, PC3–17.5%). The energy metabolism related biomarkers (LDH, IDH and ETS) loaded on PC1 with loadings larger than 0.75, while the number of dead eggs also loaded on PC1 with a factor loading score of -0.45 . The oxidative stress biomarkers SOD, LPO and DNA damage contributed to PC2 (factor loading score > 0.65) and the number of live eggs and CAT to PC3 (factor loading scores of -0.81 and 0.73 respectively) (Fig. S4). PC1 scores, which also represented the number of dead eggs, were lower in males exposed to boat noise ($t = 2.03$, $df = 48$, $P = 0.048$) pointing to a decrease of metabolism in these males associated with egg mortality (Fig. 4b). PC2 ($t = 0.86$, $df = 48$, $P > 0.05$) and PC3 ($t = 0.67$, $df = 48$, $P > 0.05$) did not differ between treatment groups. We did not find a treatment effect in any immune-related biomarker (Table 2).

We explored the relation between calling activity and stress / immune response variables. Active mean CR (Spearman correlation, $N = 10$, $R = -0.67$, $P = 0.03$) and the number of hours spent calling ($N = 10$, $R = -0.69$, $P = 0.03$) were negatively correlated with CORT levels. We did not find a significant correlation with CORT for Mean CR ($R = -0.56$, $P = 0.09$) and Max CR ($R = -0.47$, $P = 0.17$). Regarding biochemical responses, IDH was positively correlated with all acoustic variables: Mean CR (Spearman correlation, $N = 10$, $R = 0.66$, $P = 0.04$), Max CR ($R = 0.71$, $P = 0.02$), Active Mean CR ($R = 0.71$, $P = 0.02$) and number of hours spent calling ($R = 0.72$, $P = 0.02$). In contrast, MPO was negatively correlated with all acoustic variables: Mean CR ($R = 0.70$, $P = 0.03$), Max CR ($R = 0.79$, $P = 0.006$), Active Mean CR ($R = 0.81$, $P = 0.004$), except for the number of hours spent calling ($R = -0.32$, $P = 0.37$). We did not find significant correlations between the other biomarkers and the acoustic variables ($R = -0.61$ – 0.52 , $P > 0.05$).

4. Discussion

Wild Lusitanian toadfish exposed to chronic boat noise (up to 2 weeks) were negatively affected in terms of reproduction outcome, physiology and behaviour. Boat noise did not seem to change nest occupation but affected reproductive success by decreasing the likelihood of receiving eggs, decreasing the number of live eggs and increasing the number of dead eggs, compared to males exposed to ambient noise. Boat noise exposed males also showed a heightened stress response and depressed calling activity. Although we used playbacks and not real boat passages, our field experiment mimicked a typical pattern of noise exposure by common ferry boats and small outboard engine boats in the Tagus estuary, pointing to marked detrimental fitness effects, even with only up to 2 weeks of noise exposure from two boat types and without the possible effect of low frequencies below c. 100 Hz. Toadfish parental males will likely experience a higher noise exposure from boating as parental care takes up to 3 months during which males stay in the nest to care for the eggs and defend the nest from takeovers or egg predation (Félix et al., 2016). Also, depending on the breeding site, they will be exposed to more boat noise sources, such as large cruisers, cargo vessels and dredgers (Vieira et al., 2021b), which tend to be louder (Hildebrand, 2009) and likely more impacting (Putland et al., 2018), not to mention other forms of anthropogenic noise (Vieira et al., 2021b). We argue that, at least fish that cannot easily avoid noise sources due to their dependence on specific spawning sites, such as nesting areas, may incur in significant direct fitness costs due to chronic noise exposure.

4.1. Nest occupation and breeding success

In the six studied fortnights, the Lusitanian toadfish males occupied nests irrespective of playback treatment. Despite boat noise nest-set having less quality than the ambient noise, competition for appropriate spawning substrate is high during the peak of the breeding season leading to the occupation of less favourable nests (Amorim et al., 2010a), as the alternative may be losing the opportunity to reproduce at all. This is consistent with Lusitanian toadfish males decreasing in size with the progression of the reproductive season suggesting that smaller males only get access to these nests when competition starts to decrease. In line with this suggestion, Bose et al. (2019) recently found that the batrachoidid plainfin midshipman, *Porichthys notatus*, preferred to nest in the lower intertidal zone but still occupied nests in the higher levels of the intertidal gradient (especially smaller males) despite facing higher physiological stress (increased anaerobic metabolism) and greater offspring mortality. These authors also attributed this pattern of nest occupation to the high competition for nest sites in the breeding season.

Noise treatment had an effect on the chances of mating successfully. A higher number of males from environmental-noise group (control) had clutches in their nest compared to males exposed to boat-noise. Importantly, control males had more live eggs and fewer dead eggs than males exposed to boat-noise. This indicates that although boat noise exposure does not seem to affect nest occupation it yields important fitness costs in terms of viable offspring. The lower chances of mating in the boat-noise groups could be related to a diminished ability of females to locate calling males as the boat noise will mask BWs (Alves et al., 2021), or to a reduced preference for boat-noise males. Males from the boat noise treatment could be less attractive because of their lower calling rate (Vasconcelos et al., 2012) or other traits associated to heightened stress levels that were not measured, such as altered behaviour (Nedelec et al., 2017; Butler and Maruska, 2021), or because of their lower quality (noisy) nests. Because female Lusitanian toadfish are single spawners (Modesto and Canário, 2003), their reproductive success depends on a single mating decision and will profit not only from choosing a good quality male/good father but also a nest where offspring development will be maximised. In this species calling rate is associated with male quality, namely body energetic reserves that are key to parental care (Amorim et al., 2010b). In addition, nests exposed to noise will be disadvantageous for offspring growth as boat noise induces

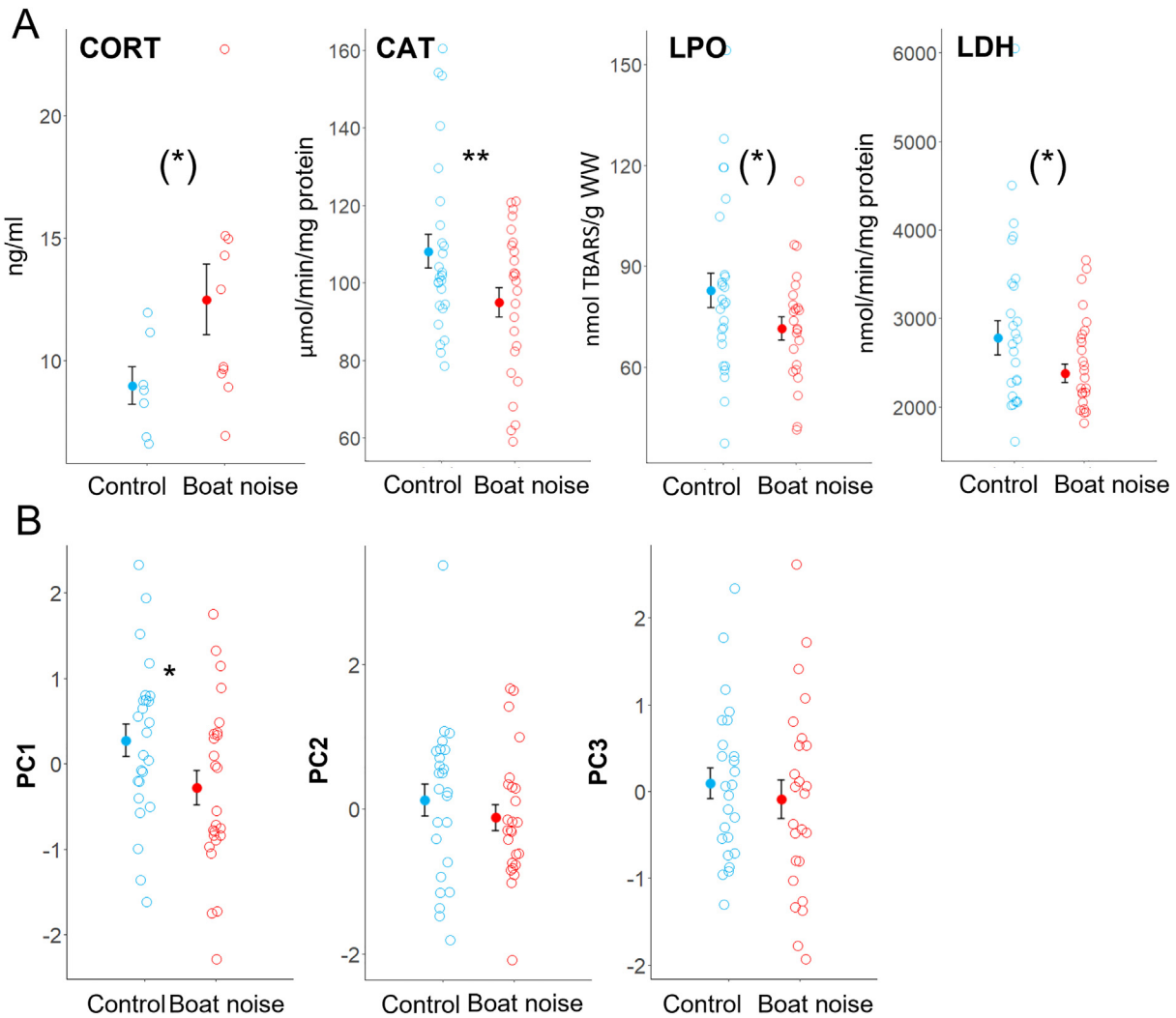


Fig. 4. A - Effect of treatment on levels of circulating cortisol (CORT), and of biomarkers related with oxidative stress (catalase – CAT and lipid peroxidation - LPO) and energy metabolism (lactate dehydrogenase - LDH) in male Lusitanian breeding males. B - Effect of treatment on the first three components of a Principal Component Analysis considering stress responses (oxidative stress and energy metabolism related biomarkers) and reproductive success (number of live and dead eggs). Data are means and S.E. ** = $P < 0.01$, * = $P < 0.05$, (*) $P < 0.1$.

Table 2

Effect of treatment on oxidative stress, energy metabolism and humoral immune response biomarkers. A – Results from linear mixed models including sampling date as a random factor. B – Results from *t*-tests. * *t*-test for separate variances.

A				
Dependent variable	Effect	df	F	P
SOD-liver	Date	4,45	3.01	0.03
	Treatment	1,45	0.39	0.53
CAT-liver	Date	4,45	8.49	<0.001
	Treatment	1,45	10.28	0.002
B				
Dependent variable	N (control)	N (boat)	t	P
DNA damage-liver	26	25	-0.18	0.86
LPO-liver	26	25	1.81	0.08
LDH-muscle	26	25	1.74*	0.08
IDH-muscle	26	25	1.36	0.18
ETS-muscle	26	25	1.11	0.27
Cholesterol-serum	20	21	0.30	0.77
Peroxidase-serum	26	25	-1.30	0.19
Lysozyme-serum	26	25	-0.34	0.73

detrimental effects on embryos and larvae stress response, and on larvae development in wild Lusitanian toadfish (Faria et al., 2022).

Why did males from the boat noise groups have a higher number of dead eggs? One possibility is that boat noise exposed males were less efficient in fertilizing eggs perhaps due to depressed activity levels associated with heightened stress levels (decreased metabolic activities suggesting a freezing behaviour, see below). Alternatively, successful fertilization success may have been impaired if sperm quality was affected by noise. Although not yet studied in fish, noise has a detrimental impact on sperm quality in other taxa (Kaiser et al., 2015; Choe et al., 2020; Abdollahi et al., 2021) with implications for fertilization success (Kowalski and Cejko, 2019). One of the mechanisms behind the decrease in sperm quality could be associated with stress responses, namely heightened cortisol or oxidative stress levels (Milla et al., 2009; Félix et al., 2021). Poorer early parental care (before the sampling day) could also be underpinning offspring survival. Several studies addressing the effect of noise exposure carried out both *in situ* (Nedelec et al., 2017; Maxwell et al., 2018; McCloskey et al., 2020) and in the laboratory (Bruinjes and Radford, 2013; Butler and Maruska, 2021) point to diminished parental care with consequences for offspring survival. This could be linked to heightened stress responses. For example, cortisol may affect parental care in fishes (Butler and Maruska, 2021), consistent with higher CORT levels being

Table 3

Correlation between activity levels of oxidative stress (OS), energy metabolism (EM) and humoral immune response (IR) biomarkers in Lusitanian toadfish males. Sample size is $N = 51$ for all biomarkers except serum biomarkers where $N = 41$. Values shown are Pearson correlation coefficients. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Biomarkers	CAT-liver	DNA damage - liver	LPO - liver	LDH - muscle	IDH - muscle	ETS - muscle	Cholesterol - serum	Peroxidase - serum	Lysozyme - serum
OS SOD -liver	- 0.03	0.28*	0.38**	0.14	-0.04	0.18	-0.06	0.08	-0.17
OS CAT -liver	-	-0.08	0.23	0.11	0.18	-0.01	-0.11	0.08	0.04
OS DNA damage - liver		-	0.63***	-0.02	0.05	0.20	0.03	-0.14	-0.08
OS LPO - liver			-	0.16	0.28*	0.37**	-0.11	-0.18	0.02
EM LDH - muscle				-	0.56***	0.26	0.14	-0.22	-0.35*
EM IDH - muscle					-	0.66***	-0.03	-0.37*	-0.20
EM ETS - muscle						-	-0.14	-0.13	-0.23
EM Cholesterol - serum							-	-0.10	-0.12
IR Peroxidase - serum								-	-0.002
IR Lysozyme - serum									-

associated with a higher number of dead eggs in the Lusitanian toadfish in the present study. Interestingly, exposure to only 3 h of noise can alter gene expression in the brain of African cichlid fish, *Astatotilapia burtoni*, and down regulate the expression of prolactin and vasotocin, both neuropeptides related to parental care (Butler and Maruska, 2021).

Our results agree with previous studies that point to a detrimental effect of noise on reproductive success in fish. Artificial noise has been shown to decrease reproductive outcome in captivity setups in two goby species, *Pomatoschistus pictus* and *P. microps* (de Jong et al., 2018a; Blom et al., 2019), in the Atlantic cod *Gadus morhua* (Sierra-Flores et al., 2015) and in the African cichlid *A. burtoni* (Butler and Maruska, 2021), by affecting mating probability (gobies), fertilization rate and egg viability (Atlantic cod) or parental care/offspring survival (cichlid). A field study has shown that playback of boat noise to spiny chromis fish, *Acanthochromis polyacanthus*, impaired parental behaviour and offspring survival, but the impact on mating successfully and on the obtained clutch size was not assessed (Nedelec et al., 2017). The only study that has thus far evaluated the effect of boat noise on reproductive success *in situ* showed that the oyster toadfish *Opsanus tau*, nesting in areas with real motorboat noise exposure had lower reproductive success as more nests had no egg clutches or the number of embryos per clutch was significantly lower than in quiet areas (Krahforst, 2017), consistent with the present study. In contrast, Brown et al. (2021) did not find an effect of exposure to artificial tonal noise on reproductive success in the plainfin midshipman fish in the field.

4.2. Calling behaviour

Males in the boat noise treatment showed a continued depressed calling activity compared to control males. A detrimental effect in calling activity has been found in other batrachoidids namely the oyster toadfish (Krahforst, 2017; Mackiewicz et al., 2021) and the plainfin midshipman (Brown et al., 2021), in sciaenids such as the black drum *Pogonias cromis* (Ceraulo et al., 2021) and the meagre *Argyrosomus regius* (Vieira et al., 2021c), and in two gobies, *Gobiusculus flavescens* and *P. pictus* (de Jong et al., 2018a). Besides from decreasing calling activity, which is essential to attract and court mates in several fish species (Amorim et al., 2015), reproduction may be further impaired by the reduction in the communication active space (Alves et al., 2016), by masking (Alves et al., 2021; Pine et al., 2021) or changing the receivers' physiology by causing stress and/or distraction (Simpson et al., 2016; Butler and Maruska, 2021). Interestingly, some species compensate for communication loss by increasing vocalization rates (such as the brown meagre *Sciaena umbra*; Picciulin et al., 2012), by changing the frequency and/or amplitude of their vocalizations (Brown et al., 2021), or by shifting to other sensory modalities (de Jong et al., 2018b).

In the present study, a continued lower calling activity was associated with higher cortisol levels, consistent with its regulatory role of reproductive functions, including sound production (Arterbery et al., 2010). Interestingly, males with a high mean calling rate had fewer dead eggs and elevated activity levels of the energy metabolism biomarker IDH (indicating increased aerobic metabolism) suggesting that calling rate could be

advertising male quality and paternal abilities as suggested by previous studies (Amorim et al., 2010b, 2013).

4.3. Stress and humoral innate immune responses

Exposure to a stressor elicits a complex physiological cascade of events in fish to allow internal homeostasis that starts with neuroendocrine responses (primary response), including the release of catecholamines and the activation of the hypothalamic-pituitary-interrenal axis, with the consequent release of steroid glucocorticoid hormones, mainly cortisol (Barton, 2002). If exposure to stressors is prolonged, the primary response mediates a secondary or even a tertiary response, respectively concerning the alteration of metabolic pathways and whole-animal activity and performance; the latter includes for example changes in condition, immunity, behaviour, and ultimately survival (Barton, 2002). In our experiment, noise treatment appears to have prompted all types of responses as exposure to boat noise elicited slightly higher levels of cortisol (primary), altered biochemical responses including metabolism related biomarkers (secondary) and changed calling behaviour (tertiary), when compared to control. In terms of primary responses, males exposed to boat noise tended to have higher circulating cortisol levels, which in turn were associated with increased egg mortality and depressed calling activity. A rise in circulating cortisol levels has been commonly reported in fishes in response to noise (e.g., Wysocki et al., 2006; Sierra-Flores et al., 2015).

Secondary stress responses commonly include oxidative stress resulting from the accumulation of reactive oxygen species (ROS) that can lead to lipid, protein, carbohydrate and DNA damage (Lesser, 2006). To combat ROS accumulation and prevent cellular damage, organisms produce antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT). In turn, these defence mechanisms at the subcellular level may translate into higher energy requirements for coping with oxidative stress reflected in the activity levels of energy metabolism-related biomarkers (Faria et al., 2018). In our study, males exposed to boat noise showed lower CAT activity compared to control males and no difference in SOD activity. This could suggest a lower ability to prevent damage due to oxidative stress. However, DNA damage did not differ between treatment and control males and lipid peroxidation levels (LPO) were even slightly lower in boat noise exposed males, suggesting lower lipid damage. LPO was positively correlated with ETS activity (a biomarker for cellular oxygen consumption), pointing to a lower metabolism and respiration levels (natural inducers of ROS and consequently damage) which is in turn associated with reduced lipid damage. Interestingly, this pattern was paralleled by a decrease in the activity of the other energy metabolism-related biomarkers. In fact, all three assessed energy metabolism biomarkers, LDH (an enzyme associated with anaerobic metabolism), IDH (an enzyme associated with aerobic metabolism) and ETS (the electron transport system of aerobic metabolism) were positively correlated, and LDH tended to be lower in males exposed to boat noise, pointing towards an overall metabolic depression. This is supported by boat noise males presenting lower values of the first principal component, which represents a composite score for the energy metabolism related biomarkers. A lower metabolism is also consistent with depressed

activity levels such as calling activity and likely parental care (associated with a higher number of dead eggs). Together, our results suggest that males exposed to boat noise adopted a freezing defensive response, a typical response to a threat (defensive modes include freezing or active fight-or-flight reactions; Roelofs, 2017).

Strategies to counter stressors can result in either increased or decreased metabolism, depending on the species and ecological trade-offs (Brown et al., 2005). This could help avoid high energetic costs of dealing with a stressor, such as counteracting oxidative damage and impacts on the immune system (the latter were not verified with the assessed biomarkers in this study). Consistently, Debusschere et al. (2016) found that juvenile sea bass *Dicentrarchus labrax* exposed to pile driving noise in the field reduced oxygen intake, which could point to a metabolic depression associated with freezing and suggested that this response was likely a useful strategy for fish to save energy. Studies in other fish species also found a decrease in activity and longer periods spent sheltering when exposed to the playback of boat noise, suggesting that reducing activity could be a common response to boat noise (Picciulin et al., 2010; Mickle et al., 2019).

These results should be taken with caution since Lusitanian toadfish were likely exposed to treatment for different periods as they occupied nests spontaneously, which could have influenced stress responses. Nevertheless, this species is gregarious and nest occupation by the first nest-holders facilitates further occupation by other males (Amorim et al., 2010a), which should have reduced variability in exposure duration among males. Also, variability in exposure duration should have increased data variability and thus hamper the ability to find patterns and not otherwise.

4.4. Final remarks

Marine traffic is the most pervasive source of continuous anthropogenic noise underwater altering soundscapes particularly at frequencies used by fishes to perceive their environment and to communicate (Popper and Hawkins, 2019). Using an integrated approach by assessing physiological, behavioural and direct fitness traits we have demonstrated that marine traffic noise can incur high fitness costs in a marine fish species by both impacting male performance and reproductive success. Although Lusitanian toadfish has particular life history traits that increase the vulnerability to the impact of boat noise (de Jong et al., 2020), such as depending on vocal communication to attract mates and low ability to avoid noise sources in all life stages (epibenthic sedentary fish with non-dispersing larvae), results can still be extrapolated to other fish species. Noise can impact individuals and populations by both bottom-up (responses at the genetic, cellular and physiological level) and top-down (responses modulated by species-specific behavioural ecology and habitat requirements) mechanisms (Solan and Whiteley, 2016). As bottom-up mechanisms are conserved they allow for broad predictions across taxa (Solan and Whiteley, 2016). Hence, stress responses and associated depressed behaviour are likely to occur in many other fish species with fitness implications, especially in fishes with swimbladders or other gas chambers, as these structures enhance the ability to detect sound pressure and increase the risk of noise exposure effects (Popper et al., 2014). Further research is needed addressing other species, particularly fish species with different reproductive strategies and pelagic larvae before we can fully understand the deleterious impacts that anthropogenic noise can have on aquatic animals.

CRediT authorship contribution statement

M. Clara P. Amorim: Funding acquisition, Project administration, Conceptualization, Methodology, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Manuel Vieira:** Investigation, Formal analysis, Writing – review & editing. **Gabriela Meireles:** Investigation. **Sara C. Novais:** Conceptualization, Methodology, Writing – review & editing. **Marco F.L. Lemos:** Methodology. **Teresa Modesto:** Methodology, Investigation. **Daniel Alves:** Investigation. **Ana Zuazu:** Formal analysis. **Ana F. Lopes:** Formal analysis. **André B. Matos:**

Investigation, Formal analysis. **Paulo J. Fonseca:** Project administration, Investigation, Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to Marília Freire for the valuable assistance during fieldwork. We thank the Air Force Base No. 6 of Montijo (Portugal) for allowing the field study in their military establishment. This study was funded by the Science and Technology Foundation, Portugal, project PTDC/BIA-BMA/29662/2017; strategic projects UID/MAR/04292/2020 to M.C.P.A. by MARE and UID/BIA/00329/2020 to P.J.F. by cE3c; grants SFRH/BD/48015/2008 and SFRH/BD/115562/2016 to D.A. and M.V., respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2022.154735>.

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