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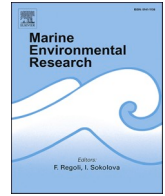
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Turning up the heat: Effects of temperature on agonistic acoustic communication in the two-spotted goby (*Pomatoschistus flavescens*)

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ABSTRACT

Acoustic communication is linked to fitness traits in many animals, but under the current scenario of global warming, sound signals can be affected by rising temperatures, particularly in ectothermic organisms such as fishes. This study examines the effect of water temperature in acoustic communication in the two-spotted goby, *Pomatoschistus flavescens*. To address this, we looked at the effect of different temperatures on the acoustic features of drums produced by males during territorial defence and related it with their auditory sensitivity. We also analysed the differences in acoustic features between male agonistic drums and previously reported male courtship sounds, to better understand how acoustic communication may be affected by different temperature conditions. We recorded two-spotted goby males during territorial intrusions for 10 min at 16 °C, 19 °C, and 21 °C in the laboratory. We found that agonistic drums were shorter, had fewer pulses and shorter pulse periods at higher temperature, in contrast with the peak frequency that remained unaffected. Male agonistic and mating drums (recorded in a previous study) at 16 °C only differed in pulse period, which was higher in mating drums. Hearing thresholds obtained with Auditory Evoked Potentials at 16 °C, revealed higher sensitivity below 400 Hz, matching the main energy of agonistic and mating sounds. Our findings suggest that increasing temperature could potentially affect acoustic communication in this species by reducing the duration of agonistic drums, which might hinder effective communication. Nevertheless, the impact may not be significant as there was a good match between the best hearing sensitivity and the peak frequency range of their calls, which was not influenced by temperature. As fish and other organisms are increasingly threatened by multiple anthropogenic stressors, including warming, future research should address how changes in water temperature impact acoustic communication within a more realistic multi-stressor scenario.

1. Introduction

Marine and coastal ecosystems are among the world's most diverse and productive environments. Yet, they are increasingly threatened by multiple anthropogenic stressors that are impacting their biodiversity and sustainability (Arora et al., 2023). For example, marine ecosystems

are experiencing unprecedented effects of global warming, with projections suggesting that ocean temperatures could increase by 4 °C by the end of the century, along with more frequent and intense marine heatwaves (IPCC, 2021). Coastal regions are more vulnerable to the effects of global warming compared to deeper offshore areas due to their lower thermal inertia (Vinagre et al., 2018). In these ecosystems,

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ectothermic species are particularly at risk due to their reliance on environmental temperature which directly affects their energy metabolism and physiology (Seebacher et al., 2015; Sokolova, 2023). This can impact important traits for their fitness, including visual and acoustic behaviour that ultimately affect reproductive success (Lopes et al., 2020; Albouy et al., 2023).

Acoustic communication is widespread in fishes and has independently evolved multiple times within Actinopterygii, or ray-finned fishes, highlighting its strong selection pressure for social communication (Rice et al., 2022) and relevance in reproductive success (Parmentier and Fine, 2016; Amorim, 2023). Representing more than half of extant vertebrates, fishes have evolved a remarkable diversity of sound production mechanisms and auditory systems (Parmentier and Fine, 2016; Ladich, 2024). Although difficult to categorise, many sound production mechanisms in fishes can be associated with either swimbladder-related mechanisms, involving the contraction of muscles directly or indirectly connected to the swimbladder, or stridulatory mechanisms, entailing the rubbing of skeletal elements, like teeth or bones (Parmentier and Fine, 2016). The peripheral auditory system (the inner ear) allows fish to detect underwater sound waves that comprise both a pressure and a particle-motion component (Wysocki, 2006; Popper and Hawkins, 2018; Putland et al., 2019). Many species have evolved accessory hearing morphological structures, such as Weberian ossicles found in otophysans. These structures convey the sound pressure induced swimbladder wall motions to the inner ear otoliths, enabling fish to detect a wider range of sound frequencies and at lower intensities (Ladich and Schulz-Mirbach, 2016). Species with such enhanced hearing abilities have been known as “hearing specialists” as opposed to “generalists” that can only detect particle motion (Popper et al., 2022).

Regardless of the particularities of the sound production or auditory systems of fish species, they are susceptible to temperature fluctuations, which can affect acoustic communication (reviewed in Ladich, 2018). Previous studies have looked into the effect of temperature on calling activity and sound characteristics in different fish families. Calling activity can either increase or remain unaffected, as it primarily depends on the motivation and physiological state of the caller (Vicente et al., 2015; Ladich, 2018). Sound characteristics may also change with temperature, but the specific effects depend on the sound production mechanism and vary among species (Ladich, 2018; Millot et al., 2023). For example, if sound pulses are directly caused by muscle contractions, pulse repetition rate and/or the fundamental frequency of sounds can increase with temperature (Ladich, 2018), due to the strong influence of temperature on the central nervous system’s activity controlling sonic muscle contraction and speed of muscle twitching (Bass et al., 2015). Studies addressing the effect of temperature on hearing sensitivity consistently reveal an increase in auditory sensitivity with increasing temperature (reviewed in Ladich, 2018; also see Schliwa and Ladich, 2021).

There is a lack of studies on how increasing temperatures affect acoustic communication in fishes (Ladich, 2018). Understanding this relationship is crucial, as acoustic communication is linked to fitness traits (Albouy et al., 2023; Amorim, 2023). Here, we investigate the effects of water temperature on the acoustic communication in a small semi-pelagic marine fish species, the two-spotted goby, *Pomatoschistus flavescens*. This species is polygamic (Amundsen, 2018) and uses low-frequency pulsed acoustic signals to attract females into the nest and as courtship signals (Albouy et al., 2023; Millot et al., 2023). The two-spotted goby was selected as a model species because it depends on acoustic communication for successful reproduction (Albouy et al., 2023) and is an established model species for studies in behavioural ecology and bioacoustics (Amundsen, 2018; de Jong et al., 2018; Millot et al., 2023). Also, water temperature has been shown to affect male mating acoustic signals (Millot et al., 2023) and spawning success (Lopes et al., 2020, 2022) in this species.

Sound production during territorial defence and hearing sensitivity

have not been studied in the two-spotted goby thus far. In this study, we characterized agonistic acoustic signals during male-male interactions under three temperatures: 16 °C, 19 °C and 21 °C, which fall within their natural thermal range (14–21 °C). To further gain insights into the effects of varying temperature conditions on the acoustic communication of this species, we compared agonistic sounds from this study with male courtship sounds previously recorded by our team (Millot et al., 2023). This comparison aimed to identify differences in acoustic features associated with different behavioural contexts that may be susceptible to temperature effects. In addition, we measured hearing thresholds in males and investigated if auditory sensitivity matches the main energy of agonistic and courtship sounds.

2. Methods

2.1. Ethical note

Experiments were authorized by the Portuguese National Authority for Animal Health - Direção Geral de Alimentação e Veterinária (DGAV reference 0421/000/000/2021) and performed in strict accordance with the EU Directive 2010/63/EU for animal experiments. The study also followed the recommendations of the Animal Care and Use Committee of the University of Lisbon (reference ORBEAFCUL 6_2022).

2.2. Study species

The Gobiidae (Amorim, 2005) family stands as one of the largest families of bony fish (Teleosts), with about 2,000 identified species (Fricke et al., 2024). They are widely distributed, thriving in diverse habitats, ranging from rivers, to mangroves, estuaries, and coastal ecosystems. Within Gobiidae, species belonging to the *Pomatoschistus* genus hold particular significance as marine coastal intermediate predators, playing a key role in ecosystem dynamics (O’Gorman et al., 2008). Within the genus, the two-spotted goby (*P. flavescens*) emerged as a reference model species in behavioural ecology and bioacoustics (Amundsen, 2018; Amorim, 2023). It is a nest spawner with paternal care, and during the breeding season males attract females to their nests using both visual and acoustic courtship/mating signals (Amundsen and Forsgren, 2001; Forsgren et al., 2004; Albouy et al., 2023). The two-spotted goby produces two sound types during mating—drums and thumps (Albouy et al., 2023; Millot et al., 2023), which are also found in other gobies of the *Pomatoschistus* genus (Amorim and Neves, 2007; Malavasi et al., 2009; Millot et al., 2023). The main (peak) frequency of mating drums ranges between 120 and 360 Hz and of mating thumps between 60 and 150 Hz at temperatures within 16–21 °C (Millot et al., 2023). Agonistic encounters are also frequent among neighbouring nest-holding males (Forsgren et al., 2004; Wacker et al., 2012) but agonistic sounds have not previously been documented. Note that within Gobiidae, sound production is not known to occur in females (e.g. Horvatić et al., 2019).

2.3. Fish collection and experimental setup

Two-spotted gobies from both sexes were collected by scuba diving at depths down to 8 m, in the Arrábida Marine Park, Portugal (38°28’N; 8°59’W), in December 2021. Upon collection, fish were transported to the fish facilities of the Animal Biology Department of University of Lisbon, where they were housed, under a natural light cycle, in two 200-L tanks filled with artificial filtered seawater and equipped with mechanical and biological filtration. Fish were initially maintained in tanks at 16 °C (controlled using a chiller, Hailea HC300A, Hailea Group Co., Ltd – China), with a salinity of 35 parts per thousand (PPT). Environmental enrichment included sand, polyvinyl chloride (PVC) shelters and artificial algae. Temperature and salinity were daily measured, whereas ammonia, nitrates and nitrites were monitored on a weekly basis and kept below critical levels. Fish were daily fed *ad libitum* with frozen

Artemia.

Males were tested for sound production in an agonistic context at three temperatures: 16 °C, 19 °C, and 21 °C, reflecting the natural thermal range experienced by the species during their breeding season (Encarnação et al., 2022; Fig. S1), which extends at least from April to August in the study area (Faria et al., 2018; Lopes et al., 2022). Prior to transferring them to the experimental tanks, fish designated for the 19 °C and 21 °C treatments underwent a gradual acclimation process, increasing by 1 °C on the first day and 2 °C on subsequent days until reaching the target temperature. Water temperature was controlled and adjusted with aquaria chillers, one per tank (Hailea HC300A). Fish were then introduced in the experimental tanks and left undisturbed at least 24 h before being tested.

The experimental tanks consisted of six 35-L units, with two replicate tanks per experimental temperature. Tanks were positioned on top of two 3-cm thick marble slabs interspaced with two levels of rubber foam shock absorbers, which significantly minimised floor-born vibrations. Each tank was partitioned into three compartments using transparent, perforated plexiglass dividers to allow for water circulation, as well as visual and olfactory communication. Each lateral compartment housed one male, while two round females were placed in the central section to promote male territorial behaviour. This setup design, also used in previous studies (e.g. Amorim et al., 2013; Vicente et al., 2015; Albouy et al., 2023; Millot et al., 2023), allows to speed up the time for males to acquire territorial behaviour. Also, note that any sounds produced by

one male in its compartment attenuates to background levels before reaching the other male (Amorim et al., 2018). Only males that showed territorial behaviour were tested, i.e. that showed brighter colours, interest in females and occupied the artificial nest added to each male compartment (Fig. 1A). Nests were made with PVC tubes (4 cm in diameter and 8.5 cm long, as in Amorim et al., 2013) and placed on top of bags of gravel (5 cm high) (as in Millot et al., 2023).

2.4. Sound recordings

Experimental sessions were carried out from April to June 2022, following the protocol of Millot et al. (2023). Before the start of experiments two hydrophones were positioned in the test aquarium: one in the nest chimney (HTI-96-Min, sensitivity of -165 dB re. $1\text{V}/\mu\text{Pa}$, High Tech Inc., Gulfport, MS), and another positioned at 5-cm distance from the nest (HTI 94 SSQ, sensitivity of -165 dB re. $1\text{V}/\mu\text{Pa}$, flat frequency response up to $6\text{ kHz} \pm 1\text{ dB}$) (Fig. 1A). All pumps and chillers were turned off before the start of recordings. An opaque partition prevented visual contact with the non-focal male compartment during recordings. An intruder male was then introduced in the compartment of the focal male, inside a plastic jar, simulating a territorial intrusion. The intruder males were not observed to make sounds. Experimental sessions lasted for 10 min, which was sufficient to elicit consistent territorial defence behaviour while minimizing the time the intruder spent in the container. Sounds were captured by the hydrophones, digitized with an audio

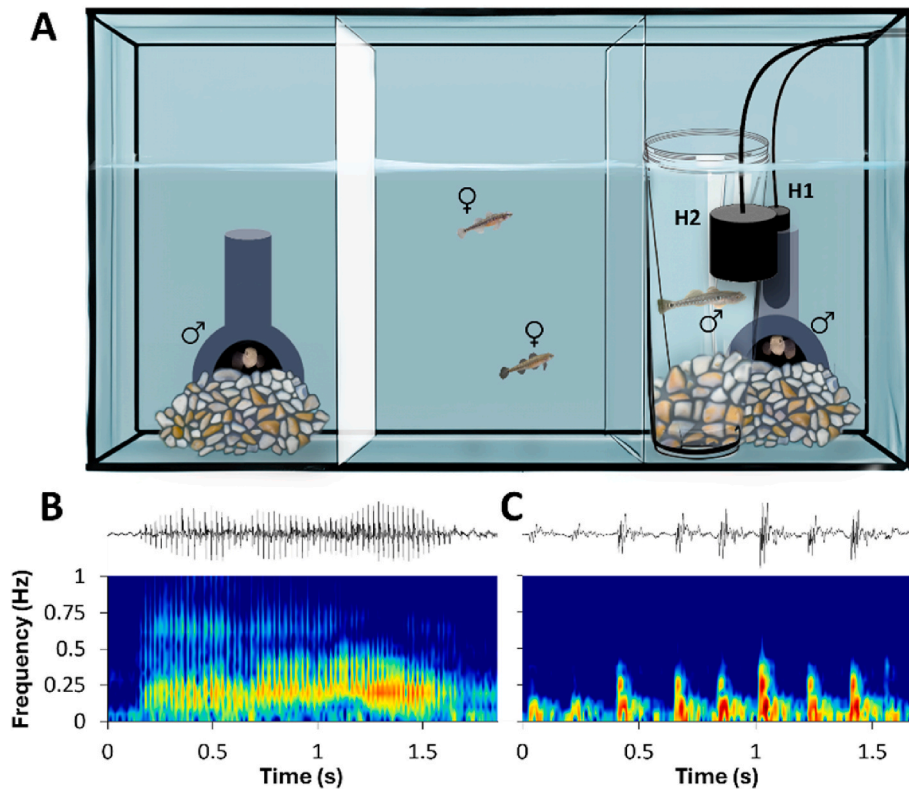


Fig. 1. Experimental setup used to record sounds during territorial intrusions in the two-spotted goby (A). The experimental tank was partitioned into three compartments using transparent, perforated plexiglass dividers to allow for water circulation, as well as visual and olfactory communication. Each lateral compartment housed one male while two round females were placed in the central section to promote male territorial behaviour. Visual and chemical contact during the acclimation phase promoted territorial behaviour in males. During recordings, an opaque partition was placed to avoid visual contact between the non-focal male and the focal male. An intruder male was placed inside a plastic jar in the compartment of the territorial focal male to elicit territorial defence. The focal male responded by approaching the intruder, and by making visual and acoustic displays. Sounds were captured using two hydrophones (H1 and H2) connected to a recording chain. Males made two types of sounds: (B) drums and (C) thumps. Spectrograms based on Fast Fourier transform (FFT): sampling frequency: 6 kHz; FFT size: 128; frequency resolution: 47 Hz; window type: Hanning; window overlap: 50%.

This setup design, also used in previous studies (e.g. Amorim et al., 2013; Vicente et al., 2015; Albouy et al., 2023; Millot et al., 2023), allows to speed up the time for males to acquire territorial behaviour.

interface (Cakewalk UA25EX, 16-bit, 6 kHz acquisition rate; Roland, Hamamatsu, Japan) to a laptop controlled by Cool Edit Pro (v2.0, Syntrillium, Phoenix, AZ, USA). In each recording day, fish from the three temperature treatments were tested. Each focal male was used in a single tested temperature, and one to four recording sessions were carried out per male (mean = 2.5). The number of males studied at 16 °C, 19 °C and 21 °C were 6, 7 and 5, respectively. Of these, 3, 4 and 3 males made sounds at 16 °C, 19 °C and 21 °C, respectively. At the end of the 10-min session, males were measured for total length (TL, mm) and weighed (g). Sound producing males measured on average 45.5 mm (\pm SD, range: \pm 3.4, 40–50) and weighed on average 1.1 g (\pm 0.2, 0.8–1.4). Fish that did not produce sounds measured on average 46.4 mm (\pm 4.6, 40–55) and weighed 1.2 g (\pm 0.3, 0.5–1.7).

2.5. Sound analysis

Only drums were considered in this study as these were the main sounds produced by males during agonistic interactions, although they also made thumps (Fig. 1B and C; Audio S1_Agonistic drum; Audio S2_Agonistic thumps). Sounds were analysed using Raven Pro software version 1.6 (Cornell Lab of Ornithology, Ithaca, NY). Temporal features were measured from oscillograms, and spectral parameters were obtained from power-spectra (FFT size 1024 points, time overlap 50%, Hanning window, Hz). Sound duration (DUR; ms), peak frequency (PF; the frequency with maximum energy, Hz), number of pulses (NP) and mean pulse period (mean duration between peak-to-peak interval of consecutive pulses, ms) were measured. Pulse rate (NP/drum duration*1000, Hz), which is a redundant metric of PP was also calculated to allow comparisons with the literature.

Agonistic drums recorded at 16 °C were compared to courtship sounds at 16 °C from a previous study (Millot et al., 2023) using the same experimental setup. Only this temperature was considered as it presented a larger sample size and allowed comparisons with other studies (Amorim et al., 2013, 2018; Bolgan et al., 2013; Pedroso et al., 2013). For this purpose, 20 courtship sounds produced by 4 males in May and June 2022 (4–6 sounds per male) were randomly selected. These males measured 47.5 mm (\pm 5.3, 41–54) and 1.1 g (\pm 0.2, 0.9–1.3).

2.6. Audiometry

Auditory sensitivity was measured at 16 °C. The Auditory Evoked Potential (AEP) recording technique used in this study followed previously established methods (Vieira et al., 2021; Wong et al., 2022). To initiate the procedure, fish were gently anaesthetized until they ceased movement in 0.003% tricaine methanesulfonate bath (MS-222, Acros Organics, NJ, USA) buffered with an equal amount of sodium bicarbonate. The specimens were then carefully positioned in a custom-designed hemi-cylinder plastic holder, which was covered with foam on the inside and partially closed with a fine net. This setup ensured overall body immobilization while leaving the opercula free for breathing, without the need for further anaesthesia. Subsequently, the holder containing the fish was submerged underwater, with the fish's head positioned just beneath the water surface and centred with the underwater speaker at about 8.5 cm distance.

The recording tank (diameter 35 cm, water depth 18 cm) was filled with saltwater from the stock tanks and the temperature adjusted and kept at 16 °C (\pm 1 °C). A custom-built sound stimulation system, optimized for lower frequencies (<300 Hz), was installed at the centre, in the bottom of the tank. This system consisted of a vibrating plexiglass disc (8 cm diameter) attached to a rod. The rod crossed the tank bottom through a water-restraining flexible device, which prevented water drainage while keeping the rod vertically aligned. The rod was driven by a mechanical wave driver (SF9324, PASCO, Roseville, CA, USA) kept below the tank (Vasconcelos et al., 2011). As the mechanical wave driver was uncoupled from the experimental tank, it avoided

transmitting extraneous mechanical noise to it. The setup was placed in a dedicated room on a vibration-damped steel table and inside a Faraday cage to shield against electromagnetic interference. Additionally, the audiometry workstation was kept outside the room to prevent noise contamination.

To record the AEP responses, a measuring electrode was positioned and slightly pressed against the skin of the mid upper surface of the head over the brainstem region, and a reference electrode positioned on the side of the body in a posterior area.

Both sound stimuli presentation and AEP recordings were accomplished using the workstation from TDT (Tucker-Davis Technologies, FL, USA). The AEP signals were fed into a low impedance head stage (Medusa 4Z, TDT) connected to a pre-amplifier (RA4PA, TDT) and then routed to a Multi-I/O processor (RZ6, TDT), digitized (16 bit, \pm 4 mV) and band-pass filtered (0.1–1 kHz). Control of sound stimuli and AEP recordings was achieved using SigGen and BioSig TDT software. The stimuli comprised tone bursts ranging from 45 to 1000 Hz, randomly presented with a duration of 20 ms and a rise/fall time of 2 ms, and presented at least 1000 times, half at opposite polarities (180° phase shifted).

Prior to each experiment, a hydrophone (8104, Brüel & Kjær, Naerum, Denmark; sensitivity: -205 dB re. 1 V μ Pa -1 ; frequency response from 0.1 Hz to 180 kHz) connected to a sound level meter (Bruël & Kjaer 2238 Mediator, Naerum, Denmark) was used for calibrating the stimuli at the position that would be occupied by the fish head in the recording tank. Additional calibration in particle motion was carried with a 3-axis accelerometer (M20-040, sensitivity 0–3 kHz, GeoSpectrum Technologies, Dartmouth, Canada) to measure particle acceleration for each pure tone stimulus in the vertical axis (the main axis of stimulation).

For each frequency, tones were initially presented at 134 dB re. 1 μ Pa and then decreased in 5 dB steps until the AEP response became less noticeable. At this point, the sound level was reduced in 2.5 dB steps to accurately determine the hearing threshold (see Fig. 6). The auditory threshold was defined as the lowest SPL at which a visible and repeatable AEP response was obtained in at least two averaged waveforms. Validation of an auditory response was based on at least two out of three criteria: (1) matching waveform shape compared to the response from the previous sound level (visual inspection); (2) increased latency in the auditory response, measured in a consistent AEP peak; and (3) presence

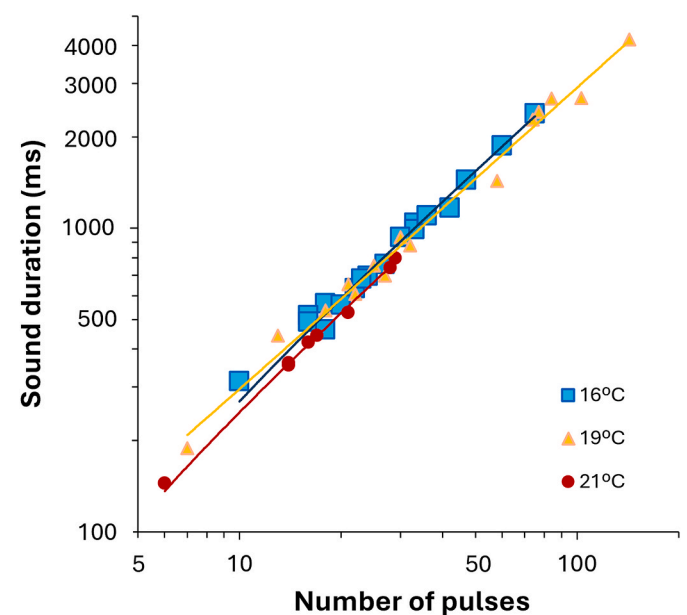


Fig. 2. Relation between number of pulses and sound duration (in log-scale) in agonistic drums of the two-spotted goby recorded during territorial intrusions at 16 °C, 19 °C and 21 °C.

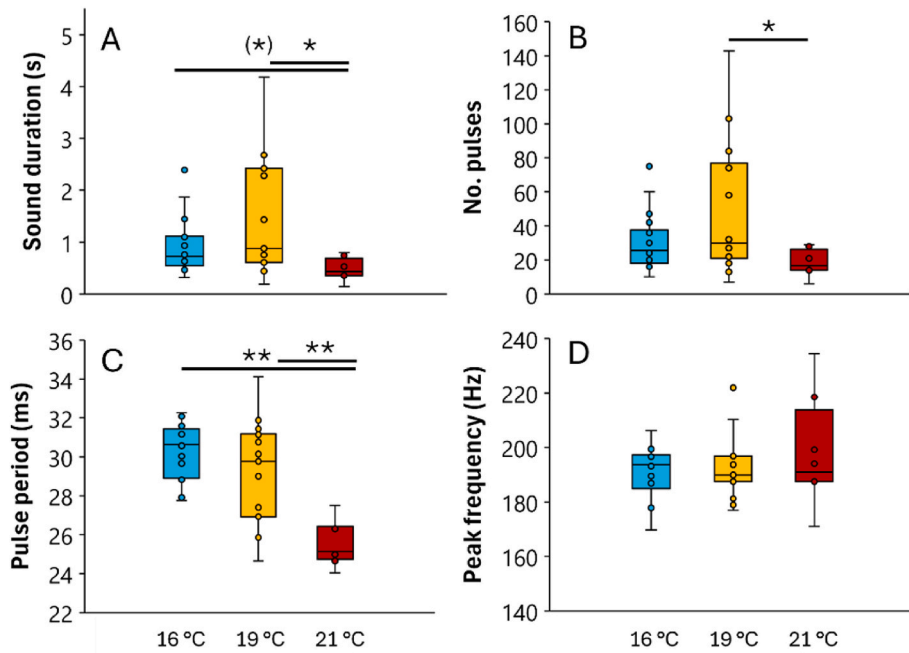


Fig. 3. Comparison of the acoustic features of agonistic drums produced by the two-spotted goby during territorial intrusions at the three test temperatures: (A) sound duration (s), (B) number of pulses, (C) mean pulse period (ms) and (D) peak frequency (Hz). Pairwise differences given by post-hoc tests for the Kruskal–Wallis: (*) = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$. Bars indicate medians \pm 95% confidence intervals and quartiles.

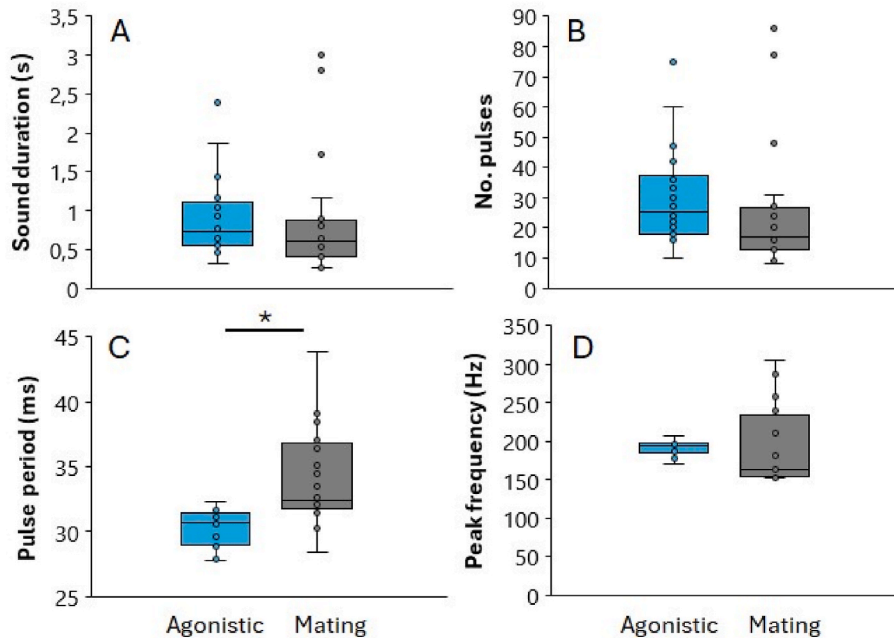


Fig. 4. Comparison of drum features produced by two-spotted goby males during agonistic (this study) and mating (Millot et al., 2023) contexts at 16 °C: (A) sound duration (s), (B) number of pulses, (C) mean pulse period (ms) and (D) peak frequency (Hz). * = $P < 0.05$. Bars indicate medians \pm 95% confidence intervals and quartiles.

of a spectral peak in the Fast Fourier Transform (FFT) analysis of the auditory response that is double the stimulation frequency.

Eight males with bright breeding colours, measuring on average 45.1 mm (\pm 4.6, 37–51) and 1.0 g (\pm 0.3, 0.6–1.4) were used for auditory sensitivity recordings. Only males were used due to logistical reasons, but measurements should be representative of the species as most fishes investigated do not show sex-specific differences in auditory sensitivity (e.g. Maruska et al., 2007). Nevertheless, we cannot rule out sex-specific differences in auditory thresholds. AEP measurements were

only done at one temperature due to limited number of specimens available.

2.7. Statistical analysis

Statistical tests were performed with Statistica 12.0 for Windows (StatSoft, Inc., Tulsa, OK, USA). The effect of temperature on agonistic sound parameters was tested with Kruskal–Wallis tests. Post hoc tests available in Statistica and described in Siegel and Castellan (1988) were

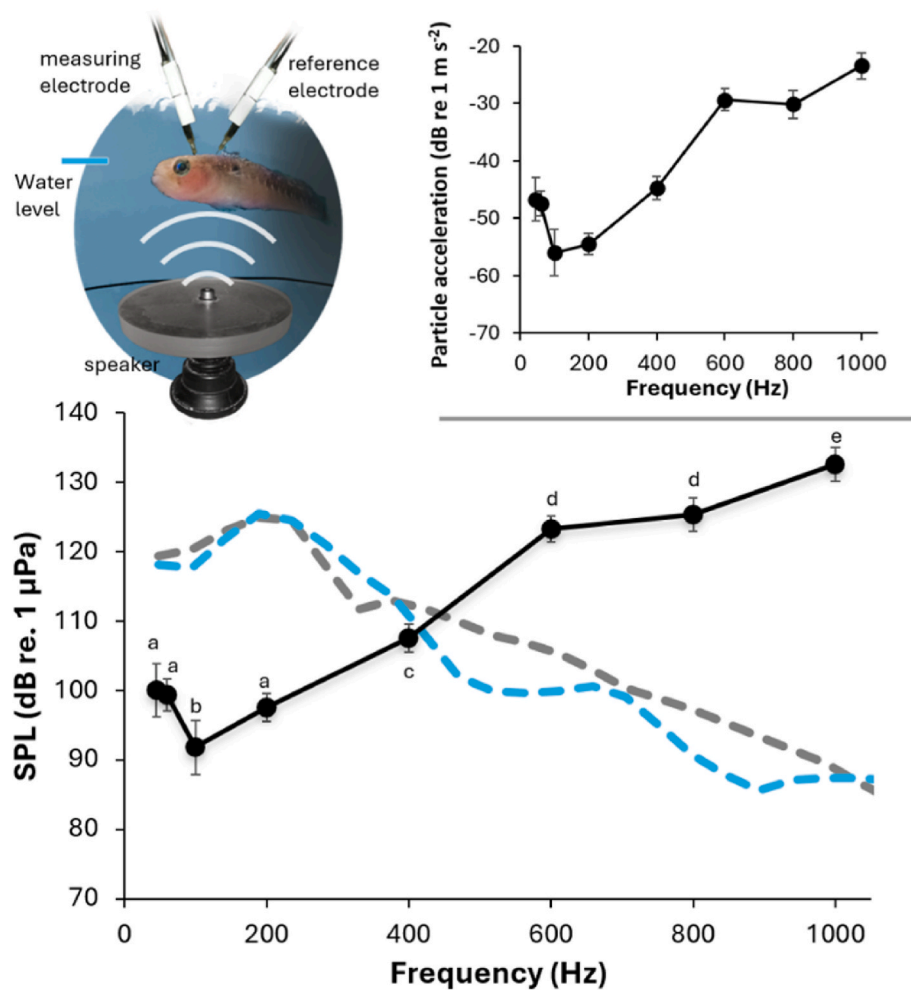


Fig. 5. Hearing thresholds (mean \pm standard deviation) of the two-spotted goby (an example of a specimen in the AEP recording setup is shown). The power spectra from an agonistic (blue; this study) and a courtship (grey; Millot et al., 2023) drum examples are also depicted. The inset depicts hearing thresholds in particle acceleration units, highlighting a similar pattern to the audiogram in sound pressure level (SPL). Note that only the vertical component (z-axis) of the particle acceleration is displayed as it had significantly larger amplitudes compared to the horizontal components (axes x and y). However, a reduction in SPL led to a corresponding decrease in particle acceleration across all three axes. Error bars represent standard deviations. Different letters denote significant pairwise differences for the post-hoc comparison tests. Spectra settings: sampling frequency: 6 kHz; FFT size: 128; frequency resolution: 47 Hz; window type: Hanning; window overlap: 50%.

used for multiple comparisons between treatments. Differences between agonistic and courtship sound parameters were tested with Mann–Whitney U-tests. Differences in hearing thresholds at different frequencies were tested with a repeated measures ANOVA considering AEP thresholds for each subject fish (within-subject factor). This analysis was followed by Tukey HSD pairwise comparisons post-hoc tests. All statistical tests assumptions were met.

3. Results

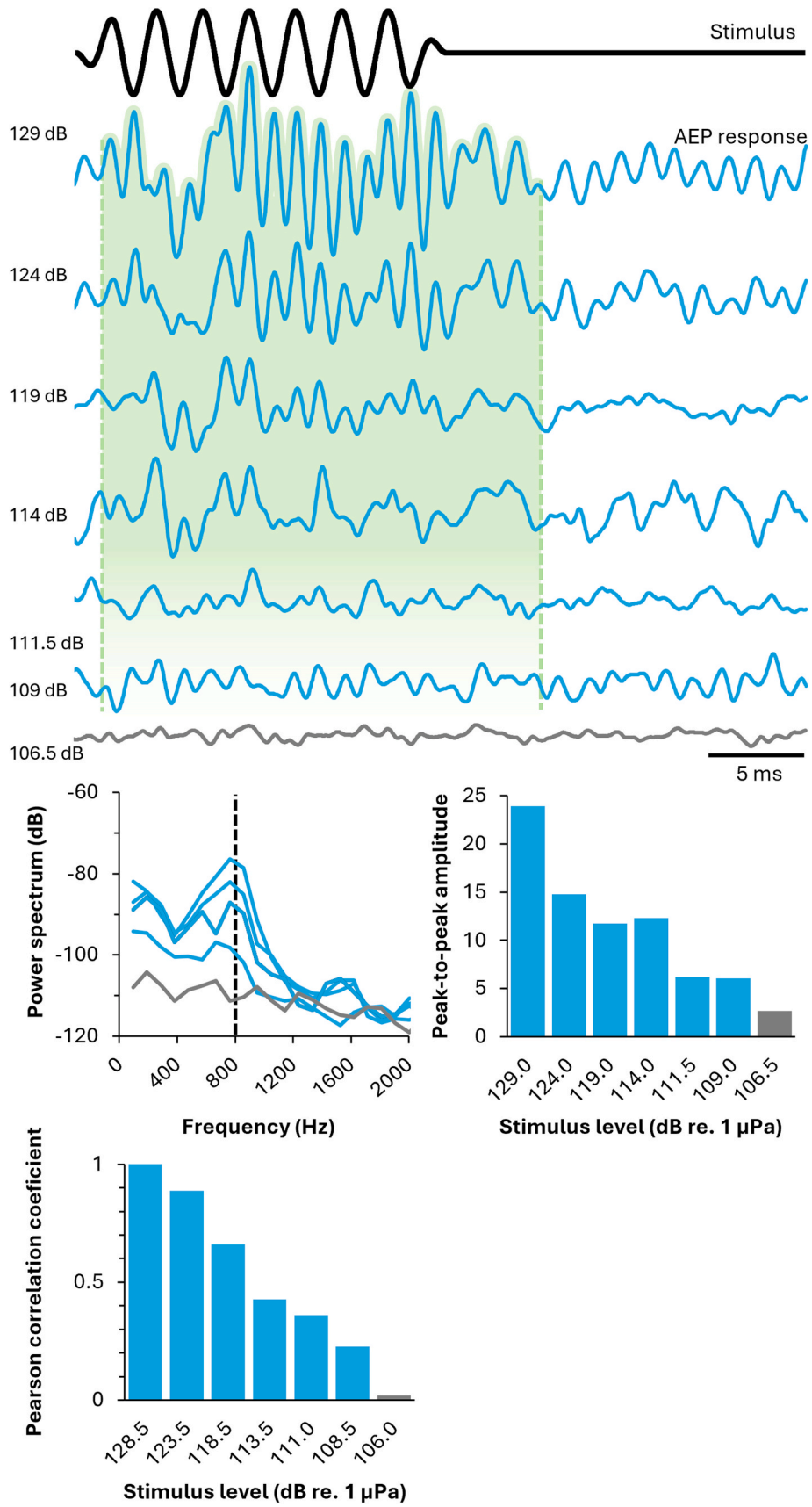
3.1. Acoustic signals

During territorial intrusions fish made both thumps and drums, the latter being more frequent. Thumps were mainly produced when the territorial male was in the nest. Agonistic drums were mostly emitted when the territorial male approached and was near the intruder male in the jar and sometimes when they were in the nest with their head out. In the recording sessions with sound production, males made an average of 6 drums (range = 1–12) per 10 min session at 16 °C, 3.8 (2–7) at 19 °C and 2.7 (2–3) at 21 °C. Due to the small sample size, sounds were grouped together for analysis based on temperature. Sample size was thus 18, 15, and 8 sounds for 16 °C, 19 °C and 21 °C (Table 1).

Agonistic drums were variable in duration ranging from 144 ms to over 4 s considering all temperatures (Table 1). Drum duration mainly depended on the number of pulses, which varied from 6 to 144 (Fig. 2). Pulse period varied from 24.1 to 34.1 ms and peak frequency from 179 to 234 Hz in all recorded agonistic sounds (Table 1).

There was a significant effect of temperature on sound duration (Kruskal-Wallis test: $N = 41$, $H = 8.62$, $P < 0.05$ and number of pulses ($H = 6.55$, $P < 0.05$), with drums at 21 °C being shorter and with fewer pulses than at 19 °C (Fig. 3A and B). The difference in drum duration between 16 °C and 21 °C was nearly significant (post-hoc test, $P = 0.06$). Mean pulse period also differed among temperature levels ($H = 16.41$, $P < 0.001$), being significantly shorter at 21 °C than at 16 °C and 19 °C (Fig. 3C). In contrast, peak frequency did not vary among temperatures ($H = 0.30$, $P > 0.05$; Fig. 3D).

Agonistic and mating drums recorded at 16 °C did not differ in sound duration, number of pulses or peak frequency (Mann-Whitney test, $N_{\text{agonistic}} = 18$, $N_{\text{mating}} = 20$, DUR: $U = 134.0$, $P > 0.05$; NP: $U = 116.0$, $P > 0.05$; PF: $U = 131.0$, $P > 0.05$; Fig. 4A,B,D). However, mean pulse period was higher in mating drums ($U = 42.0$, $P < 0.001$; Fig. 4C) than in agonistic drums.



(caption on next page)

Fig. 6. Example of Auditory Evoked Potential (AEP) response curves to 400 Hz pure tone stimuli with decreasing amplitudes. The auditory threshold was determined as the lowest stimulus amplitude at which a consistent response curve pattern could be observed. The double frequency response is evident in the first 20 ms of the AEP responses to higher amplitude stimuli (e.g., response at 129 dB). The green shadow highlights the biological response but should only be considered as a reference and not an accurate measure of the AEP response duration. A decrease in the overall peak-to-peak amplitude and the presence of a spectral peak in the Fast Fourier Transform analysis of the auditory response (twice the stimulation frequency) are also shown below. Decreasing Pearson correlation coefficients are also provided, which match the visual inspection of matching waveform patterns. Note that validation of an auditory response was based on at least two out of three criteria (see Methods). Blue indicates positive AEP responses, while grey indicates recordings below the threshold.

Table 1

Acoustic features of drums produced during agonistic interaction by territorial males of the two-spotted goby *P. flavescens*. Descriptive statistics are provided for sounds produced by 11 different males: 3, 4 and 4 males at 16 °C, 19 °C and 21 °C. N = number of sounds.

Sound feature	T (°C)	Mean	SD	Range	Min	Max	N
Sound duration (ms)	16 °C	923.2	533.7	312.6–2389.3	312.6	2389.3	18
	19 °C	1425.0	1151.5	188.5–4184.1	188.5	4184.1	15
	21 °C	473.4	213.5	144.3–797.8	144.3	797.8	8
Number of pulses	16 °C	30.6	16.7	10.0–75.0	10.0	75.0	18
	19 °C	48.9	39.4	7.0–143.0	7.0	143.0	15
	21 °C	18.1	7.7	6.0–29.0	6.0	29.0	8
Pulse period (ms)	16 °C	30.2	1.5	27.8–32.3	27.8	32.3	18
	19 °C	29.2	2.7	24.7–34.1	24.7	34.1	15
	21 °C	25.5	1.1	24.1–27.5	24.1	27.5	8
Pulse rate (Hz)	16 °C	33.5	2.1	30.9–38.8	31.0	38.8	18
	19 °C	34.5	3.2	29.3–40.6	29.3	40.6	15
	21 °C	38.8	1.6	36.3–41.6	36.3	41.6	8
Peak frequency (Hz)	16 °C	191.0	9.5	169.7–206.2	169.7	206.2	18
	19 °C	192.5	11.7	176.9–221.9	176.9	221.9	15
	21 °C	197.5	20.0	171.1–234.4	171.1	234.4	8

3.2. Auditory sensitivity

Auditory thresholds obtained from males with AEPs under quiet laboratory conditions and at 16 °C indicated higher sensitivity below 400 Hz, with the best hearing frequency at 100 Hz and a gradual sensitivity decrease towards the higher tested frequencies (repeated measures ANOVA, $F_{7,42} = 221.5$ $P < 0.001$; Figs. 5 and 6). Within the best hearing range, hearing thresholds increased from (means \pm SD): 91.3 ± 4.2 dB re. 1 μ Pa at 100 Hz to 99.5 ± 3.8 dB re. 1 μ Pa at 45 Hz and 97.0 ± 2.0 dB re. 1 μ Pa at 200 Hz. Hearing thresholds at 45, 60 and 200 Hz were similar, (Tukey HSD tests, $P > 0.05$), but thresholds at 100 Hz were significantly lower than the remaining frequencies ($P < 0.01$). At frequencies above 200 Hz, hearing thresholds were higher than 100 dB re. 1 μ Pa, showing a gradual but significant increase from 107.0 ± 2.0 dB re. 1 μ Pa at 400 Hz to 132.0 ± 2.4 dB re. 1 μ Pa at 1000 Hz. Pairwise differences in hearing thresholds between consecutive tested frequencies from 100 to 1000 Hz were significant ($P < 0.001$) except between 600 and 800 Hz ($P > 0.05$). The audiograms shown in sound pressure and particle motion were similar in shape (Fig. 5). We only display the vertical component (z-axis) of particle acceleration as it had significantly larger amplitudes compared to the horizontal components (axes x and y). However, a reduction in sound pressure level led to a corresponding decrease in particle acceleration across all three axes.

4. Discussion

Fishes typically produce sounds in association to reproduction, particularly during agonistic interactions (to establish a territory or assert social status), and during courtship and spawning (Ladich and Myrberg, 2006; Lobel et al., 2010; Amorim et al., 2015). The relation of sound production with reproductive success has been established in a few species highlighting its role in fish fitness (Vasconcelos et al., 2012; Oliver and Lobel, 2013; Albouy et al., 2023). However, the ability to communicate effectively with other individuals is subject to ecological constraints, such as temperature fluctuations, which are particularly relevant for ectotherms (Ladich, 2018; Vieira et al., 2022). Here, we investigated whether two-spotted goby males use acoustic signals to defend their territories and further studied the effect of temperature on agonistic sound production across three temperatures that are within the

species natural range. Importantly, we characterise this species' hearing sensitivity (also in males) for the first time and investigate if the best hearing frequencies match the frequencies with main energy of agonistic sounds produced under different temperatures.

We found that two-spotted goby nest-holders produce drums and thumps when confronted with a territorial intruder, mirroring the findings of Millot et al. (2023) and Albouy et al. (2023) regarding courtship and mate attraction contexts. Other gobies are known to produce both drums and thumps in a reproductive context (Amorim and Neves, 2007; Malavasi et al., 2009; Millot et al., 2023) but agonistic thumps have not been previously described in species of the sand goby group (*sensu* Horvatić et al., 2023). As the emission of thumps is associated with nest display, where the male stays in the nest with its head out making downward thrusts with the head accompanied by thumps (present study; Amorim and Neves, 2007), this multimodal signal likely advertises nest ownership and can serve for both territorial intruder deterrence and mate attraction.

Temperature had a significant effect on agonistic drum features with sounds being shorter, with fewer pulses and shorter pulse periods at higher temperatures. Similarly, the pulse period of courtship drums in the two-spotted goby decreased with rising temperature but drum duration and the number of pulses was not affected by temperature in the study of Millot and colleagues (Millot et al., 2023). The number of pulses in a sound and consequently sound duration is highly dependent on motivation (e.g. Myrberg et al., 1978; Hawkins and Amorim, 2000) and other factors such as fish size (Colley and Parmentier, 2012; Amorim et al., 2013) thus masking possible effects of temperature. Consistently, there are mixed results in the literature with some species showing an increase, others a decrease and still others no differences of sound duration in warmer waters (Ladich, 2018). In contrast, temperature dependence of pulse rate (or pulse period) is common in fish species where pulse rate emission is dependent on the rhythmicity of the vocal central pattern generator located in the central nervous system and driving fast muscle contraction, such as in swimbladder drumming sounds (Parmentier and Fine, 2016; Ladich and Mauditsch, 2020). In these species pulse rate increases with temperature. This pulse rate - temperature relation is also found in gobies in which the sound-producing mechanisms involve pectoral sonic muscles associated with the pectoral girdle (Torricelli et al., 1990; Vicente et al., 2015;

Parmentier et al., 2013, 2017), such as in the congeneric painted goby, *P. pictus* (Vicente et al., 2015).

It is possible that shorter drums with fewer pulses found in higher temperatures are less effective for communication than longer ones as their detection could be hindered. The threshold for detecting brief sounds deteriorates as the sound duration shortens, since shorter sounds have less overall energy (integration over time of kinetic and potential energy components of sound) and provide less time for neural integration (Fay and Simmons, 1999). This suggests that in warmer waters, sounds would need to be louder for detection thresholds to stay the same. However, research on the closely related painted goby suggests that temperature does not impact drum amplitude (Vicente et al., 2015). Nevertheless, agonistic drums in the two-spotted gobies are likely long enough to be detected even at higher temperatures. Indeed, in goldfish (*Carassius auratus*), detection thresholds stabilise with sound durations longer than ca. 400 ms (Fay and Coombs, 1983). Acoustic communication could however still be hindered at higher temperatures as shorter sounds could be masked more easily by transient background noises (Fay and Simmons, 1999).

Peak frequency of agonistic drums did not vary with temperature, consistent with the findings of Millot et al. (2023) for courtship drums produced by the two-spotted goby. Similar results were found in the painted goby, indicating a lack of temperature effect on peak frequency (Vicente et al., 2015). As there seems to be no relationship between pulse rate and peak frequency in these species, it suggests that the sound's main energy is influenced by the vibration properties of sound-producing structures rather than muscle contraction rate. If sounds are produced by muscles associated with the pectoral girdle and amplified by the radials at the level of the fins, as suggested by Parmentier et al. (2013, 2017), then peak frequency is likely more dependent on fish size than temperature, as found for the painted goby (Vicente et al., 2015). Similarly, a lack of temperature dependency was found for stridulatory sounds of the Amazonian Pictus catfish, *Pimelodus pictus* (Ladich and Maiditsch, 2020) and the striped Raphael, *Platydoras armatulus* (Papes and Ladich, 2011). Despite this lack of relation with temperature in some species, in others, peak frequency has been found to increase in warming waters (Fine, 1978; Connaughton et al., 2000; Amorim, 2005; Ladich and Schleinzner, 2015).

Auditory evoked potentials indicated that the two-spotted goby has its best auditory sensitivity at frequencies below 400 Hz, matching the main energy of agonistic and courtship drums, which presented similar peak frequencies. Although auditory sensitivity was measured in males, sex-specific differences in auditory thresholds are not expected (e.g. Maruska et al., 2007), though we cannot rule them out. Hearing curves were also similar to the painted and the marbled (*P. marmoratus*) gobies (Amorim et al., 2018), suggesting similar hearing abilities across the genus. Although we did not test the effect of temperature on auditory sensitivity, different studies consistently point to an improvement with increasing temperature (Ladich, 2018 and references within). It is thus likely that the two-spotted goby will have improved hearing sensitivity in warmer waters, leading to a better ability to detect their acoustic signals. Also, a mismatch of best hearing sensitivity and sound spectra is unlikely with warming as drum peak frequency seems unaffected by temperature. This hypothesis remains however to be investigated.

In conclusion, our study revealed that agonistic drums get shorter in warmer temperatures potentially impacting communication effectiveness, particularly in the presence of fluctuating background noise from abiotic (waves, wind) and human sources. Nevertheless, the impact may not be significant as there was a strong match between the best hearing sensitivity and sound frequencies, which were not influenced by temperature. In addition, any potential decrease in detection due to shortening of sounds could be compensated by a potential improvement in hearing with warming. Hearing is important in various situations beyond communication, playing a crucial role in survival by enabling the detection of sounds from predators, prey, or sounds from abiotic or anthropogenic origin (Hawkins and Popper, 2017). Hence, studying the

impact of temperature on acoustic communication, addressing both acoustic signalling and hearing abilities within a species becomes increasingly crucial in the context of global warming, and should be a focus of future research. In addition, it is critical to understand how changes in water temperature impact acoustic communication and the ability to retrieve acoustic information from the environment in a more realistic multi-stressor scenario.

Data availability

Data is in the supplementary material.

CRediT authorship contribution statement

Jorge Penim: Investigation, Formal analysis. **Marilyn Beauchaud:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Morgane Millot:** Writing – review & editing, Investigation, Formal analysis. **Ana M. Faria:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Manuel Vieira:** Writing – review & editing, Visualization, Validation, Methodology. **Paulo J. Fonseca:** Writing – review & editing, Validation, Resources, Methodology. **Raquel O. Vasconcelos:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **M. Clara P. Amorim:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization.

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.

Data availability

Data is available as a supplementary file

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Appendix A. Supplementary data

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