

# Endemic fish calling: acoustics and reproductive behaviour of the Neretva dwarf goby *Orsinigobius croaticus*

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## Abstract

The Neretva dwarf goby *Orsinigobius croaticus* (Gobiiformes, Gobionellidae) is an endemic fish native to the freshwaters of the Adriatic Basin in Croatia and Bosnia and Herzegovina. Due to its limited distribution range, specific karst habitat and endangered status, laboratory studies on reproductive biology are scarce. We investigated the sound production and acoustic behaviour of this species during reproductive intersexual laboratory encounters. We performed dissection and micro-computed tomography ( $\mu$ CT) scanning of the pectoral girdle to explore the anatomy of its putative sound producing mechanism. To study interspecific acoustic differences and determine whether acoustic features can discriminate among species, comparative analysis was conducted on sounds produced by closely related soniferous sand gobies. Our results indicate that males of the *O. croaticus* emit pulsatile sounds composed of a variable number of short ( $\sim 15$  ms) consecutive pulses when interacting with females, usually during the pre-spawning phase in the nest, but also during courtship outside the nest. Pulsatile sounds were low-frequency and short pulse trains ( $\sim 140$  Hz,  $< 1000$  ms), and spectro-temporal parameters were correlated with physical traits and water temperature. Male visual behaviour rate was higher when co-occurring with sounds and females entered the male's nest significantly more frequently when sounds were present. Male sound production was accompanied by movements such as head thrust and fin spreading.  $\mu$ CT scans and dissections suggest that *O. croaticus* shares certain anatomical similarities of the pectoral girdle (osseous elements and arrangement of levator pectoralis muscles) to previously studied sand gobies. Multivariate comparisons, using sounds produced by eight soniferous European sand gobies, effectively distinguished soniferous (and sympatric) species based on acoustic properties. Discrimination success decreased when temperature-dependent features (sound duration and pulse repetition rate) were excluded from analysis. Therefore, we suggest both spectral and temporal features are important for acoustic differentiation of sand gobies.

## INTRODUCTION

The Mediterranean Biodiversity Hotspot (MBH) is a widely renowned region for its significant environmental diversity and endangered wildlife (Myers et al., 2000; Darwall et al., 2014). The rivers of the Adriatic Sea Basin in Croatia are part of the MBH and Dalmatian freshwater ecoregions (Abell et al., 2008), and are especially rich in freshwater endemic fish, with 40 species, or almost 30% of the total Croatian ichthyofauna, endemic to this area (Myers et al., 2000; Kottelat & Freyhof, 2007; Čaleta et al., 2015, 2019). This endemism is a feature of the habitats of the Dinaric karst that covers roughly 54% of Croatian territory, with the presence of numerous caves, sinkholes, cold seasonal wells, and underground rivers (Kutle et al. 1999; Mrakovčić et al., 2006).

Among these endemic fish species, *Orsinigobius croaticus* (Mrakovčić, Kerovec, Mišetić & Schneider, 1996)

formerly described as *Knipowitschia croatica*, is a small benthic and short-lived (less than two years) sand goby confined to the Dinaric karst of the Dalmatian ecoregion. It can be found exclusively in the freshwaters of Croatia and Bosnia and Herzegovina (Abell et al., 2008; Zanella et al., 2011; Horvatić et al., 2017; Čaleta et al., 2019; Tutman et al., 2020). In Croatia, this species inhabits the Eastern part of the Adriatic Basin, and has a naturally fragmented distribution range that includes the Neretva River, Matica River, the Vrgoračko Polje and Rastočko Polje fields, and Baćina Lakes, some of which are NATURA 2000 sites (Mrakovčić et al., 1996; Zanella et al., 2011, 2017; Čaleta et al., 2015; see Horvatić et al., 2017 for the map with distribution area). In its natural habitat, *O. croaticus* occupies sandy bottoms with occasional stones/pebbles in karst rivers, slow-flowing streams and oligotrophic lakes (Zanella et al., 2011; Horvatić et al., 2017). During winter and early spring, *O. croaticus* thrives in small rivers and streams whereas during the summer period, this goby survives in small karst underground ponds and refuges, when the watercourses completely dry out (Miller, 2004; Mrakovčić et al., 2006). On the IUCN Red List, *O. croaticus* is assessed globally as vulnerable (VU, B2ab(iii); D2, ver. 3.1.), but regionally as endangered (EN) due to its highly limited/fragmented habitat and declining habitat quality (Crivelli, 2006, 2018; Mrakovčić et al., 2006; Horvatić et al., 2017). However, this endangered status is also due to a lack of knowledge regarding its biological traits. There is little published data on the ecology or biology of *O. croaticus* (Mrakovčić et al., 2006; Zanella et al., 2011, 2017; Horvatić et al., 2017). Like other sand gobies, *O. croaticus* is a polygamous multiple spawner species that achieves sexual maturity quite early (within its first year), with a reproduction season from March to November, although most spawning occurs from April to September (Mazzoldi & Rassotto, 2001; Kottelat & Freyhof, 2007; Zanella et al., 2011, 2017).

The sand gobies are a monophyletic gobiiform group (Gobionellidae, Gobiiformes) of about thirty species (in the genera *Knipowitschia*, *Pomatoschistus*, *Economidichthys*, *Ninnigobius* and *Orsinigobius*) (Thacker, 2009; Nelson, Grande & Wilson, 2016; Betancur-R et al., 2017; Thacker et al., 2018; Tougard et al. 2021). They inhabit intertidal marine and coastal freshwater ecosystems with muddy-to-pebble bottom across Europe, including the waters of the Mediterranean, Ponto-Caspian and Northeast Atlantic regions (Miller, 2004; Šanda & Kovačić, 2009; Kovačić & Patzner, 2011; Freyhof, 2011). Recent phylogenetic studies found evidence that sound production is widespread among actinopterygian fishes, suggesting that acoustic behaviour evolved independently multiple times in unrelated clades, and that there is a strong selection for the use of sound production as a behavioural trait across vertebrate evolution (Fine & Parmentier, 2015; Rice et al., 2020, 2022). Communicative sound emission in fish is usually linked to courtship and spawning or aggressive behaviour (Amorim, 2006; Myrberg & Lugli, 2006; Mann et al., 2008). In fish bioacoustics, acoustic signals associated with reproductive intersexual interactions have been the most commonly studied types of sounds (Amorim, 2006), since it is believed that these sounds serve to attract potential mates (Parmentier et al., 2010; Longrie et al., 2013), to synchronise spawning activities at aggregation sites (Lobel, 1992; Rowell et al., 2015; Erisman & Rowell, 2017; Jublier et al., 2019), or to synchronise gamete release by conspecifics (Hawkins & Amorim, 2000; Lobel 2002). Sand gobies are a common model group among soniferous actinopterygian fishes for sound production and have long been utilised in ethological and comparative bioacoustics studies. The acoustic abilities in sand gobies have been intensively investigated in the last 30 years, especially in the species of the genera *Pomatoschistus*, *Knipowitschia* and *Orsinigobius* (Torricelli, Lugli & Pavan, 1990; Lugli et al., 1997; Malavasi et al., 2008, 2009; Amorim & Neves, 2007; Bloom et al., 2016; Zeyl et al., 2016; Parmentier et al., 2017). In eight sand goby species, two different types of acoustic signals (pulsatile and thump sounds) have been recorded to date (Amorim & Neves 2007; Blom et al. 2016; de Jong et al., 2016; Zeyl et al., 2016), while in *Economidichthys pygmaeus* (Holly, 1929), sounds were not detected during behavioural experiments (Gkenas et al., 2010). Most of our understanding regarding the acoustic abilities of Mediterranean sand gobies stems from the common, widely distributed and non-threatened species assigned to the least concern (LC; IUCN Red List) category (Amorim et al. 2013; Blom et al. 2016; de Jong et al., 2016; Zeyl et al., 2016). However, since *O. croaticus* was regionally classified as a vulnerable species with a very restricted distribution (Crivelli, 2006, 2018; Horvatić et al., 2017), this research is a first study of the acoustic communication of this endangered Mediterranean sand goby.

The main goal of this study was to empirically investigate acoustic communication of *O. croaticus* and

the anatomy of its sound-producing mechanism. Specifically, our aims were to: i) investigate the sound production of captive *O. croaticus* males and quantify acoustic parameters of the sounds; ii) examine the reproductive behaviour of soniferous males and its association to sound production; iii) provide insight into the putative sound-producing mechanism by exploring the anatomy of the pectoral girdle, and iv) explore the acoustic diversification of soniferous sand gobies by quantitatively comparing acoustic signals between the study species and previously recorded Mediterranean sand gobies (genera *Ninnigobius*, *Pomatoschistus*, *Knipowitschia* and *Orsinigobius*).

## MATERIAL AND METHODS

### Fish sampling, laboratory housing and experimental design

*Orsinigobius croaticus* was sampled using electrofishing (Hans Grassl, model: EL65 IIGI, power: 13 kW) from a boat during spring 2019. Sampling was performed on the Matica River in Croatia (near the village Vina 43°10'30.33" N, 17°23'12.36" E). In total, we collected 25 individuals (15 males and 10 females) from the main river channel at a depth of 0.5 – 2 m. Fish were transferred to large plastic water containers equipped with aerators and transported to the laboratory. At the laboratory, fish were sexed based on urogenital papilla and body coloration (Miller, 1984) and housed in appropriate community rectangular tanks (120 L). After an acclimatization period of 5 – 8 days, prospective soniferous males were chosen for subsequent laboratory acoustic-visual recordings based on coloration (complete or partially darken body, fins and head; Zanella et al., 2011), aggressiveness and territoriality. Eight males ( $x \pm s.d. = 49.21 \pm 0.8$  mm total length,  $L_T$ ; range: 48.07 – 50.06 mm;  $41.07 \pm 1.01$  mm standard length,  $L_S$ ; range: 38.96 – 42.29 mm;  $1.24 \pm 0.11$  g weight,  $W$ ; range: 1.10 – 1.40 g) exhibiting typical reproductive behaviour were chosen for the experiments. Females ( $N = 5$ ;  $36.75 \pm 5.10$  mm  $L_S$ ; range: 28.75 – 41.27 mm) were chosen for the recording sessions according to yellow belly coloration, luminescent green spot on the first dorsal fin and dark eyes, all indicators of female readiness for spawning (Zanella et al., 2011; Bloom et al., 2016). Measurements were made using digital callipers CD-15APX with a precision of 0.01 mm (Mitutoyo, Japan) and a digital scale (0.1 g precision). As a metric of male body condition, we calculated the condition factor [Fulton's  $K$ , where  $K = (W/L_S^3) * 10^5$ ].

We followed the acoustic-visual recording protocol established by previous authors (Amorim & Neves, 2007; Amorim et al., 2013; Pedroso et al., 2013), where experimental tanks were divided into three compartments separated by removable partitions. Each lateral compartment housed one territorial male with a nest (artificial pvc shelter), while the middle section (“arena”) was occupied by a ripe female. The female compartment was not provided with a nest. Briefly, our experimental rectangular tanks were 120 L (60 x 48 x 35 cm) in volume and were divided into three unequal sections by means of two opaque removable acrylic partitions to prevent intersexual (visual) interactions. The tanks had a 5 cm thick layer of fine sand or gravel and each male in each section was provided with a water pump system and aeration. The experiments were performed from mid-April to October, at random times. The reproductive behaviour of resident males was elicited by introducing one ripe female into the “arena”. Before experiments, each female was left 12 – 24 h in the experimental tank for acclimatization. Eight territorial males were kept in the experimental tanks until the end of the recording sessions in October, after which they were returned to male community tanks. Prior to recordings (approx. 15 min), electricity, water pumps and aeration were switched off to minimise ambient noise. Between recording sessions of the same resident male, 15 – 30 min pauses were included. In addition, to reduce substrate-born noise, the experimental tanks were placed onto three cm thick rubber foam shock absorbers. Fish were kept at natural photoperiod and fed daily *ad libitum* with *Daphnia*. Water temperature, monitored with a thermometer (AQUATERRA, Garešnica, Croatia), was maintained between 18 – 22°C. The male-female trials lasted approximately 30 min, and began by removing one of the lateral partitions, allowing intersexual interaction. To eliminate the noise from the room light system, recording sessions were performed in the dark, with the experimental tank illuminated by LED light from the side of the aquarium. This procedure had no noticeable effects on fish behaviour.

### Acoustic recordings and sound analysis

Acoustic experiments lasted from mid-April to October, which overlaps the natural reproduction season of

*O. croaticus* . During acoustic recording sessions, a hydrophone (H2A-XLR hydrophone, Aquarian Audio & Scientific, Anacortes, WA, USA; sensitivity:  $-180$  dB re.  $1$  V  $\mu\text{Pa}^{-1}$ ; frequency range  $\pm 4$  dB from  $0.01$  to  $100$  kHz), was placed in the centre of the experimental tank above the shelter, and connected to a IRIG PRE preamplifier (Aquarian Audio & Scientific). Sounds were recorded using a ZOOM H4n portable digital audio recorder ( $16$  bit/ $44.1$  kHz sample rate; ZOOM, Tokyo, Japan). The hydrophone was placed within the attenuation distance from the emitter (less than five cm), and we obeyed the laboratory protocol for minimum resonant frequency for small glass tanks (*e.g.* ,  $2.7$  kHz for  $170$  L tanks, according to Akamatksu et al., 2002). Sounds, monitored with headphones and noted by the observer, were stored on the recorder memory card as .wav files. Recordings were later band-pass filtered ( $0.05 - 3$  kHz) to improve S/N ratio and subsampled at  $4$  kHz, and further amplified ( $10$  dB) for better auditory and visual inspection of the audio tracks. Digitalised sounds were analysed using Avisoft - SASLab Pro 5.2 Software ( $1024$ -point FFT, FlatTop window;  $100\%$  frame; Avisoft Bioacoustics, Berlin, Germany). Ten audio recordings ( $2.5$  per male, each lasting approx.  $30$  min) were aurally and visually inspected. Each *O. croaticus* sound was labelled using the “insert label” function of Avisoft - SASLab Pro. In this study, we recorded  $367$  pulsatile sounds from four soniferous males, but not all sounds presented a good signal to noise ratio (S/N) for acoustic analysis. From ten recordings presenting the best S/N ratio, we analysed  $20$  randomly selected sounds. Temporal features were measured from oscillograms, while frequency-related variables were obtained from the logarithmic power spectra (FlatTop window,  $512$ -points FFT,  $96.87\%$  overlap; resolution  $8$  Hz). For sounds, we measured the following acoustic properties (**Figure A1**): (1) sound rate (SR, number of sounds emitted in  $1$  min); (2) sound duration (DUR, total length of the call, measured in milliseconds); (3) number of pulses (NP); (4) pulse repetition rate (PRR; NP divided by DUR and multiplied by  $1000$ ; Hz); (5) pulse duration (PD; ms); (6) pulse period (PP; average peak-to-peak interval of consecutive pulses, ms); (7) fatigue (FAT; ratio between the average PP of the last three and first three pulses representing the decrease in pulse emission rate possibly due to muscle fatigue, following Amorim et al., 2013); (8) frequency modulation (FM, after the sound has been divided into three temporally identical sections, FMi - initial, FMm - middle and FMf - final - see **Figure A1** , frequency modulation was calculated as the difference between the final and initial pulse repetition rate and expressed in Hz; FMi, pulse repetition rate of the initial section of a sound and FMf, pulse repetition rate of the final section of a sound); (9) peak frequency (PF, the peak with the highest energy from the logarithmic power spectrum function, Hz). In order to follow the previous recording protocols as closely as possible (Amorim & Neves, 2007; Amorim et al., 2013), we also calculated the vocal activity parameters per male: (I) sound rate (number of sounds produced per min), (II) maximum sound rate (maximum number of sounds emitted in  $1$  min) and (III) calling effort (percentage of time spent calling, *i.e.*, seconds of sound production divided by the duration of the recording in seconds). Despite the fact that the variables PP and PRR indicate the pulse repetition pattern, they were deliberately indicated separately here in order to facilitate comparisons with the goby literature on sound production.

### Video recordings and ethological analysis of behavioural categories

During acoustic-visual recordings, a second hydrophone (HTI-96-Min, High Tech Inc., Long Beach, MS, USA, sensitivity:  $-201$  dB re.  $1$  V  $\mu\text{Pa}^{-1}$ , frequency response  $2$  Hz to  $30$  kHz), placed less than three cm from the nest opening, was connected directly to a video camcorder (Canon Legria FS200,  $41\times$  digital zoom,  $25$  frames/sec, Tokyo, Japan) to directly synchronise acoustic and visual signals into a uniform dataset (.mod format) for subsequent analysis. By including or excluding different females into a male’s territory, we could control the experimental variables of interest under standardised conditions and study multimodal communication in males. The camcorder was mounted on a stand and positioned approx.  $40$  cm from the front of the experimental glass tanks. Quantitative ethological analysis of the courtship, pre-spawning and spawning phases was performed by calculating the number of sounds co-occurring with each behavioural category. Courtship behaviour began when the females entered the male territory at a distance of  $<5$  cm from the male’s nest, while the pre-spawning phase was observed when the ripe female entered the male’s nest. Spawning was observed beginning with the female turning upside-down in the nest numerous times in short succession (assuming *oviposition* ) and started circling the ceiling. Male behaviours and the associated sound emissions were observed in four soniferous males during seven recording sessions and analysed using Solomon

Coder (ver. beta 19.08.02). Again, the ripe females were chosen for the recording sessions according to two indicators of their readiness for spawning (belly and eye coloration). Behavioural categories (and behavioural acts per category) expressed by the males were classified and scored according to our observations and the literature (Amorim & Neves, 2007, 2008; Amorim et al., 2013; Malavasi et al., 2009). In Solomon Coder, two datasets were analysed separately and then compared. Firstly, behavioural coding was performed by re-watching the video recordings with sound production involving five females ( $N = 5$ ;  $37.23 \pm 4.29$  mm  $L_S$ ; range: 31.62 – 43.76 mm) and documenting the frequency ( $n \text{ min}^{-1}$ ) and duration (in sec) of behavioural categories, calculating their total occurrence and percentage. In addition, we analysed eight video recordings (two per male) containing the behaviours of the same four tested males, but performed with six different females ( $N = 6$ ;  $37.23 \pm 4.29$ ; range: 31.62 – 43.76 mm) when they did not co-occur with sound production (*i.e.*, males were mute for the entire recording period). By having these two datasets, we investigated the differences in frequencies of behavioural categories in males when they engaged in sound production and when they did not. In total, we used a total of eight males in our experiments, but four were unresponsive (*i.e.*, did not perform courtship behaviour or sounds), resulting in insufficient data for their further analyses. We analysed videos using Solomon Coder for the following behavioural parameters: male behaviour rate (the total number of behavioural acts per min) (1) co-occurring with sounds or (2) not co-occurring with sounds; number of times a female entered the male’s nest accompanied with sounds (3) or (4) without sounds; (5) total behaviours (number of behavioural categories per video recording).

### Anatomical analysis

For anatomical dissections, additional *O. croaticus* individuals were collected in October 2020 from the same watercourse near the village Brečići (43°7'11.30"N, 17°29'4.03"E) using electrofishing. Five individuals were collected, of which three males (40 – 50 mm  $L_T$ ) were immediately euthanised with an overdose of MS-222 (tricaine methane sulphonate; Pharmaq, Overhalla, Norway), and stored for one week in 7% formaldehyde fixative solution and then transferred to 70% ethanol. Specimens were dissected and examined with a Wild M10 binocular microscope (Leica Camera, Leica, Wetzlar, Germany) equipped with a camera lucida to study the anatomy of the putative sound producing mechanism. Since earlier research on gobies highlighted the role of the pectoral girdle and (pectoral) fins in sound production, dissections primarily addressed the muscles related to this body part. The nomenclature used to designate muscular parts was based on earlier research (Winterbottom, 1974; Adriaens et al., 1993; Parmentier et al., 2013, 2017). Additionally, one specimen was subjected to micro-computed tomography ( $\mu$ CT) scanning to visualise the fish skeleton at the level of the neurocranium and pectoral girdle. Scanning was completed using a RX EasyTom (RX Solutions, Chavanoz, France; <http://www.rxsolutions.fr>), with an aluminium filter. Images were generated at 75 kV and 133  $\mu$ A, with a frame rate of 12.5, 5 average frames per image. This procedure generated 2897 images at a voxel size of 10  $\mu$ m. Reconstruction was performed using X-Act software from RX Solutions. Segmentation, visualisation, and analysis were performed using Dragonfly software (Object Research Systems (ORS) Inc, Montreal, Canada, 2019; software available at <http://www.theobjects.com/dragonfly>). Three-dimensional (3D) 16-bit images were produced and subsequently converted into 8-bit voxels using ImageJ (Abràmoff et al., 2014). Three-dimensional processing and rendering were obtained after semi-automatic segmentation of the body using a ‘generated surface’, according to the protocols described by Zanette et al. (2014). Direct volume renderings (iso-surface reconstructions) were used to visualise a subset of selected voxels of the anterior skeleton in AMIRA 2019.2.

### Acoustic comparison among soniferous sand gobies

The sounds of seven soniferous sand gobies, *Knipowitschia panizzae* Verga, 1841, *Ninnigobius canestrinii* (Ninni 1883), *Orsinigobius punctatissimus* (Canestrini 1864), *Pomatoschistus marmoratus* (Risso 1810), *P. pictus* (Malm, 1865), *P. microps* (Krøyer, 1838) and *P. minutus* (Pallas 1770), were previously recorded and characterised (Lugli et al., 1995; Lugli & Torricelli, 1999; Malavasi et al., 2008; Amorim et al., 2013, 2018; Bolgan et al., 2013; Pedroso et al., 2013). However, these acoustic data were never combined into a single phylogenetic dataset and analysed interspecifically. Therefore, we studied their interspecific acoustic variability (*P. marmoratus* was separated geographically into two populations, Italian and Portuguese). Briefly, the

species were sampled either from brackish habitats in north Adriatic Sea (*K. panizzae*, *P. marmoratus* and *N. canestrinii*), from freshwaters of north-west part of Reggio Emilia Romagna, Italy (*O. punctatissimus*) (Lugli et al., 1995, 1997; Lugli & Torricelli, 1999; Lindström & Lugli, 2000), from Portuguese marine/brackish waters (Amorim et al., 2013, 2018; Bolgan et al., 2013) or the west coast of Sweden (Pedroso et al., 2013). Sound recordings gathered from the previously conducted laboratory experiments were re-analysed to allow for interspecific comparison with a minimal measurement experimental error. All investigated sand gobies produced pulsatile sounds, thus enabling acoustic interspecific comparisons. The dataset was composed of 36 individuals of eight soniferous sand gobies including *O. croaticus* (min – max: 3 – 5 individuals, except for a single individual of *P. microps*), with at least three sounds recorded per individual. In total we calculated the means for five acoustic variables (temporal: DUR in ms, NP, PRR in Hz; spectral: PF and FM, both in Hz) for each individual. Since gobies included in the current study were recorded at different water temperatures (range: 15.8 – 22.6°C) and it is well known that the ambient water temperature affects fish acoustic signals (Vicente et al., 2015; Ladich, 2018), we conducted two separate multivariate analyses: the first involving the complete dataset (all five acoustic features for each species), and the second excluding the temporal features (DUR and PRR) known to be influenced by water temperature (Lugli et al., 1996; Vicente et al., 2015).

### Statistical analysis

Each sound produced by a different *O. croaticus* male was assumed to be an independent acoustic unit, and the statistical analyses were performed by combining the sounds from multiple individuals into a single dataset. In the intraspecific analyses, the *O. croaticus* individuals were utilised as a grouping variable, to explore acoustic variation between males. For each spectral and temporal variable of the sound, the descriptive statistics are presented. Outliers and extremes were detected visually from the boxplot and were eliminated from the dataset if necessary. In order to test the assumption of normality, we initially performed Shapiro-Wilk normality test on a raw intraspecific dataset. Since the assumption of normality was not met for some variables, the overall acoustic dataset was then transformed (either using log or square root functions) followed by a Box-cox function to estimate the transformation parameter by maximum likelihood estimation. The acoustic dataset was re-examined for distribution fitting, and since assumptions of normality (Shapiro - Wilk test,  $P < 0.05$ ) and homoscedasticity (Bartlett test,  $P < 0.05$ ) of the variances were not achieved, we continued with non-parametric tests. For pairwise comparisons between soniferous *O. croaticus* males, we employed the non-parametric Kruskal-Wallis rank sum test  $H$  followed by pairwise Dunn's multiple comparison test with Bonferroni correction for the  $P$  values. To investigate the mutual relationship between mean individual acoustic variables, we utilised non-parametric *Spearman* correlation tests. The association between acoustic variables with body characteristics ( $L_S$ ,  $W$  and Fulton's  $K$ ) and water temperature ( $T$ , in °C) was performed with *Spearman* correlations. Additionally, the *Chi*-square ( $\chi^2$ ) was used to test for independence of behaviour (expressed as behavioural categories) from sound production. In this test, the residuals from the  $\chi^2$  were used to determine which behaviours were positively related to sound production. Kruskal-Wallis  $H$  test was used to compare the mean behavioural variables (calling rate, behaviour rate, n. of female nest entrances) between soniferous males. Wilcoxon signed-rank test was performed to compare the two dependent samples, *i.e.*, mean behavioural variables (behaviour rate and female nest entrance) of males when they produced sounds and when they were mute. Additionally, Wilcoxon test was used to compare the frequency and duration of courtship and pre-spawning phases between males.

For the interspecific comparisons, the means of individual acoustic properties of soniferous sand gobies were compared with the non-parametric Kruskal-Wallis  $H$  test. To quantify interspecific acoustic variability among the soniferous sand gobies (genera *Knipowitschia*, *Orsinigobius*, *Pomatoschistus* and *Ninnigobius*), we used a multivariate approach. Principal component analysis (PCA) is a commonly used method in the bioacoustics literature for detecting the variables that explain the most variance among soniferous fish species or populations. PCA, based on the correlation matrix, was performed on transformed and standardised individual means of five sound variables (temporal: DUR, NP, PRR; spectral: PF and FM) to assess overall acoustic variability between sand gobies, and additionally to recognise acoustic variables explaining the observed variance. To assess the percentage of successful classification of the sounds assigned to the correct goby species, and to maximise the separability among taxa, we used linear discriminant analysis (LDA). Two

different LDAs were performed, first with the complete dataset (five acoustic variables for each species) and then removing the temperature-dependent features (DUR and PRR). Our results were presented as means ( $\bar{x}$ )  $\pm$  standard deviation (*s.d.*), while the level of significance for inter- and intraspecific comparisons was 5% ( $\alpha = 0.05$ ). Statistical analyses were performed in STATISTICA® (v. 13.6.0., TIBCO, USA), Past (v. 4.11) and R Studio (2022.07.0) software.

## Permits

*Orsinigobius croaticus* is legally protected by law as an endangered taxon in Croatia (Official Gazette of the Republic of Croatia, 2016). In addition, it is an endemic species with very limited distribution. As a result, the number of individuals employed in the laboratory experiments was kept to a minimum (less than 15) to prevent possible effects on the natural population of this species. The sampling by electrofishing for scientific purposes in the natural habitat was approved by the Ministry of Agriculture (permit number 525-13/0545-19-2), while all laboratory experimental protocols were approved by the Bioethics and Animal Welfare Committee of the Faculty of Science, University of Zagreb (permit number 251-58-10617-21-147).

## RESULTS

### Sound production and intraspecific sound signal structure

Males of *O. croaticus* produced a single type of acoustic signal, named pulsatile sound, during intersexual (male-female) interactions conducted within the reproductive season (April – October). Four resident males ( $\bar{x} \pm s.d. = 49.1 \pm 0.8$ ; range: 48.0 – 50.0 mm  $L_T$ ;  $40.9 \pm 1.8$ ; range: 38.9 – 42.2 mm  $L_S$ ;  $1.2 \pm 0.1$ ; range: 1.1 – 1.4 g  $W$ ;  $1.7 \pm 0.1$ ; range: 1.5 – 1.9 Fulton's  $K$ ) produced sounds when interacting with females, while the other four males remained mute and did not court. We recorded 372 sounds produced by the four males (mean 93.0 sounds per male). These signals were reminiscent of brief felid purrs. They were produced in a relatively irregular repetition pattern, at a rate of 4.7 sounds per min, while the maximum recorded rate was 10 sounds per min ( $7.7 \pm 1.4$ ; range: 6 – 10) (**Figure 1, Table 1**). Structurally, the sounds are short duration signals, lasting around 450 ms ( $442.0 \pm 132.6$ ; range: 156.8 – 952.8 ms), and composed of a variable number of short pulses ( $14.2 \pm 4.0$ ; range: 5 – 32) of around 15 ms ( $14.5 \pm 1.9$ ; range: 9.7 – 22.9 ms) (**Figure 2a-e, Table 1**). The individual pulse unit structure differed markedly between sounds, exhibiting one to three peaks with variable amplitude. Generally, the amplitude of a sound changed gradually, first increasing and then steadily decreasing throughout the sound, with the first two or three pulses being the loudest (**Figure 2a-e**). The pulse repetition rate varied from 26.0 to 38.0 Hz ( $32.5 \pm 1.6$  Hz), while the sound pulse period (PP) averaged 32 ms ( $31.9 \pm 1.4$ ; range: 27.6 – 37.5 ms). PP changed with water temperature, with higher values occurring at lower temperatures (**Figure 3**). Fatigue averaged  $1.1 \pm 0.08$  (range: 0.9 – 1.4). The sound peak frequency varied from 89 to 340 Hz ( $137.4 \pm 38.3$  Hz), although several higher frequency components were also present, especially in the range 0.5 - 1.5 kHz. Energy extended from 0.05 to 2 kHz (**Figure 2a-e, Table 1**), with most of the sound energy within 0.05 – 0.6 kHz. Frequency modulation of the sounds ranged from 0.7 – 1.1 Hz. Additionally, the calling effort varied between males from 0.37 to 0.60 ( $0.49 \pm 0.09$ ), indicating that some individuals emitted sounds more frequently than others (**Table 1**). Interestingly, sounds were never organised in bursts, which are usually composed of several consecutive sounds produced with regular inter-sound interval, as observed in some sand gobies.

Intraspecifically, soniferous *O. croaticus* males differed significantly in all acoustic features (Kruskal-Wallis  $H$  test,  $\chi^2 = 8.53 - 41.97$ ; d.f. = 3;  $N = 80$ ;  $P < 0.05$ ) except for calling effort (Kruskal-Wallis  $H$  test,  $\chi^2 = 5.67$ ; d.f. = 3;  $N = 7$ ;  $P > 0.05$ ) (**Table 1**). Some acoustic features were significantly correlated (*Spearman* rank correlations;  $rS = -0.90 - 0.97$ ;  $P < 0.05$ ), while the strongest correlation was observed between NP and DUR ( $rS = 0.97$ ), PRR with FMf ( $rS = 0.7$ ), and PRR with PP ( $rS = -0.9$ ). Note that for pairwise correlation between sound variables, we only considered  $rS > 0.7$  due to the limited sample size. These relations are expected as DUR results in part from NP, PRR and PP are redundant metrics, and FMf is the PRR of the initial part of the sound. In addition, some acoustic features were also correlated with physical features  $L_S$  (PF and FMi,  $rS = -0.53$ ;  $P < 0.05$  for both variables),  $W$  (DUR and NP,  $rS = 0.53$  and  $0.55$ ;  $P < 0.05$ ), and water temperature (PRR, PP, PF and FMi,  $rS = -0.67 - 0.69$ ;  $P < 0.05$ ) (**Figure 4**).

## Reproductive ethology and association with sounds

We identified nine male behavioural categories within three distinct reproductive phases in *O. croaticus*: Chase, Lead, Approach and Circling (courtship phase), Nest display, Frontal display, Nest rubbing, Pre-mating (pre-spawning phase) and Spawning (spawning phase). The category Pre-mating included all behaviours that occurred after the female entered the male nest and prior to belly-up position (*oviposition*). Spawning was not observed, but we observed the female's repeated upside-down or belly-up position (an indication of *oviposition*), during which she probably laid eggs on the nest ceiling. Briefly, the behaviours Chase, Lead, Approach and Circling were performed by the male outside the nest (recognised as the "courtship" phase), while Nest display, Frontal display, Nest rubbing, Pre-mating and Spawning were performed within the nest ("pre-spawning" and "spawning" phases). In some cases, Nest display and Frontal display were performed by the male occupying the nest with or without a female inside. However, Nest rubbing, Pre-mating and Spawning were always performed by the male when the female was inside the nest. The frequency, duration and overall percentage of these categories were scored during ethological analysis. The first dataset included seven video recordings (210 min) where at least one sound occurred per recording. Overall, we observed 410 behavioural acts (102.5 per male). The behavioural categories Nest display (29.3%), Pre-mating (22.7%) and Approach (19.1%) were most frequently observed, while Circling (1.2%), Chase (1.0%) and Lead (0.7%) were rarely recorded (**Figure 5a-d**). Concerning the association of sound production with behavioural acts, of the 410 behavioural acts, 99 acts (24.1%) were accompanied by sound production. The behaviours Pre-mating (303 sounds), Nest display (27 sounds), Frontal display (20 sounds), Nest rubbing (16 sounds) and Spawning (5 sounds) were coincided with sounds, while Approach, Circling and Lead never co-occurred with sound production (**Figure 5a**). During four Chase acts, only one sound was recorded. Only one spawning act (Spawning) was observed in this study, during which five sounds were produced. Unfortunately, spawning sounds were not used in the comparative purposes due to their limited occurrence. The chi-square ( $\chi^2$ ) test of independence indicated that behavioural categories Nest display and Pre-mating were significantly associated with sound production ( $\chi^2 = 138.3$ ; d.f. = 5;  $P < 0.05$ ; residual score: 1.5 and 41.5, respectively), while other categories failed to support this hypothesis (**Figure 5a**).

To compare male behaviour when soniferous or mute, a second dataset of eight video recordings was considered (190.5 min) of the same four males but in which no sound production was documented. In these recordings, we have observed 324 male behavioural acts (averaging 81.0 per male), of which Approach (38.3%), Lead (31.8%) and Nest display (20.1%) were the most frequent categories (**Figure 5b**). Contrary, Chase, Circling, Pre-mating or Spawning were not documented within these recording sessions. In general, there is an obvious dissimilarity between the frequency of the behavioural categories that were or were not accompanied by sounds. Specifically, Pre-mating, one of the two behaviours significantly associated with sound production, decreased from an average of 22.7% in the trials with sounds to 0% in the trials without sound. In addition, Nest display, Nest rubbing and Frontal display acts produced during sound emission decreased from 29.3%, 13.9% and 12%, to 20.1%, 3.7% and 6.2% in frequency in experiments without the sounds, respectively. On the other hand, Approach and Lead were more frequent during the mute behaviours (31% and 38%, respectively) than during sound production (19% and 0.7%, respectively) (**Figure 5b**). Overall, the behavioural rate decreased from 55.8% to 44.1% when males produced sounds in comparison to when they were mute (soniferous *vs.* mute males: means 2.79 *vs.* 1.55), though the differences were not significant (*Wilcoxon* signed-rank test,  $P > 0.05$ ) (**Figure 6a**). Importantly, the number of times the females entered the male nest differed significantly between the two datasets (means 3.71 *vs.* 0.71), as female nest entrance was more frequent when males produced sound than when they were mute (*Wilcoxon* signed-rank test,  $P < 0.05$ ) (**Figure 6b**).

When producing sounds, the occurrence frequency between the courtship (Chase, Lead, Approach and Circling) and pre-spawning (Nest display, Frontal display, Nest rubbing, Pre-mating) phases of reproduction did not differ between soniferous males (*Wilcoxon* signed-rank test,  $P > 0.05$ ), though their duration did (*Wilcoxon* signed-rank test,  $P < 0.05$ ). Generally, males exhibited courtship-related behaviours less frequently and for a shorter period compared to pre-spawning behaviours ( $x = 22.5$  *vs.* 79.7 for frequency and  $x = 173.4$  *vs.* 2393.3 for duration).

## Anatomical findings and movements during sound production

The pectoral girdle of *O. croaticus* was subjected to  $\mu$ CT scanning and anatomical dissection to identify the various osseous structures and muscles that may be involved in sound generation. From  $\mu$ CT scans, three functional units were distinguished in the skeletal part of the pectoral girdle of *O. croaticus*: the shoulder girdle (composed of the post-temporal, the supracleithrum and the cleithrum bones) dorsally attached to the neurocranium, the shoulder plate (i.e., four large radials), and the fin plate, made up of fin rays articulated with the shoulder plate (**Figure 7a-b**). On the dorsal tip of the cleithrum, anterior and posterior processes are present. The supracleithrum articulates with the post-temporal and the cleithrum, connecting with the cleithrum bone at its dorsal tip. The post-temporal is made up of a basal plate and two rostrally oriented processes (a “fork”) with dorsal and lateral attachments to the neurocranium. The rostral tip of the dorsal process is flattened and firmly attached to the epiotic bone. Putative sound producing muscles were observed during the dissection and were found originating on the neurocranium and inserting on the pectoral girdle (**Figure 7a-b**). The *levator pectoralis* muscle is divided into two bundles: the *pars lateralis* and the *pars medialis*. The *pars lateralis* originates on the posterior part of the pterotic and inserts on the anterior dorsal process of the cleithrum. The *pars medialis* is the thicker of the two muscles. It originates on the posterior part of the basioccipital and inserts on the medial part of the posterior dorsal process of the cleithrum.

Moreover, video recordings allowed to highlight characteristic fish movements during sound production, especially concerning the head region and fins. During sound production, soniferous males would usually stop swimming and would suspend the body on the fused pelvic fins. In addition, the pectoral fins were abducted, and the rays spread during sound emission. The male performed a lateral body quiver starting from the head to the tail (including dorsal fins), while the dorsal fins (both first and second) were erected prior to the production of the first pulse. Then the male would rapidly elevate the head and perform lateral head motions, while spreading the buccal and opercular cavities. The mouth was closed during the period of emission, though the anterior part of the branchial basket was slightly uplifted.

## Interspecific acoustic diversity in soniferous sand gobies

Eight soniferous sand gobies, namely *Ninnigobius canestrinii*, *Pomatoschistus marmoratus* (two populations; five individuals originating from Venice lagoon, Italy, and three from Portugal), *P. minutus*, *P. microps*, *P. pictus*, *Orsinigobius punctatissimus*, *O. croaticus* and *Knipowitschia panizzae*, produce pulsatile sounds, thus enabling acoustic interspecific comparisons. Interspecific pairwise comparisons revealed interspecific differences in the acoustic features DUR, NP, PRR, and FM (Kruskal-Wallis  $H$  test,  $\chi^2 = 15.97$ – $30.19$ ; d.f. = 8;  $N = 36$ ;  $P < 0.05$ ), while they did not differ in PF (Kruskal-Wallis  $H$  test,  $\chi^2 = 11.54$ ; d.f. = 8;  $N = 36$ ;  $P > 0.05$ ) (**Figure 8a-f**). On average, *P. marmoratus* (Portuguese), *O. punctatissimus*, *P. microps* and *K. panizzae* were the smallest in size (34–43 mm  $L_T$ ), while *P. minutus*, *N. canestrinii* and *P. marmoratus* (Italian) were the largest species (50–59 mm  $L_T$ ). In most cases, *K. panizzae* differed significantly from other species, especially in DUR and NP (Dunn’s multiple comparison test,  $P < 0.05$ ). Regarding PF, *P. microps* had the highest mean values, alongside with *K. panizzae* (Dunn’s multiple comparison test,  $P < 0.05$ ). Finally, *P. marmoratus* (Italian population) and *P. pictus* differed significantly from the rest of the species having lower values of FM, while other species presented upward or downward modulated sounds (Dunn’s multiple comparison test,  $P < 0.05$ ) (**Figure 8a-f**).

Results of multivariate statistics were summarised by a PCA and LDA, based on the five transformed ( $\log_{10}$  or square root) and standardised acoustic features (DUR, NP, PRR, PF and FM), highlighting the acoustic differences (heterogeneity) between species according to sound feature. Due to the FM’s negative raw values, we introduced a positive factor so that we could use this feature in the analyses. In PCA, the first two principal components of the PCA explained cumulatively 69.79% of the variation, with PC1 and PC2 explaining 39.28% and 30.51% of the variation, respectively. On the PC1 and PC2 scatterplot, although several species are clearly separated based on the acoustic features of their sounds, most of the plots overlap (**Figure A2**). PC1 was strongly associated with DUR (-0.69) and NP (-0.67), while PRR (-0.65) and PF (0.58) mostly contributed to PC2. We performed two LDA analysis, first with the complete dataset (five acoustic variables DUR, NP, PRR, PF and FM) and the second excluding the temperature-

dependent features (DUR and PRR), to test for sound classification into correct groups (*i.e.*, species). In the first LDA, the first two axes accounted for a discrimination of 83.36%, with LD1 accounting for 61.09% and LD2 for 22.27%. LDA successfully attributed the most sounds of a sand goby to the correct species according to five acoustic parameters, with a correct interspecific classification rate of 86.11%. For some goby species, a contingency table supports the 100% level of correct classification of sounds (*N. canestrinii*, *O. croaticus*, *O. punctatissimus*, *P. pictus* and *P. microps*), while for the remaining species lower levels were achieved (67% for *K. panizzae*, 80% for Italian and 67% for Portuguese *P. marmoratus*, 60% for *P. minutus*). In the LDA bi-plot, species clusters overlap, but not significantly, with some taxa occupying relatively isolated positions along the LD axes (**Figure 9**). LD1 supports differentiation of *N. canestrinii* and *P. marmoratus* (Italian) from the rest of the species within this acoustic space, while LD2 separated *K. panizzae* and *P. pictus* from the other taxa. Plots of other species means show overlapping patterns (**Figure 9**). Interestingly, two populations of *P. marmoratus* (Italian and Portuguese) overlap substantially on the scatterplot, even though the LDA successfully separated the species. LD1 was significantly loaded with FM (0.32), while LD2 with PRR (-0.47) and PF (-0.28). To explore the effect of water temperature on the interspecific acoustic classification success by LDA, we carried out a second LDA, including only the three acoustic features that are known to be unaffected by water temperature, namely NP, PF and FM. In this second LDA, axis 1 and 2 accounted for 95.49% of discrimination, with LD1 axis accounting for 66.88% and LD2 for 28.61%. However, the second LDA was less successful than the first LDA in accurately classifying the sounds of sand gobies, with a 69.44% rate of correct interspecific classification. Again, some species (*K. panizzae*, *P. microps* and *N. canestrinii*) achieved 100% classification, while the remaining species were misidentified in different percentages in comparison to first LDA (75% for *O. croaticus* and 80% *O. punctatissimus*, 66% for *K. panizzae*, 40% for Italian and 33% for Portuguese *P. marmoratus*, 40% *P. minutus* and 80% for *P. pictus*).

## DISCUSSION

Freshwater sand gobies are considered important indicators for the conservation of Mediterranean inland aquatic ecosystems due to their wide range of habitats and high level of endemism (Vanhove, Kovačić & Zogaris, 2016). Our study investigated, for the first time, the sound production and reproductive intersexual behaviour of a freshwater endemic Mediterranean goby, the Neretva dwarf goby *Orsinigobius croaticus*, under laboratory conditions. In addition, we investigated multimodal signalling, using both acoustic and visual signals, in this protected and geographically restricted sand goby. The results of this study are consistent with prior research suggesting that gobies employ sound production, along with visual or chemical communication, as an important sensory modality for intraspecific communication (Lugli et al., 1995; Amorim & Neves, 2007; Malavasi et al., 2009; Amorim et al., 2013; Bolgan et al., 2013; Blom et al., 2016). *O. croaticus* is the ninth acoustically investigated European sand goby, excluding *Economidichthys pygmaeus*, which was mute during the experiments. However, it is the only one with a high IUCN Red List classification status, listed as vulnerable (Crivelli, 2006, 2018).

Males of *O. croaticus* produced pulsatile sounds when interacting with females, during courtship, pre-spawning and spawning phases of the reproductive behaviour. Males did not produce sounds in all trials and calling rate varied between males and with female proximity. When males were in close contact with females or the prospective female approached/entered the nest, the calling rate would significantly increase from a few up to 10 sounds per min. Structurally, the pulsatile sounds in sand gobies are composed from a variable number (range 5 – 32) of pulses (organised in pulse trains), which are considered the fundamental units of this acoustic signal (Lindström & Lugli, 2000; Zeyl et al., 2016). *Orsinigobius croaticus* acoustic signals are short and low-frequency sounds (< 500 ms, ~ 140 Hz) composed from a short number of sound pulses with an average duration and period of around 15 ms and 32 Hz, respectively.

In this study, PD and PP differed significantly among males. In pulsed acoustic signals, PD can be related to body size and condition (Amorim et al., 2010) or temperature (Vicente et al., 2015), while PP is often dependent on temperature, but also reflects phylogenetic affinities in fish groups such as pomacentrids, cichlids, and sand gobies (Myrberg et al., 1978; Amorim et al., 2008, 2013; Vicente et al., 2015). Here, PP

in *O. croaticus* increased with decreasing water temperature ( $\sim 37$  ms at  $19^\circ\text{C}$  vs.  $\sim 29$  ms at  $21.1^\circ\text{C}$ ). In *P. pictus* water temperature significantly influenced pulse period and explained 83% of its variability (Amorim et al., 2013). In ectothermic animals (such as fish), temperature-dependence in sound-producing central and peripheral mechanisms is corroborated, since it results from muscle activation (Bennett, 1985; Feher et al., 1998; Rome & Lindstedt, 1998; Kéver, Boyle, Parmentier, 2015; Vicente et al., 2015; Ladich, 2018).

Pulsatile sounds of *O. croaticus* males differed in all acoustic features except calling effort. These acoustic differences amongst soniferous males highlights the unique intraspecific acoustic variability of their reproductive sounds. In addition, several acoustic features were shown to be correlated to physical characteristics. We found an inverse effect of male size on sound frequency, since these two features were significantly and negatively related. This effect has previously been recognised in acoustic studies on sand gobies (Lindström & Lugli, 2000; Malavasi et al., 2008; Amorim et al., 2013). In terms of relationships, DUR and NP were highly and positively correlated in our study, whereas PRR and PP had a negative association. The strong correlation between DUR and NP suggests that the sound-producing mechanism is based on a fixed motor pattern (Parmentier & Lecchini, 2022).

In this study, *O. croaticus* males exhibited nine (visual) behavioural acts, confined to three distinct reproductive phases. The sound production in males was mostly associated with pre-spawning behaviours. Also, males exhibited courtship-related behaviours less frequently and for a shorter period than pre-spawning behaviours. These findings imply that the sound production is key in the mating process in *O. croaticus* and that it is likely efficient in transmitting information at only short-range distances (within one body length). Regarding the multimodal communication, soniferous *O. croaticus* males differed in the frequency and occurrence of displayed behavioural categories (and their acts) when producing sounds and when they were mute, since most of the categories in the mute experiments were related to the courtship phase (outside the nest). Some behavioural acts, such as Pre-mating, Chase, Circling and Spawning, were completely absent from mute experiments. When producing sounds, Pre-mating and Nest display were the most frequent categories, indicating that males modulate their behaviour according to mate attraction investment. These findings could indicate that the multimodal signals, as produced by *O. croaticus* males, could convey a wider set of information to the prospective breeding females, rather than using only one signal type. Indeed, males of different species, such as *P. pictus*, make a suite of signals from one or more modalities that females may use in mating decisions (Amorim & Neves, 2007; Amorim et al., 2013; Bro-Jørgensen, 2010). Multimodal signals, which are used by many species to communicate, contain components that can be analysed by multiple sensory channels (Otovic & Partan, 2009). Fish communicate through visual, chemical and acoustic signals often operating simultaneously to improve the chances of mating success, by indicating the physical quality or the motivation of the emitter (e.g., Levine, Lobel & MacNichol, 1980; Liley, 1982; Heuschele et al., 2009; Amorim et al., 2013). It has been suggested that this acoustic modality is highly advantageous for territorial species, in which the nest site is frequently hidden, and the male is out of sight from the prospective mate (Myrberg, 1981).

Another significant finding from the current study is that females entered the male's territory, particularly the nest hollow, more frequently when accompanied by sound production than when the males were mute. In this study, the two males who received the most female entries were the largest. These two males exhibited the sounds with highest values of NP, FMi and PRR, suggesting that these acoustic features might be used to communicate important information to potential mates. Other studies suggest that different acoustic traits or morphological features could advertise male quality (genetic or phenotypic), serving as honest signals of different aspects of male quality in sand gobies (Knapp & Kovach, 1991; Amorim et al., 2013). According to Amorim et al. (2013), successful breeding *P. pictus* males produced more sounds and with a higher number of pulses than unsuccessful males.

Our findings indicate there are anatomical similarities in the musculo-skeletal system of the pectoral girdle between the previously studied *Pomatoschistus* gobies and *O. croaticus* (Adriaens et al., 1993; Parmentier et al., 2017). Our study provided the first anatomical dissections and  $\mu\text{CT}$  scans of the *O. croaticus* pectoral girdle and neurocranium. However, it is hypothesised that the Bauplan of soniferous gobies does not show

deep significant modifications, meaning that the anatomy of soniferous species appears to be comparable to that of their mute relatives (Parmentier & Fine, 2016). To investigate the anatomy of the sound producing mechanism in gobies, Parmentier et al. (2013, 2017) undertook two empirical studies in two European gobies, gobiid *Gobius paganellus* (Gobiidae) and sand goby *P. pictus* (Gobionellidae), with the goal of testing the hypothesis of contraction of the pectoral girdle muscles. These multidisciplinary studies suggested strong similarities between the two gobies, and that sounds might be generated by the contraction of the *levator pectoralis* muscle. These results suggested that the pectoral girdle is most likely involved in sound production. It is worth noting that sound production was coupled with nodding in *G. paganellus* or with lateral head movements in *P. pictus* (Parmentier et al., 2013, 2017). However, this does not mean head movements are responsible for the sound production. In this study, the pectoral girdle of *O. croaticus* consists of three functional osseous parts, with main elements present as in other dissected sand gobies (Adriaens et al., 1993; Parmentier et al., 2013, 2017). In addition, the *levator pectoralis* muscles, divided into two bundles (*pars lateralis* and *pars medialis*), were also found in *O. croaticus*, originating on the neurocranium and inserting onto the pectoral girdle. Four large radial bones were also present, forming the shoulder plate in *O. croaticus*. Lastly, the males performed lateral head movements during sound emission. Some authors suggest that certain sound characteristics are positively correlated with temperature if pulses are directly based on sonic muscle contractions (Ladich, 2018). Although our study did not include methodologies such as muscle histology, high-speed video, or electromyography to fully corroborate the findings from earlier research, we believe there is sufficient evidence to hypothesise that the assumed sound producing mechanism in *O. croaticus* could be related with the contractions of the *levator pectoralis* (*pars lateralis* and *medialis*) muscles. Our assumptions are based on: 1) the observed anatomical similarities (i.e., muscle organization) between *O. croaticus* and other tested sand gobies, 2) prominent temperature-dependence of the peripheral (muscular) part of the sonic mechanism (as seen from the correlation of acoustic features with water temperature, and PP variation), and 3) head lateral movements observed during sound emission. Interestingly, in some situations, males were observed to perform body movements (lateral movements, head uplift, erection of fins), but without sound production, indicating that sound production requires more than just body movements. This supports the hypothesis that sounds are intentional and not only a by-product of other activities such as breathing, feeding or swimming.

Sand gobies are highly similar morphologically (Kovačić, 2008) and frequently live in sympatry (Miller, 1986), making their discrimination difficult. Several discrimination techniques have previously been proposed for gobioids, such as mitochondrial/nuclear DNA markers (Agorreta et al., 2013; Vanhove et al., 2012; Thacker et al., 2018), otoliths in the inner ear (Lombarte et al., 2018) and behaviour (Malavasi et al., 2012). Recently, the sounds (and their acoustic features) have become a useful parameter in determining the phylogenetic relationships in fish (Rice & Bass, 2009; Parmentier et al., 2009; Mélotte et al., 2016; Bolgan et al., 2020), particularly in gobies (Malavasi et al., 2008; Horvatić et al., 2021). The aim of this study was not to infer the phylogenetic relationships between sand gobies, but rather to investigate how the species can be separated according to their acoustic features, and how well the sounds can be classified for each taxon. However, qualitatively, the pulsatile sounds of soniferous sand gobies from this study are similar in that they are composed of a series of pulses (**Figure 9**), though when examined quantitatively (using a multivariate approach), they were discriminated according to their spectral and temporal acoustic parameters. In the present study, we found interspecific differences among the sand gobies species based on acoustic properties. The LDA assigned each sound produced by sand gobies to the correct species with a discrimination rate of 86%. On the scatterplot, acoustic variables NP and FM contributed to the separation of species in the negative direction, while DUR, PF and PRR separated species in the positive direction. Note, however, that species were recorded at different temperatures and results should be taken with caution. The observed interspecific differences, although based on a limited dataset, shed light on the taxonomic position and affinities of the genera *Orsinigobius* and *Ninnigobius*, relative to the rest of the sand gobies. *Ninnigobius canestrinii* and *K. panizzae*, along with *P. pictus*, were the species most separated from the other taxa on the LDS bi-plot. Some authors have opposed the separation of *O. croaticus* and *O. punctatissimus* into the genus *Orsinigobius*, and the isolation of *N. canestrinii* from the genus *Pomatoschistus* (Thourgard et al., 2021). On the LDS bi-plot, the two *Orsinigobius* taxa were closely situated. *Pomatoschistus* taxonomy

is currently complicated, but *P. minutus* from our study was in the close proximity of the two *Orsinigobius* taxa. Interestingly, the hulls of the two populations of *P. marmoratus* overlapped in LDA, despite the fact they encompass individuals from a wide geographic area (the Po River delta in Italy and Parede/Arrábida in Portugal). However, the Italian population appeared partially isolated from the rest of the species. When applying the reduced dataset, the classification rate in LDA decreased from 86% to 69%, which is not an unacceptable outcome, though it implies that interspecific discrimination becomes more difficult without certain acoustic features, such as temperature-dependent DUR and PRR in our case.

## CONCLUSION

In conclusion, our study demonstrates that the threatened and geographically restricted freshwater sand goby, *Orsinigobius croaticus*, produces pulsatile sounds during intersexual laboratory experiments. These sounds were produced during courtship, pre-spawning and spawning phases of the reproduction interactions with females. In addition, our results suggest that *levator pectoralis* muscles could be responsible for pulse emission. Finally, at the interspecific level, acoustic signals produced by soniferous sand gobies appear to be sufficiently different and species-specific to enable the discrimination of species.

## AUTHOR CONTRIBUTIONS

**Sven Horvatić** : conceptualization (lead); data curation (lead); formal analysis (equal); investigation (equal); methodology (equal); visualization (lead); software (lead); writing - original draft (lead); writing - review & editing (equal). **Eric Parmentier** : formal analysis (equal); investigation (equal); methodology (equal); visualization (equal); software (equal); writing - original draft (equal); writing - review & editing (equal). Stefano Malavasi: conceptualization (equal); data curation (equal); writing - original draft (equal); supervision (equal); writing - review & editing (equal). **Maria P. Clara Amorim** : conceptualization (equal); data curation (equal); writing - original draft (equal); writing - review & editing (equal). **Paulo J. Fonseca** : conceptualization (equal); data curation (equal); writing - original draft (equal); writing - review & editing (equal). **Davor Zanella** : data curation (equal); supervision (equal); writing - original draft (equal); writing - review & editing (equal); resources (lead).

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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## DATA AVAILABILITY STATEMENT

Audio files and Excel tables are available from the Figshare data repository (<https://doi.org/10.6084/m9.figshare.22787093.v1> and <https://doi.org/10.6084/m9.figshare.22786952.v1>).

## REFERENCES

Abell, R., Thieme, M. L., Revenga, C., Bryer, M., Kottelat, M., Bogutskaya, N., Coad, B., Mandrak, N., Contreras Balderas, S., Bussing, W., Stiassny, M. L. J., Skelton, P., Allen, G. R., Unmack, P., Naseka, A., Ng, R., Sindorf, N., Robertson, J., Armijo, E., Higgins, J. V., Heibel, T. J., Wikramanayake, E., Olson, D., López, H. L., Reis, R. E., Lundberg, J. G., Sabaj Pérez, M. H., & Petry, P. (2008). Freshwater Ecoregions of the World: A New Map of Biogeographic Units for Freshwater Biodiversity Conservation. *BioScience*, 58(5), 403–414. <https://doi.org/10.1641/B580507>

Abramoff, M. D., Magalhaes, P. J., & Ram, S. J. (2014). Image processing with ImageJ. *Biophoton*

- Adriaens, D., Decleyre, D., & Verraes, W. (1993). Morphology of the pectoral girdle in *Pomatoschistus lozanoi* De Buen, 1923 (Gobiidae), in relation to pectoral fin adduction. *Belgian Journal of Zoology*, 123, 137-157.
- Agorreta, A., San Mauro, D., Schliewen, U.K., Van Tassell, J.L., Kovačić, M., Zardoya, R., & Rüber, L. (2013). Molecular phylogenetics of Gobioidae and phylogenetic placement of European gobies. *Molecular Phylogenetics and Evolution*, 69(3), 619-633. <https://doi.org/10.1016/j.ympev.2013.07.013>
- Akamatsu, A., Okumura, T., Novarini, N., & Yan, H. Y. (2002). Empirical refinements applicable to the recording of fish sounds in small tanks. *Journal of the Acoustical Society of America*, 112, 3073-3082. doi: 10.1121/1.1518218
- Amorim, M.C.P. (2006). Diversity of sound production in fish. In F. Ladich (Ed.), *Communication in Fishes*, Vol. 1 (pp. 71-105). Science Publishers.
- Amorim, M. C. P., & Neves, A. S. M. (2007). Acoustic signalling during courtship in the painted goby, *Pomatoschistus pictus*. *Journal of the Marine Biological Association of the United Kingdom*, 87, 1017-1023. <https://doi.org/10.1017/S0025315407056822>
- Amorim, M. C. P., Neves, A. S. M. (2008). Male painted goby (*Pomatoschistus pictus*) vocalize to defend territories. *Behaviour*, 145, 1065-1083.
- Amorim, M. C. P., Simões, J. M., Mendonça, N., Bandarra, N. M., Almada, V. C., & Fonseca, P. J. (2010). Lusitanian toadfish song reflects male quality. *Journal of Experimental Biology*, 213(17), 2997-3004. <https://doi.org/10.1242/jeb.041020>
- Amorim, M. C. P., Pedroso, S. S., Bolgan, M., Jordão, J. M., Caiano, M., & Fonseca, P. J. (2013). Painted gobies sing their quality out loud: acoustic rather than visual signals advertise male quality and contribute to mating success. *Functional Ecology*, 27, 289-298. doi: 10.1111/1365-2435.12032
- Amorim, M. C. P., Vasconcelos, R. O., Bolgan, M., Pedroso, S. S., & Fonseca, P. J. (2018). Acoustic communication in marine shallow waters: testing the acoustic adaptive hypothesis in sand gobies. *Journal of Experimental Biology*, 221(Pt 22), jeb183681. <https://doi.org/10.1242/jeb.183681>
- Bennett, A. F. (1985). Temperature and muscle. *Journal of Experimental Biology*, 115, 333-344.
- Betancur-R, R., Wiley, E. O., Arratia, G., Acero, A., Bailly, N., Miya, M., Lecointre, G., & Ortí, G. (2017). Phylogenetic classification of bony fishes. *BMC Evolutionary Biology*, 17(1), 162. <https://doi.org/10.1186/s12862-017-0958-3>
- Blom, E. L., Mück, I., Heubel, K., Müller, R., Schulz-Mirbach, T., & Ladich, F. (2016). Acoustic and visual courtship traits in two sympatric marine Gobiidae species – *Pomatoschistus microps* and *Pomatoschistus minutus*. *Environmental Biology of Fishes*, 99, 999-1007. <https://doi.org/10.1007/s10641-016-0550-5>
- Bolgan, M., Pedroso, S. S., Picciulin, M., Fonseca, P. J., & Amorim, M. C. P. (2013). Differential investment in acoustic communication during social interactions in two closely-related sand goby species. *Behaviour*, 150(2), 133-152. <https://doi.org/10.1163/1568539X-00003041>
- Bolgan, M., Crucianelli, A., Mylonas, C. C., Henry, S., Falguière, J. C., & Parmentier, E. (2020). Calling activity and calls' temporal features inform about fish reproductive condition and spawning in three cultured Sciaenidae species. *Aquaculture*, 524, 735243.
- Bro-Jørgensen, J. (2010) Dynamics of multiple signalling systems: animal communication in a world in flux. *Trends in Ecology & Evolution*, 25, 292-300.
- Crivelli, A. J. (2018). *Orsinigobius croatica* (amended version of 2006 assessment). The IUCN Red List of Threatened Species 2018: e.T11031A136231892. <https://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T11031A136231892.en>

- Ćaleta, M., Buj, I., Mrakovčić, M., Mustafić, P., Zanella, D., Marčić, Z., Duplić, A., Mihinjač, T., & Katavić, I. (2015). Endemic Fish of Croatia. Agencija za zaštitu okoliša, Zagreb, 116pp. (in Croatian)
- Ćaleta, M., Marčić, Z., Buj, I., Zanella, D., Mustafić, P., Duplić, A., & Horvatić, S. (2019). A review of extant Croatian freshwater fish and lampreys. Annotated list and distribution. *Croatian Journal of Fisheries*, 77(3), 136–232. <https://doi.org/10.2478/cjf-2019-0016>
- Darwall, W., Carrizo, S., Numa, C., Barrios, V., Freyhof, J., & Smith, K. (2014). Freshwater Key Biodiversity Areas in the Mediterranean Basin Hotspot: Informing species conservation and development planning in freshwater ecosystems. Cambridge, UK and Malaga, Spain: IUCN. x + 86pp.
- de Jong, K., Amorim, M. C. P., Fonseca, P. J., Klein, A., & Heubel, K. U. (2016). Noise affects acoustic courtship behavior similarly in two species of gobies. *Proceedings of Meetings on Acoustics*, 27, 010018. <https://doi.org/10.1121/2.0000272>
- Erisman, B. E., & Rowell, T. J. (2017). A sound worth saving: Acoustic characteristics of a massive fish spawning aggregation. *Biology Letters*, 13, 20170656. <https://doi.org/10.1098/rsbl.2017.0656>
- Feher, J.J., Waybright, T.D., Fine, M.L., 1998. Comparison of sarcoplasmic reticulum capabilities in toadfish (*Opsanus tau*) sonic muscle and rat fast twitch muscle. *J. Muscle Res.* 19, 661–674.
- Fine, M.L., & Parmentier, E. (2015). Mechanisms of Fish Sound Production. In F. Ladich (Ed.), *Sound Communication in Fishes (Animal Signals and Communication, Vol. 4, pp. 39–62)*. Springer Vienna. [https://doi.org/10.1007/978-3-7091-1846-7\\_3](https://doi.org/10.1007/978-3-7091-1846-7_3)
- Freyhof, J. (2011). Diversity and distribution of freshwater gobies from the Mediterranean, the Black and Caspian Seas. In R. A. Patzner, J. L. Van Tassell, M. Kovačić, & B. G. Kapoor (Eds.), *The Biology of Gobies* (pp. 279–288). Science Publishers, CRC Press
- Gkenas, C., Malavasi, S., Georgalas, V., Leonardos, I. D., & Torricelli, P. (2010). The reproductive behaviour of *Economidichthys pygmaeus*: secondary loss of sound production within the sand goby group? *Environmental Biology of Fishes*, 87, 299–307. doi: 10.1007/s10641-010-9597-x
- Hawkins, A.D. & Amorim, M.C.P. (2000). Spawning sounds of the male haddock, *Melanogrammus aeglefinus*. *Environmental Biology of Fishes*, 59, 29–41.
- Heuschele, J., Mannerla, M., Gienapp, P., & Candolin, U. (2009). Environment-dependent use of mate choice cues in sticklebacks. *Behavioral Ecology*, 20(6), 1223–1227. <https://doi.org/10.1093/beheco/arp123>
- Horvatić S., Marčić Z., Mrakovčić M., Mustafić P., Buj I., Ćaleta M. & Zanella D. (2017). Threatened fishes of the world: *Orsinogobius croaticus* (Mrakovčić, Kerovec, Mišetić & Schneider, 1996) (Teleostei: Gobiidae). *Croatian Journal of Fisheries*, 75, 30–35.
- Horvatić, S., Malavasi, S., Vukić, J., Šanda, R., Marčić, Z., Ćaleta, M., Lorenzoni, M., Mustafić, P., Ivana Buj, I., Lucija Onorato, L., Lucija Ivić, L., Francesco Cavarro, F., Zanella, D. (2021). Correlation between acoustic divergence and phylogenetic distance in soniferous European gobiids (Gobiidae; Gobiidae lineage). *PloS one*, 16(12), e0260810. <https://doi.org/10.1371/journal.pone.0260810>
- IUCN. (2006). IUCN Red List of Threatened Species. Retrieved from [www.iucnredlist.org](http://www.iucnredlist.org). Accessed on 17th January 2023.
- IUCN. (2018). IUCN Red List of Threatened Species. Retrieved from [www.iucnredlist.org](http://www.iucnredlist.org). Accessed on 17th January 2023.
- Jublier, N., Bertucci, F., Kéver, L., Colleye, O., Ballesta, L., Nemeth, R.S., Lecchini, D., Rhodes, K.L., Parmentier, E. (2019). Passive monitoring of phenological acoustic patterns reveals the sound of the camouflage grouper, *Epinephelus polyphkadion*. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 1-11. <https://doi.org/10.1002/aqc.3242>

- Kéver, L., Boyle, K. S., & Parmentier, E. (2015). Effects of seawater temperature on sound characteristics in *Ophidion rochei* (Ophidiidae). *Journal of Fish Biology*, 87(2), 502-509. <https://doi.org/10.1111/jfb.12730>
- Knapp, R.A., & Kovach, J.T. (1991). Courtship as an honest indicator of male parental quality in the bicolor damselfish, *Stegastes partitus*. *Behavioral Ecology*, 2, 230–295. <https://doi.org/10.1093/beheco/2.3.230>
- Kottelat, M., & Freyhof, J. (2007). *Handbook of European freshwater fishes*. Cornol, Switzerland and Berlin, Germany: Freyhof.
- Kovačić, M. (2008). The key for identification of Gobiidae (Pisces: Perciformes) in the Adriatic Sea. *Acta Adriatica*, 49, 245–254.
- Kovačić, M., & Patzner, R. A. (2011). North-Eastern Atlantic and Mediterranean gobies. In Patzner, R. A., Van Tassell, J. L., Kovačić, M., & Kapoor, B. G. (Eds.), *The Biology of Gobies* (pp. 177–193). Science Publishers, CRC Press.
- Kutle, A. (Ed.). (1999). *Pregled stanja biološke i krajobrazne raznolikosti Hrvatske sa strategijom i akcijskim planovima zaštite* [Review of the state of biological and landscape diversity of Croatia with strategy and action plans for protection]. Državna uprava za zaštitu prirode i okoliša, Zagreb.
- Ladich, F. (2018). Acoustic communication in fishes: temperature plays a role. *Fish and Fisheries*, 19(4), 598–612. <https://doi.org/10.1111/faf.12272>
- Levine, J.S., Lobel, P.S., MacNichol, E.F. (1980). Visual Communication in Fishes. In: Ali, M.A. (eds) *Environmental Physiology of Fishes*. NATO Advanced Study Institutes Series, vol 35. Springer, Boston, MA. [https://doi.org/10.1007/978-1-4899-3659-2\\_17](https://doi.org/10.1007/978-1-4899-3659-2_17)
- Liley, N.R. (1982). Chemical Communication in Fish. *Canadian Journal of Fisheries and Aquatic Sciences*, 39, 22-35.
- Lindström, K., & Lugli, M. (2000). A quantitative analysis of the courtship acoustic behaviour and sound patterning in male sand goby, *Pomatoschistus minutus*. *Environmental Biology of Fishes*, 58(4), 411–424. <https://doi.org/10.1023/A:1007695526177>
- Lobel, P. (1992). Sounds produced by spawning fishes. *Environmental Biology of Fishes*, 33, 351–358. <https://doi.org/10.1007/bf00010947>
- Lobel, P. S. (2002). Diversity of fish spawning sounds and the application of passive acoustic monitoring. *Bioacoustics*, 12, 286–289. <https://doi.org/10.1080/09524622.2002.9753724>
- Lombarte, A., Miletić, M., Kovačić, M., Otero-Ferrer, J. L., & Tuset, V. M. (2018). Identifying sagittal otoliths of Mediterranean Sea gobies: variability among phylogenetic lineages. *Journal of Fish Biology*, 92, 1768–1787. <https://doi.org/10.1111/jfb.13615> PMID: 29756341
- Longrie, N., Poncin, P., Denoël, M., Gennotte, V., Delcourt, J., & Parmentier, E. (2013). Behaviours associated with acoustic communication in Nile tilapia (*Oreochromis niloticus*). *PLoS ONE*, 8, e61467. <https://doi.org/10.1371/journal.pone.0061467>
- Lugli, M., Pavan, G., Torricelli, P., & Bobbio, L. (1995). Spawning vocalizations in male freshwater gobiids (Pisces, Gobiidae). *Environmental Biology of Fishes*, 43, 219–231. <https://doi.org/10.1007/BF00005853>
- Lugli, M., Torricelli, P., Pavan, G., & Miller, P. J. (1996). Breeding sounds of male *Padogobius nigricans* with suggestions for further evolutionary study of vocal behaviour in gobioid fishes. *Journal of Fish Biology*, 49(4), 648–657.
- Lugli M, Torricelli P, Pavan G, Mainardi D (1997) Sound production during courtship and spawning among freshwater gobiids (Pisces, Gobiidae). *Mar Freshw Behav Physiol* 29:109–126. doi:10.1080/10236249709379003

- Lugli, M., & Torricelli, P. (1999). Prespawning sound production in Mediterranean sand-gobies. *Journal of Fish Biology*, 54, 691–694.
- Malavasi, S., Collatuzzo, S., & Torricelli, P. (2008). Interspecific variation of acoustic signals in Mediterranean gobies (Perciformes, Gobiidae): Comparative analysis and evolutionary outlook. *Biological Journal of the Linnean Society*, 93, 763–778. <https://doi.org/10.1111/j.1095-8312.2008.00947.x>
- Malavasi, S., Valerio, C., & Torricelli, P. (2009). Courtship sounds and associated behaviours in the Cane-strini's goby *Pomatoschistus canestrinii*. *Journal of Fish Biology*, 75, 1883–1887.
- Malavasi, S., Gkenas, C., Leonardos, I., Torricelli, P., & McLennan, D. A. (2012). The phylogeny of a reduced 'sand goby' group based on behavioural and life history characters. *Zoological Journal of the Linnean Society*, 165(4), 916–924. <https://doi.org/10.1111/j.1096-3642.2012.00832.x>
- Mann, D.A., Hawkins, A.D., & Jech, J.M. (2008). Active and Passive Acoustics to Locate and Study Fish. In J.F. Webb, R.R. Fay, & A.N. Popper (Eds.), *Fish Bioacoustics* (Springer Handbook of Auditory Research, Vol. 32, pp. 301–327). Springer. [https://doi.org/10.1007/978-0-387-73029-5\\_9](https://doi.org/10.1007/978-0-387-73029-5_9)
- Mazzoldi, C., & Rassotto, M. B. (2001). Extended breeding season in the marbled goby *Pomatoschistus marmoratus* (Teleostei: Gobiidae), in the Venetian Lagoon. *Environmental Biology of Fishes*, 61(2), 175–183. <https://doi.org/10.1023/A:1011030408898>
- Melotte, G., Vigouroux, R., Michel, C., & Parmentier, E. (2016). Interspecific variation of warning calls in piranhas: A comparative analysis. *Scientific Reports*, 6. <https://doi.org/10.1038/srep36127> PMID: 27782184
- Miller, P. J. (1986). Gobiidae. In P. J. P. Whitehead, M. L. Bauchot, J. C. Hureau, J. Nielsen, & E. Tortonese (Eds.), *Fishes of the North-eastern Atlantic and the Mediterranean*, Volume 3 (pp. 1019–1085). Paris: UNESCO.
- Miller, P. J. (2004). The freshwater fishes of Europe. Gobiidae 2. AULA-Verlag.
- Mrakovčić, M., Kerovec, M., Mišetić, S., & Schneider, D. (1996). Description of *Knipowitschia punctatissima croatica*, (Pisces: Gobiidae), a new freshwater goby from Dalmatia, Croatia. In A. Kirchhofer & D. Hefti (Eds.), *Conservation of Endangered Freshwater Fish in Europe* (pp. 311–319). Birkhäuser Verlag.
- Mrakovčić, M., Brigić, A., Buj, I., Čaleta, M., Mustafić, P., & Zanella, D. (2006). Red Book of Freshwater Fishes of Croatia. Ministarstvo kulture, Državni zavod za zaštitu prirode, Zagreb, 253pp. (in Croatian with English abstract)
- Myers, N., Mittermeier, R., Mittermeier, C., Fonseca, G.A.B., & Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature*, 403, 853–858. <https://doi.org/10.1038/35002501>
- Myrberg, A. A., Spanier, E., & Ha, S. J. (1978). Temporal patterning in acoustical communication. In *Contrasts in Behavior* (pp. 137–179). Wiley.
- Myrberg, A.A., Jr. (1981). Sound communication and interception in fishes. In W.N. Tavolga, A.N. Popper, & R.R. Fay (Eds.), *Hearing and Communication in Fishes* (pp. 395–424). New York, NY: Springer-Verlag.
- Myrberg, A.A., Jr, & Lugli, M. (2006). Reproductive behaviour and acoustical interactions. In F. Ladich, S.P. Collin, P. Moller, & B.G. Kapoor (Eds.), *Communication in Fishes*, Vol. 1 (pp. 149–176). Science Publishers.
- Nelson, J. S., Grande, T. C., & Wilson, M. V. H. (2016). *Fishes of the World* (5th ed.). John Wiley and Sons. <https://doi.org/10.1002/9781119174844>
- Otovic P, Partan S. 2009. Multimodal signaling in animals. In: Squire LR, editor. *Encyclopedia of neuroscience*. London (UK): Academic Press. p.1095–1105.
- Parmentier, E., Lecchini, D., Frederich, B., Brie', C., & Mann, D. (2009). Sound production in four damselfish (*Dascyllus*) species: Phyletic relationships. *Biological Journal of the Linnean Society*, 97, 928–940.

- Parmentier, E., Kéver, L., Casadevall, M., & Lecchini, D. (2010). Diversity and complexity in the acoustic behaviour of *Dacyllus flavicaudus* (Pomacentridae). *Marine Biology*, 157, 2317–2327. <https://doi.org/10.1007/s00227-010-1498-1>
- Parmentier, E., Kéver, L., Boyle, K., Corbisier, Y., Sawelew, L., & Malavasi, S. (2013). Sound production mechanism in *Gobius paganellus* (Gobiidae). *Journal of Experimental Biology*, 216, 3189–3199. <https://doi.org/10.1242/jeb.087205>
- Parmentier, E., & Fine, M.L. (2016). Fish sound production: insight. In R. Suthers, T.C. Fitch, A.N. Popper, & R.R. Fay (Eds.), *Vertebrate Sound Production and Acoustic Communication* (pp. 19-49). New York, NY: Springer.
- Parmentier, E., Petrinisec, M., Fonseca, P. J., & Amorim, M. C. P. (2017). Sound-production mechanism in *Pomatoschistus pictus*. *Journal of Experimental Biology*, 220, 4374–4376.
- Parmentier, E., & Lecchini, D. (2022). Sound Communication. In Laudet, V., & Ravasi, T. (Eds.). *Evolution, Development and Ecology of Anemonefishes: Model Organisms for Marine Science* (1st ed.). CRC Press. <https://doi.org/10.1201/9781003125365>
- Pedroso, S. S., Barber, I., Svensson, O., Fonseca, P. J., Amorim, M. C. P., & Almada, V. C. (2013). Courtship sounds advertise species identity and male quality in sympatric *Pomatoschistus* spp. gobies. *PLoS One*, 8, e64620. doi: 10.1371/journal.pone.0064620
- Tutman, P., Zanella, D., Horvatić, S., Hamzić, A., Adrović, A., Dulčić, J., & Glamuzina, B. (2020). Freshwater gobies (Gobiidae) of Bosnia and Herzegovina: a review of the current status and distribution. *Journal of Vertebrate Biology*, 69(4), 1-15. <https://doi.org/10.25225/jvb.20046>
- Rice, A. N., & Bass, A. H. (2009). Novel vocal repertoire and paired swimbladders of the three-spined toadfish, *Batrachomoeus trispinosus*: insights into the diversity of the Batrachoididae. *Journal of Experimental Biology*, 212, 1377–1391. <https://doi.org/10.1242/jeb.028506> PMID: 19376959
- Rice, A.N., Farina, S.C., Makowski, A.J., Kaatz, I.M., Lobel, P.S., & Bemis, W.E. (2020). Evolution and Ecology in Widespread Acoustic Signaling Behavior Across Fishes. *bioRxiv*. <https://doi.org/10.1101/2020.09.14.296335>
- Rice, A.N., Farina, S.C., Makowski, A.J., Kaatz, I.M., Lobel, P.S., & Bemis, W.E. (2022). Evolutionary Patterns in Sound Production across Fishes. *Ichthyology & Herpetology*, 110(1), 1–12. <https://doi.org/10.1643/i2020172>
- Rome, L.C., Lindstedt, S.L. (1998). The quest for speed: muscles built for high-frequency contractions. *News Physiol. Sci.* 13, 261–268.
- Rowell, T., Nemeth, R., Schärer, M., & Appeldoorn, R. (2015). Fish sound production and acoustic telemetry reveal behaviors and spatial patterns associated with spawning aggregations of two Caribbean groupers. *Marine Ecology Progress Series*, 518, 239–254. <https://www.int-res.com/abstracts/meps/v518/p239-254/>
- Šanda, R., & Kovačić, M. (2009). Freshwater gobies in the Adriatic drainage basin of the Western Balkans. *Annales. Series Historia Naturalis*, 19(1), 1–10.
- Thacker, C. E. (2009). Phylogeny of Gobioidae and placement within Acanthomorpha with a new classification and investigation of diversification and character evolution. *Copeia*, 2009, 93–104.
- Thacker, C. E., Gkenas, C., Triantafyllidis, A., Malavasi, S., Leonardos, I., & Leonardos, I. (2019). Phylogeny, systematics and biogeography of the European sand gobies (Gobiiformes: Gobiionellidae). *Zoological Journal of the Linnean Society*, 185(1), 212–225. <https://doi.org/10.1093/zoolinnean/zly026>
- Toricelli, P., Lugli, M., & Pavan, G. (1990). Analysis of sounds produced by male *Padogobius martensi* (pisces, Gobiidae) and factors affecting their structural properties. *Bioacoustics* 2:261–275. doi:10.1080/09524622.1990.9753141

Tougaard, C., Vukić, J., Ahnelt, H., Buj, I., Kovačić, M., Moro, G., Tutman, P., & Šanda, R. (2021). Quaternary climatic cycles promoted (re)colonization and diversification events in Adriatic sand gobies. *Journal of Zoological Systematics and Evolutionary Research*, 59(3), 777–788. <https://doi.org/10.1111/jzs.12468>

Vanhove, M.P.M., Economou, A.N., Zogaris, S., Larmuseau, M.H.D., Giakoumi, S., Kalogianni, E., Volckaert, F.A.M., & Huyse, T. (2012). Phylogenetics and biogeography of the Balkan sand gobies' (Teleostei: Gobiidae): Vulnerable species in need of taxonomic revision. *Biological Journal of the Linnean Society*, 105, 73–91. <https://doi.org/10.1111/j.1095-8312.2011.01781.x>

Vanhove, M., Kovačić, M., & Zogaris, S. (2016). A distinct island population of threatened freshwater fish: to split or lump? *Hydrobiologia*, 777(1), 79–93. <https://doi.org/10.1007/s10750-016-2765-z>

Vicente, J. R., Fonseca, P. J., & Amorim, M. C. P. (2015). Effects of temperature on sound production in the painted goby *Pomatoschistus pictus*. *Journal of Experimental Marine Biology and Ecology*, 473, 1–6.

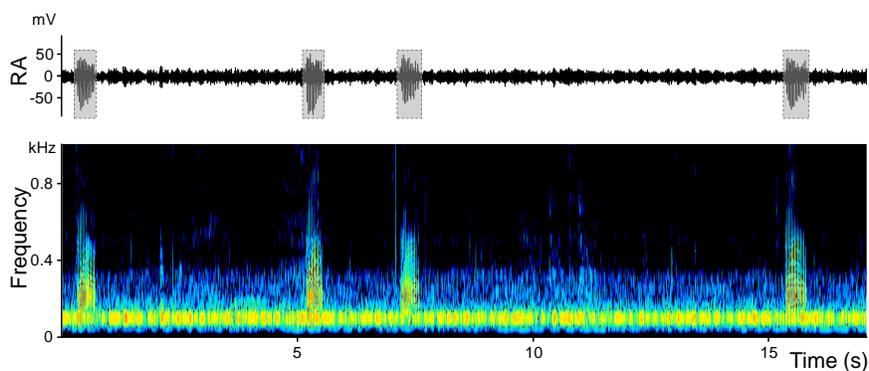
Winterbottom, R. (1974). A descriptive synonymy of the striated muscles of the Teleostei. *Proceedings of the Academy of Natural Sciences of Philadelphia*, 125, 225–317.

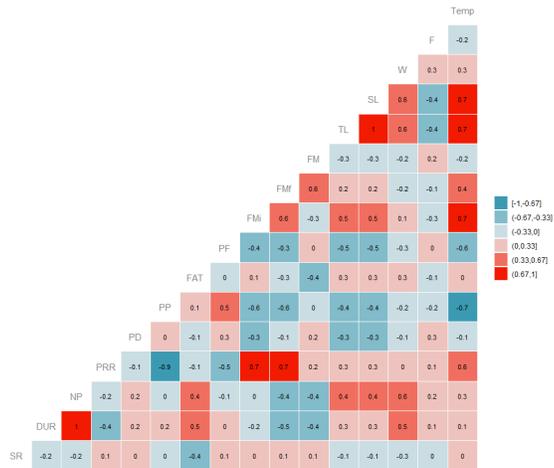
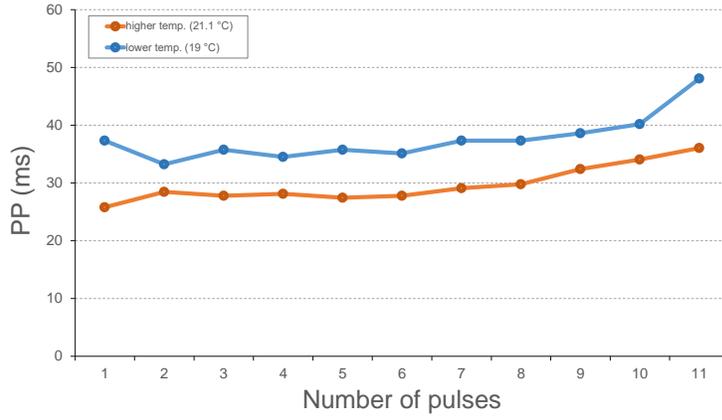
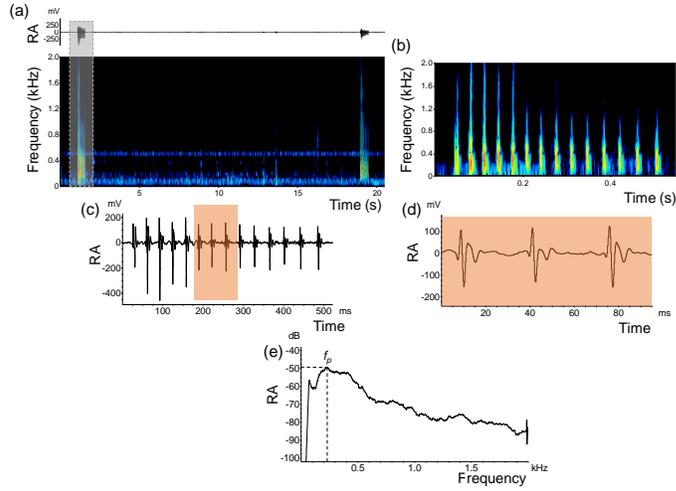
Zanella, D., Mrakovčić, M., Zanella, L. N., Miletić, M., Mustafić, P., Čaleta, M., & Marčić, Z. (2011). Reproductive biology of the freshwater goby *Knipowitschia croatica* Mrakovčić, Kerovec, Mišetić & Schneider 1996 (Actinopterygii, Gobiidae). *Journal of Applied Ichthyology*, 27, 1242–1248.

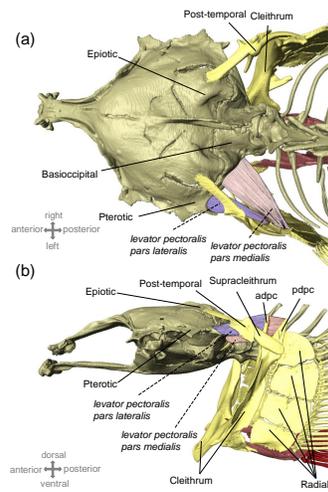
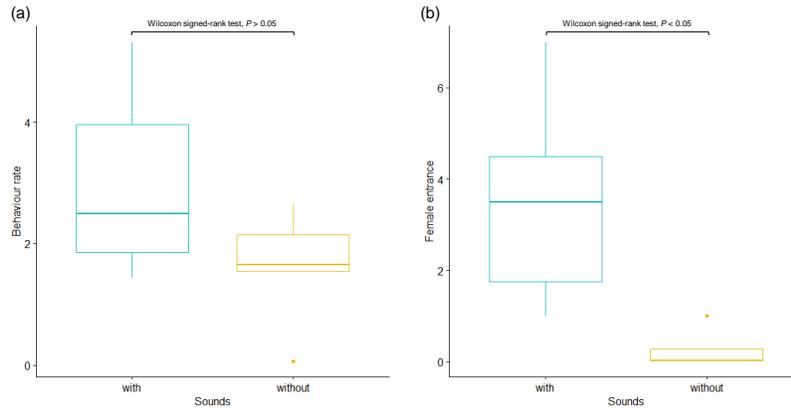
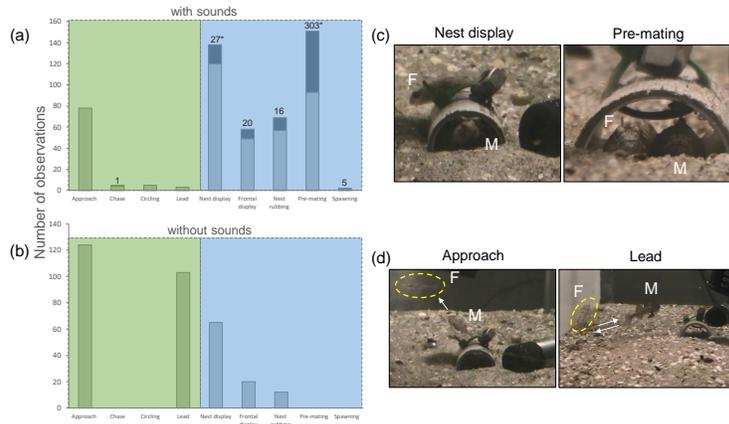
Zanella, D., Marčić, Z., Čaleta, M., Buj, I., Zrnčić, S., Horvatić, S. & Mustafić, P. (2017). Early development of the freshwater goby *Orsinogobius croaticus* endemic to Croatia and Bosnia-Herzegovina. *Cybium*, 41(4), 335–342.

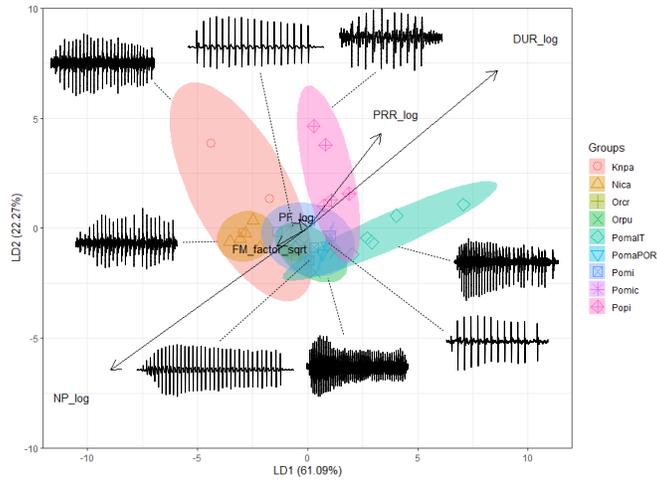
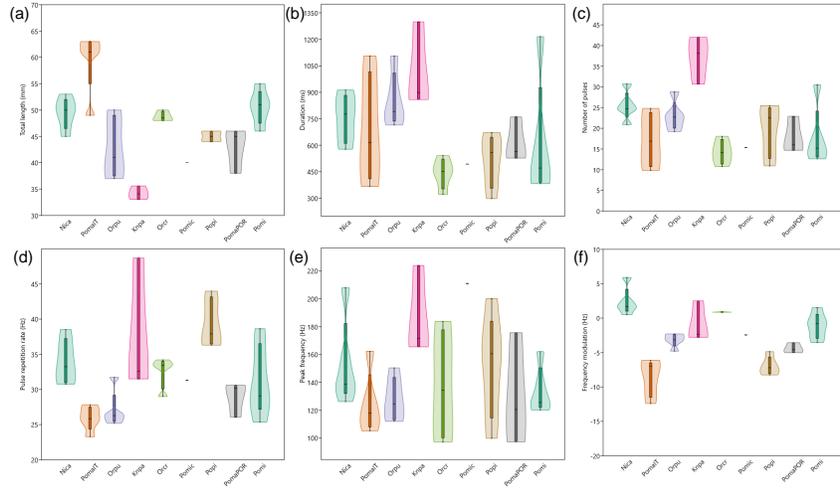
Zanette, I., Daghfous, G., Weitkamp, T., Gillet, B., Adriaens, D., & Langer, M. (2013). Looking inside marine organisms with magnetic resonance and X-ray imaging. In E. G. Reynaud (Ed.), *Imaging marine life*. <https://doi.org/10.1002/9783527675418.ch7>

Zeyl, J. N., Malavasi, S., Holt, D. E., Noel, P., Lugli, M., & Johnston, C. E. (2016). Convergent aspects of acoustic communication in darters, sculpins and gobies. In A. J. Sisneros (Ed.), *Fish Hearing and Bioacoustics: An Anthology in Honor of Arthur N. Popper and Richard R. Fay* (pp. 93–120). Springer.









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