# Vocal Behavior During Territorial Intrusions in the Lusitanian Toadfish: Boatwhistles Also Function as Territorial 'Keep-Out' Signals

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**Abstract**

Male signals are frequently studied in a single behavioral context, but in some cases they may assist multiple functions, namely for both male–male competition and female mate choice. Boatwhistles are known as the mate attraction calls of toadfishes typically produced during the breeding season. However, recent observations with the Lusitanian toadfish *Halobatrachus didactylus* (Batrachoididae) indicate that the emission of boatwhistles is not restricted to this period, which suggests a function in other behavioral contexts such as agonistic territorial interactions. We experimentally manipulated the social context of toadfish males to investigate whether boatwhistles are produced during territorial defense, by introducing 'intruders' in an experimental tank containing nesting 'resident' males. Furthermore, we examined whether parental care (eggs in the nest) affected the behavioral responses of resident males during territorial defense. Resident males defended their shelters producing sounds, mostly boatwhistles, towards intruders. Parental males revealed higher aggression levels, exhibiting additional threatening and attack behaviors. Boatwhistles registered during agonistic events were compared with the mate advertising boatwhistles recorded from small aggregations of nesting males in a natural breeding intertidal area. Agonistic boatwhistles were produced in lower and variable calling rates comparing with the advertising ones that were typically emitted in long series of calls. Agonistic boatwhistles were similar in duration and frequency harmonic structure (with a middle tonal phase) to the advertising calls, but presented less amplitude modulation, and lower dominant and fundamental frequencies. These acoustic differences were probably related to differences in calling rates and broadcast demands associated to the distance to the intended receiver. We provide first evidence that, apart from attracting mates, the toadfish boatwhistles also function as active 'keep-out' signals during territorial defense.

## Introduction

In many species, male signals used in agonistic contests differ from those used during mating interactions (Gerhardt 1982; Schmitt et al. 1994; Maruska et al. 2007). Some cases, however, point to the use of similar signaling traits across different behavioral contexts, such as status indicators used during male agonistic interactions and motivation and/or quality indicators used by females in mate choice. Examples

of male dual-function signals have been broadly described in various taxa such as mammals, birds, anurans and arthropods, and may include visual (Berglund et al. 1996; Pope 2000; Delaney et al. 2007) as well as acoustic signals (Bailey 1991; Stebbins & Cohen 1997; Beebee 2004).

Function duality of signaling traits has also been reported in fishes (Berglund et al. 1996), and seems to be common among territorial nest-guarding males (e.g. visual signals in blennies, Patzner et al. 1986). In fishes, acoustic signals are used in a variety of behavioral contexts, including reproduction and agonistic interactions during territorial defense (e.g. Sparkes et al. 2002; Tricas et al. 2006). Many species exhibit stereotyped vocalizations associated with these specific contexts (Amorim 2006) and there are only few examples where acoustic signals may serve multiple functions (Berglund et al. 1996).

Species from the Batrachoididae family (Teleostei, Actinopterygii), which includes toadfishes and the plainfin midshipman fish, are notable sound producers that typically emit two vocalizations highly divergent in their temporal properties – the mating boatwhistle (or hum in midshipman fish) and the agonistic grunt (Bass & McKibben 2003). Behavioral observations along with playback experiments support the hypothesis that these sounds are used to attract ripe females for spawning and during nest defense, respectively (Fish 1972; Ibara et al. 1983; Brantley & Bass 1994). Congruently, the Lusitanian toadfish *Halobatrachus didactylus* (Bloch and Schneider 1801) produces these distinct calls, boatwhistle and grunt train, most likely associated with mating activities and agonistic interactions (dos Santos et al. 2000; Amorim et al. 2006; Vasconcelos & Ladich 2008). Recent observations, however, indicate that boatwhistling is not restricted to the mating season in this species, since it has been detected all year round when water temperature remained higher than 19°C (Amorim et al. unpublished data). This suggests that, besides attracting gravid females to the male's nest, the boatwhistle may have other functions such as territorial defense.

The aim of this study was to test whether the boatwhistle is also produced during territorial defense in the Lusitanian toadfish. We carried out territorial intrusion experiments to simulate a male–male competition context. Because the existence of parental care typically increases levels of aggression by invaded territorial males (Östlund-Nilsson 2002), we also conducted intrusions in parental males' territories to investigate how the presence of eggs/embryos in the nest affects behavioral responses during territorial defense. Moreover, we recorded typical mate advertising boatwhistles from small aggregations of confined nesting males in a natural breeding area, in order to compare acoustic features of boatwhistles produced in the two behavioral contexts.

## Materials and Methods

### Study Species

The Lusitanian toadfish *H. didactylus* is a benthic fish which inhabits estuaries and coastal zones of the Eastern Atlantic and the Mediterranean, and is usually found partly buried in soft sediment or concealed in rock crevices (Roux 1986). During the reproductive season, that lasts from May to July in Portugal (Modesto & Canário 2003a), eggs are deposited in the roof of a nest where they attach by an adhesive disk and are guarded by a male until the offspring are free-swimming (dos Santos et al. 2000; personal observations). Like other batrachoidids, this species presents sexual polymorphism with a nest-guarding male ('type I') and a sneaking ('type II') male morphotypes that differ in size, gonadosomatic indices and development of the sonic muscles (Modesto & Canário 2003a,b). Only the type I males establish nests under rocks in shallow waters during the breeding season and are able to emit the advertisement tonal call (boatwhistle) to attract females at distance (Amorim & Vasconcelos 2008). The Lusitanian toadfish exhibits an unusual large acoustic repertoire composed of four commonly produced sounds: boatwhistle, grunt train, long grunt train and double croak, and other less frequent sound emissions such as croak and mixed croak–grunt calls (dos Santos et al. 2000; Amorim et al. 2008). These vocalizations are generated by vibration of the swimbladder caused by the contraction of embedded (intrinsic) sonic muscles (dos Santos et al. 2000).

### Test Subjects and Maintenance

Prior to the onset of the breeding season we placed 60 artificial concrete nests (internal dimensions: 50-cm long, 30-cm wide and 20-cm height) with a hemicylinder shape and closed at one end, approx. 1.5 m apart, in three rows, along an intertidal area of the Tagus River estuary (Military Air Force Base, Montijo, Portugal; 38°42′N; 8°58′W). Fish spontaneously occupied these shelters and we were able to access the animals at low tides during the whole period between May and July. We used 42 of these specimens (total length, TL = 38–52 cm; body

mass = 985–2452 g) for testing behavioral responses during territorial intrusions and 16 males for recording mate advertising sounds (TL = 35–48 cm; body mass = 963–1819 g). In the territorial intrusion experiments, the fish used within each trial were similar in size with residents and intruders differing in 0.25–3.0 cm TL, with the exception of one trial where the size differed in 7.8 cm TL. All animals used were type I males, which were easily identified on the basis of size (Modesto & Canário 2003a). Type II males and females caught in the study area were typically much smaller (generally TL < 30 cm, body mass < 500 g, personal observations) than the tested type I males and gravid females additionally differ by their larger abdomens. Moreover, the morphotypes' identity could be confirmed by gently pressing the males' abdomen since type I males have larger accessory glands (Modesto & Canário 2003a) and release a dark-brown seminal fluid (personal observations).

We maintained males to use in the territorial intrusion experiments in round stock tanks (plastic swimming-pools, 2-m diameter and water depth c. 0.5 m) near the intertidal toadfish nesting area where subjects were collected. The stock tanks were equipped with roof tiles as shelters (internal dimensions: 44-cm long, 18-cm wide and 8- to 10-cm-height). We placed the tanks on the sand just above the high tide shoreline in previously excavated depressions to protect the tanks from wind and to reduce temperature fluctuations. Water temperature was stabilized by keeping the tanks in the shadow provided by shelters made of a dark green net supported by wooden poles. These measures proved to be effective as water temperature varied between 19.5 and 21.5°C throughout the study. We renovated the water of the tanks every 2–3 d by pumping directly from the estuary. Fish were kept in the tanks for 2–5 d. A natural light cycle was maintained as the stock tanks were outdoors.

All specimens tested in this study were measured and weighed after the experiments. Some fish were labeled with marks in the fins (small cut between the fin rays) when used for different trials. The parental fish were released in the estuary along with their respective nest immediately after testing.

During trials in the experimental tank, confrontations between resident and intruder males included escalated behaviors such as biting. However, this occurred only in five out of the 15 trials and just for brief periods. The attacked fish typically swam away from the opponent, thus avoiding damaging combats. Fish always behaved normally after the experiments, suggesting that they were not exposed to abnormal stressful situations.

## Testing Behavior Interactions During 'Territorial Defense'

We carried out experiments with resident and intruder fish to simulate a context of male–male competition during territorial defense. Prior to testing, we placed two males in the experimental tank provided with two shelters for at least 12 h. The experimental tank was a 3-m diameter round tank similar to and fitted as the stock tanks (Fig. 1a). All specimens readily occupied the empty shelters and spent most of the time inside them. This allowed fish to become resident and to display territorial behavior. The nests (roof tiles or concrete shelters, see previous description) were placed approx. 50 cm apart and c. 20 cm away from the tank's border. We placed one hydrophone (High Tech 94 SSQ, Gulfport, MS, USA; frequency range: 30 Hz–6 kHz, ± 1 dB; voltage sensitivity: −165 dB re. 1 V/$\mu$Pa) in front of each nest at about 10 cm from its entrance (and from the tank bottom) attached to an wooden rod positioned over the tank. Simultaneous two channel recordings were made to a laptop connected to a USB audio capture device (Edirol UA-25, Roland, Japan; 16 bit,
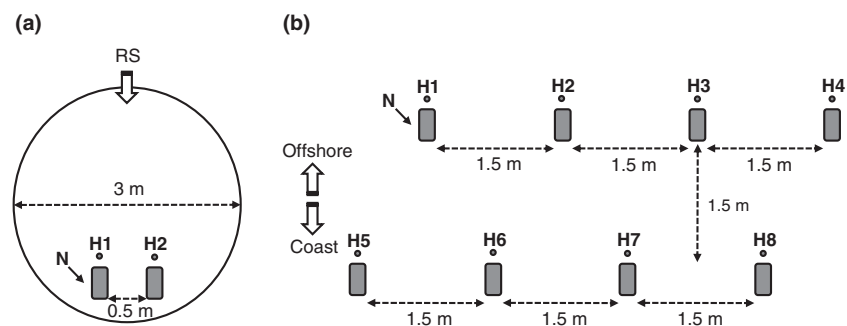


Fig. 1: Diagrams of the setups used for territorial intrusion experiments (a) and to record mate advertising vocal behavior (b). Grey rectangles depict nests (N) with resident fish inside; H, hydrophone position; RS, release site of fish intruders.

6 kHz acquisition rate per channel), controlled by Adobe Audition 2.0 (Adobe Systems Inc., San Jose, CA, USA).

In each trial, two male intruders were placed sequentially in the experimental tank with an interval of 30 min between intrusions and in the side opposite to the shelters. Behavioral interactions were registered for 60 min beginning with the introduction of the first male. The number of different agonistic behavioral patterns was tallied. These included threatening visual displays (mouth opening with the extension of pectoral fins and opercula) and attack (chase followed with bite or bite attempt). The vocal activity was also registered during the experiments by the sound recording system. We conducted a total of 15 trials (with two residents and two intruders each). In some trials the specimens used as residents (n = 6 fish) were the intruders in the previous experiment. Seven trials were performed with two non-parental resident males, that is, specimens that were captured without eggs/embryos. The other eight trials were carried out with parental males. In these cases, the shelters used during trials were the concrete nests that were deployed in the intertidal zone which contained their eggs/embryos. Once identified, the parental males and the respective nests were immediately placed in the experimental tank and tested following the aforementioned procedure.

### Recording Sound Production During 'Mate Advertisement'

We created an aggregation of shelters, readily occupied by type I males during the breeding season, similar to the natural aggregations where toadfish males emit advertising boatwhistles in choruses to attract mates (Amorim & Vasconcelos 2008). We used a group of eight concrete nests placed in the intertidal study area of the Tagus estuary to confine males that spontaneously occupied these shelters and record their vocal activity (see experimental setup in Fig. 1b). Nest's entrances were closed with a plastic net to prevent fish from escaping and to ensure male identity throughout the recordings. Plastic nets did not affect acoustic signals and allowed possible visual interactions with free-swimming conspecifics, as well as the entrance of prey items in the nest. All unoccupied nests within 15 m from a subject male were also wrapped in plastic nets to prevent further occupations during the study. We recorded two groups of eight males for an average of 36 h (range: 11–56 h) per fish, over a period of 8 d, during the peak of the breeding season

(June–July). One hydrophone (High Tech 94 SSQ) was placed at about 10 cm from the entrance of each experimental nest (and from the bottom), firmly attached to an iron rod partially buried in the sand substrate. The recording chain also included audio capture devices Edirol UA-25 connected to a laptop to perform simultaneous multi-channel recordings, which were controlled with Adobe Audition 2.0. Estuary water temperature during the recording period ranged between 19.5 and 24°C and the water level varied approx. from 0.5 to 2.8 m.

The recordings were always performed at the same distance (10 cm) to the nest entrance (and to the bottom) in the 'territorial defense' and 'mate advertisement' setups. In both situations, the short recording distance allowed us to minimize the spreading loss that typically increases with the distance to the sound source, especially in lower water levels (Mann 2006). Previous observations of sound recordings obtained in the same intertidal study area using the same setup (Amorim et al. unpublished data) confirmed that spreading loss was minimum and that sound recordings were unaffected by water level variations, suggesting that recording conditions are comparable in both setups used in this study.

### Sound Analysis

All sound recordings were analyzed and the different types of vocalizations identified based on dos Santos et al. (2000) and Amorim et al. (2008). Acoustic analysis was performed using Raven 1.2 for Windows (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, NY, USA).

During territorial intrusion trials, 22 specimens produced sounds (TL = 40.5–52.0 cm; body mass = 985–2452 g) which were classified as boatwhistles, grunts and 'other sounds' that occurred less frequently. All 16 fish used in the intertidal nest aggregation showed vocal activity that included several vocalizations, but we only considered the mate advertising boatwhistles for analysis. These advertising sounds were identified based on a higher and/or more constant emission rate during long periods of time. Only boatwhistles emitted in series with more than 15 sounds and/or produced constantly during 1 h (namely c. 4–10 calls per min on average), have been selected and classified as reproductive boatwhistles. These criteria were based on previous observations of the Lusitanian toadfish breeding chorus behavior. Moreover, in other batrachoidids, Winn (1972) reported a calling rate of three boatwhistles per minute exhibited by motivated courting

males and McKibben & Bass (1998) described that higher calling rates may induce female phonotaxis. The vocal behavior with relatively constant and high emission rate has never been observed during territorial intrusions (see Section 'Results'), which allowed us to discriminate advertising from eventual agonistic boatwhistles also recorded in the intertidal nest aggregations.

Sounds recorded in the field could be attributed to particular nest holders due to the close proximity of the hydrophones to the subject males and because of the high sound attenuation along short distances with low water depth (Mann 2006), exceeding c. 27 dB between occupied nests. In the experimental tank, we could also assign sounds to specific resident males due to the high attenuation (more than 6 dB) registered between the two nests. During territorial intrusions, only resident males produced sounds typically inside or at the entrance of their nests. The sounds registered during the brief periods when both resident and intruder males were inside the nest, namely 'agonistic boatwhistles' (see below), showed acoustic features identical to the ones produced when the fish were apart and therefore could be attributed to the resident male. According to Amorim & Vasconcelos (2008), the boatwhistles of the Lusitanian toadfish present individual-specific acoustic features which allow the identification of different fish. Moreover, later experiments to analyze sonic muscles' contraction activity using electrodes positioned directly in the swimbladder muscles confirmed that the sound producers during territorial intrusions are typically the resident fish (Jordão et al. unpublished data).

In both simulated social contexts, territorial defense and advertisement, we verified the production of boatwhistles hereafter referred to as agonistic boatwhistles (AB) and reproductive boatwhistles (RB), respectively. To compare these sounds, we analyzed 8–10 ABs per male from 12 males (7 parental and 5 non-parental) and another 8–10 RBs per male from 13 fish. Sounds presenting a high signal-to-noise ratio were selected randomly (but within the criteria described above for the RBs). For the acoustic analysis, we adopted the classification used by Amorim & Vasconcelos (2008) that considers three distinct phases in the boatwhistle [beginning (P1), middle (P2 or tonal phase) and end (P3)], based on differences in pulse period and dominant frequency. The acoustic parameters measured were total duration (ms), from the start of the first pulse to the end of the last pulse; amplitude modulation, by dividing the mean (RMS) amplitude measured in

P1 by the one measured in P2; dominant frequency, as the highest energy component within the sound power spectrum of the P2 (sampling frequency 8 kHz, Hamming window, filter bandwidth 10 Hz); fundamental frequency, calculated as the inverse of the mean pulse period (average time period between six consecutive pulses) measured in the P2 (since in the batrachoidids the fundamental frequency is determined by the sonic muscle contraction rate, Skoglund 1961; Fine et al. 2001).

## Statistical Analysis

Means of the acoustic parameters measured in the boatwhistles were calculated for each specimen and used for statistical analyses. Mann–Whitney $U$ tests were used to compare RBs with ABs for all acoustic parameters. $U$ tests were also considered while comparing the number of visual displays and total sounds produced per trial during territorial intrusions between the two test groups – parental and non-parental resident males, as well as to compare ABs produced by both groups. Non-parametric tests were used since data were not normally distributed and variances were not homogeneous. The statistical tests were performed with Statistica 8.0 for Windows (StatSoft, Inc., Tulsa, OK, USA).

## Results

### Agonistic Behavior During Territorial Defense

All intruding males swam towards the shelters and tried to enter them. The approach time varied from 1 s up to 23 min (n = 15 trials). In most cases (75%), however, the intruder approached the shelter in less than 3 min.

The resident males always responded towards the intruders by producing sounds (93% of the trials) and/or exhibiting visual displays (53%) (see Table 1). Vocal activity, only detected in residents, varied between 1 and 47 sounds per specimen and mainly included boatwhistles (92% of the calls), but also grunt trains (7%) and other less frequent vocalizations (1%). A total of 301 sounds were recorded from all males. Some of the boatwhistles (10 from a total of 278 sounds, detected in four out of the 15 trials) were followed by a grunt train resulting in a mixed call. The AB calling rates were low and irregular varying between 1 and 44 sounds/h per fish (total mean calling rate = 9.3 AB/h, n = 22).

The association between sounds and other specific behavioral pattern was not evident, as residents

**Table 1:** Descriptive statistics (mean ± SD and range) of the number of occurrences of threatening visual displays, attacks (chase and bite) and acoustic signals exhibited by parental and non-parental resident males in a total of 15 trials of territorial intrusions

| Eggs in the nest | N | No of aggressive behaviors | | No of acoustic signals | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | Visual displays | Attack | AB | Grunt | Other |
| Yes | 8 | 0.50 ± 0.76 (0–2) | 1.75 ± 3.06 (0–9) | 21.88 ± 23.49 (0–58) | 1.25 ± 1.91 (0–5) | 0.25 ± 0.46 (0–1) |
| No | 7 | 0 | 0 | 14.71 ± 17.01 (1–48) | 1.57 ± 3.74 (0–10) | 0 |

N, number of trials performed for each test group (eggs vs. no eggs in the nest). AB: agonistic boatwhistle.

vocalized mostly inside the shelters. However, some males often came out of the shelter showing threatening visual displays and attacks to deter and keep away the opponents and, in those cases, the agonistic interactions were usually not accompanied by vocalizations. Aggressive behavioral patterns included mostly attacks (78%) but also threatening displays such as mouth opening with extension of pectoral fins and opercula (22%). The attacks performed by residents consisted in chasing the intruders with consecutive bite attempts. Previous observations of this agonistic behavior associated with territorial defense also showed one episode of jaw locking between resident and intruder fish. The two types of aggressive behaviors observed, attacks and threatening displays, were never detected in the same experimental trial. Several resident males attacked the intruders once or twice during the entire experiment (just one specimen showed that behavior more frequently – nine times). The intruding males approached and swam away from the shelters after being rebuffed repeatedly during the whole trial. In each approach the intruders kept their body perpendicular to the shelter's entrance or kept their head towards its entrance without making any particular visual display or acoustic signal. Both intruders from the same trial showed similar behaviors and generally tried consecutive attempts to occupy the two nests. In some cases, the intruder was able to occupy the nest (33% of trials) and repel the resident.

### Comparison Between Agonistic and Advertising Boatwhistles

The boatwhistles produced by territorial males during confrontation with intruders (AB) were similar in duration ($U$ test: $U = 53$, $n_{AB} = 12$, $n_{RB} = 13$, $p > 0.05$) and presented a harmonic structure with a middle tonal phase identical to those emitted by males to attract females to their nests (RB) (Figs 2 and 3a). These sounds differed considerably from the other vocalizations of the species repertoire.

However, we found significant differences between ABs and RBs, recorded at the same distance from the nest entrance (see Section 'Methods'), in several other acoustical parameters, namely ABs revealed less amplitude modulation, that is, amp P1/amp P2 ~1 ($U$ test: $U = 6$, $n_{AB} = 12$, $n_{RB} = 13$, $p < 0.001$, Fig. 3b). The dominant (DF) and fundamental (FF) frequencies were significantly lower in the AB ($U$ test: DF, $U = 11$, $n_{AB} = 12$, $n_{RB} = 13$, $p < 0.001$; FF, $U = 31$, $n_{AB} = 12$, $n_{RB} = 13$, $p = 0.010$, Fig. 3c, d). In the ABs, the spectral energy was almost evenly distributed within the first three harmonics but coincided predominantly with the fundamental frequency; whereas, in the RB the energy peaked mostly at the second harmonic (Fig. 2). In six of 12 fish (50%), the dominant frequency of ABs varied within the same individual between the first and the second harmonics. On the contrary, the dominant frequency of RBs was detected in the first, second or fourth harmonics in seven of 13 fish (54%). Most of ABs were emitted singly, but infrequently we observed ABs produced in series of up to 15 boatwhistles. A series of 15 ABs was detected only once.

### Effects of Parental Care in Territorial Aggression

As expected, parental males with eggs and/or embryos in the nest were more aggressive during territorial invasions by conspecific intruders than the non-parental ones. This was clearly indicated by the visual threatening displays and attacks that were exclusively exhibited by parental males ($U$ test: $U = 63$, $n_{non-parental} = 14$, $n_{parental} = 16$, $p = 0.006$) – see Table 1. Although parental males emitted a larger number of sounds, namely ABs, in comparison with the other males, no significant differences were found between the two test groups in terms of total number of vocalizations and of ABs ($U$ test: $U = 109–111$, $n_{non-parental} = 14$, $n_{parental} = 16$, $p > 0.05$). Likewise, there were no statistical differences between ABs emitted by parental and non-parental males in any acoustic parameter ($U$ test: $U = 14–17$,
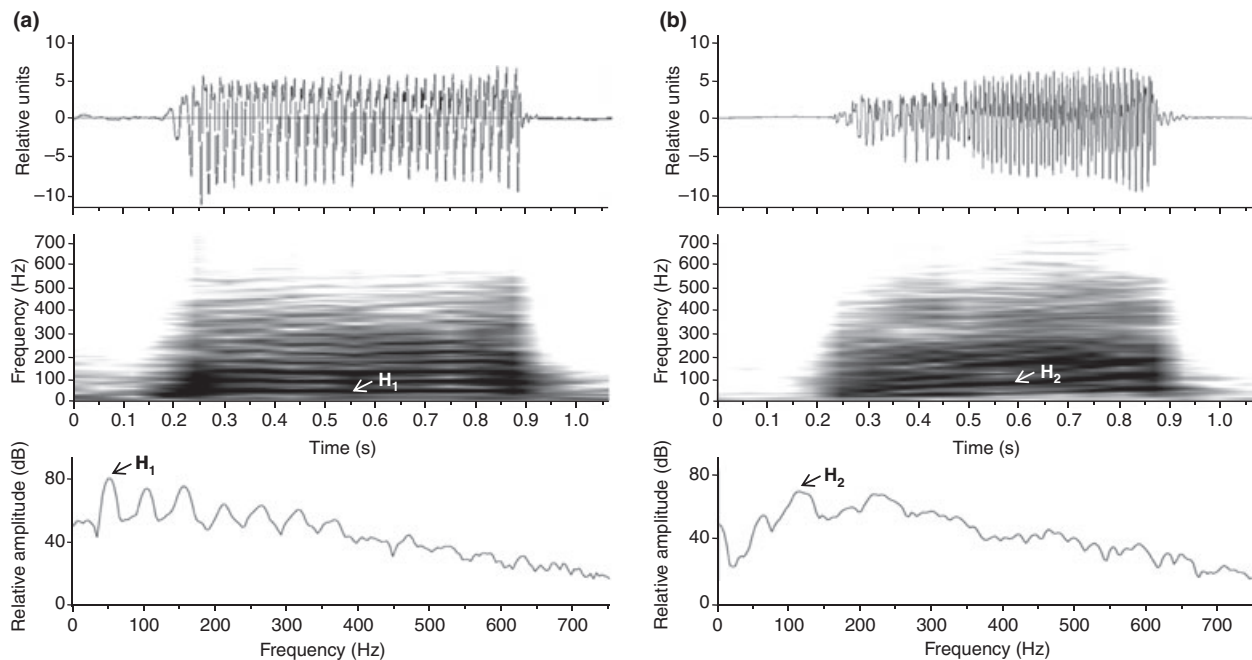
**Fig. 2:** Oscillogram, sonogram and power spectrum (of the middle tonal phase) of typical agonistic (a) and mating (b) boatwhistles emitted by the Lusitanian toadfish. The spectral energy of the agonistic boatwhistle was almost evenly distributed within the first three harmonics but with a higher peak at the fundamental frequency ($H_1$), as indicated; whereas the dominant frequency of the mating boatwhistle corresponded to the second harmonic ($H_2$). Sampling frequency 8 kHz, filter bandwidth 10 Hz (sonogram and power spectrum), 50% overlap, Hamming window.

$n_{non-parental} = 5$, $n_{parental} = 7$, $p > 0.05$). Some of the ABs were followed by grunt trains and these were mostly produced by parental males (in three of the four trials).

## Discussion

### Toadfish Boatwhistle as a Territorial 'Keep-Out' Signal

Traditionally, secondary sexual traits are thought to have evolved through sexual selection into either armaments or ornaments arising from male–male competition and female choice, respectively (Darwin 1871). More recently, however, it has become evident that many ornamental traits are also often used in aggressive displays and vice versa (reviewed in Berglund et al. 1996). Many examples of function duality of acoustic signals in which males call both to interact with males and attract females can be found among insects, anurans (Bailey 1991; Gerhardt 1994), but mostly in songbirds that provide classic examples of such dual-function traits (Searcy & Andersson 1986). For example, Beebee (2004), using song playback experiments, reported that male yellow warblers *Dendroica petechia* use two singing

modes to interact with both males and females, which do not have distinct sex-specific functions as previously thought.

In fishes there are only few examples of acoustic signals that serve multiple functions (Berglund et al. 1996). For instance, the sand goby *Pomatoschistus pictus* produces drums in both courtship and territorial defence contexts (Amorim & Neves 2007, 2008). Courtship drums, however, differ from agonistic drums as they are longer, present a larger number of pulses, shorter pulse periods and have higher dominant frequencies than the latter (Amorim & Neves 2008). In the present study, we address the hypothesis that the boatwhistle of toadfishes typically used for mate attraction may also be used in a male–male competition context. If this is the case then we would expect to observe boatwhistling during agonistic events, such as territorial defense.

Our study is the first to experimentally demonstrate that the toadfish boatwhistle can also function in active territorial defense as a 'keep-out' signal. In fact, the boatwhistle has never been explicitly associated with agonistic behavior in this group. During the territorial intrusion experiments, nest-holding males defended their territories in face of intruders, using recurrently acoustic signals during the exhibition
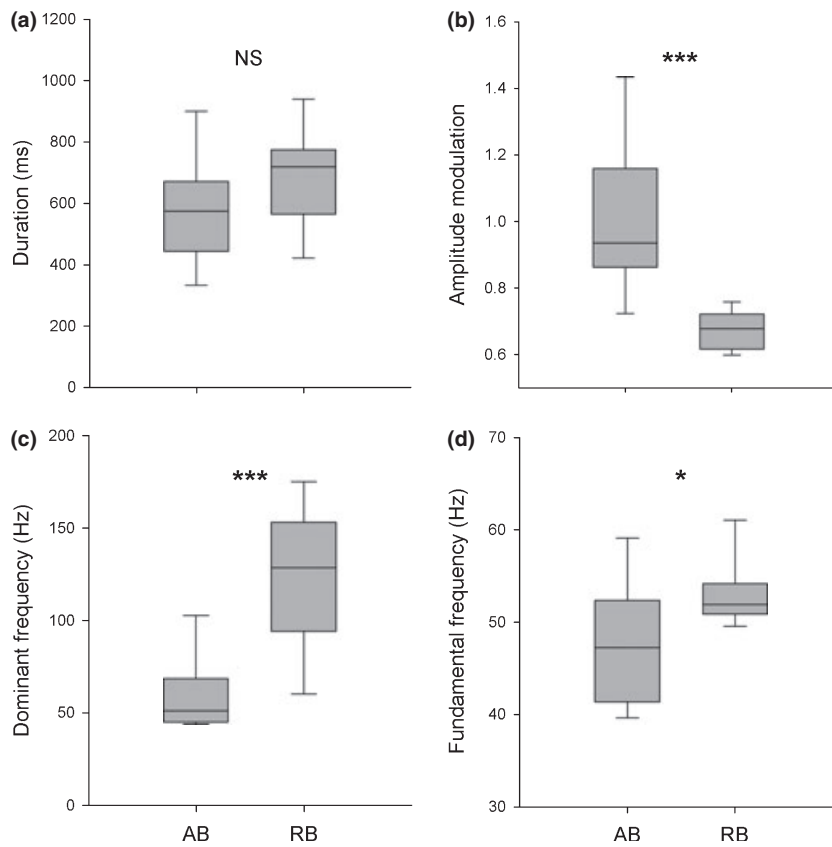
**Fig. 3:** Median of total sound duration (a), amplitude modulation (b), dominant frequency (c), and fundamental frequency (d) of agonistic (AB) and reproductive (RB) boatwhistles. Plots show 10th, 25th, 75th and 90th percentiles as boxes and whiskers. *p < 0.05, ***p < 0.001, NS: non significant, Mann–Whitney $U$ tests.

phase of contests. The boatwhistle was the most frequent vocalization produced by resident males (see Table 1) making up 92% of the registered calls, in contrast with the 78% observed in an advertisement context (see Amorim et al. 2008). Intruder males often responded to resident male sounds by swimming away from the occupied shelters. Therefore, this 'keep-out' signal seems to reduce confrontation between conspecifics and related costs associated with escalated behaviors in a male–male competition context. Such a functional role of a vocal signal has been reported in different taxa (Krebs et al. 1978; Myrberg & Riggio 1985). Previous studies with Batrachoididae only associated the boatwhistle produced by nesting type I males to the attraction of ripe females to their nest sites during the spawning season (Brantley & Bass 1994; McKibben & Bass 1998). Winn (1967) and Fish (1972) suggested that the boatwhistle may also have a role in spacing nesting males by advertising territorial ownership. However, this hypothesis was never confirmed with behavioral experiments and does not consider short-distance agonistic interactions.

In general, the boatwhistles produced during territorial intrusions and the field-recorded advertising calls were almost indistinguishable to the human ear, despite some examples which showed clear differences in amplitude modulation and frequency content. These calls revealed identical temporal structure with a middle tonal phase and similar sound duration. All boatwhistles recorded differed considerably from the other pulsed vocalizations of the species repertoire (grunt trains, croaks, double croaks, mixed grunt–croak call, long grunt train; see detailed description in dos Santos et al. 2000; Amorim et al. 2008) in terms of temporal patterning (sound duration, pulse period), amplitude and frequency content. However, we have observed that the social context affects some acoustic parameters of boatwhistles. Agonistic boatwhistles presented less amplitude modulation and generally had lower dominant and fundamental frequencies. This last parameter indicates that the agonistic calls are produced at slower muscle contraction rate (Fine et al. 2001). In particular, the spectral energy of agonistic boatwhistles was almost evenly distributed within the first three harmonics but the dominant frequency usually corresponded to the fundamental frequency, whereas in reproductive boatwhistles most of the sound energy generally appeared in the second harmonic (see Figs 2 and 3).

These differences in signal acoustic features probably relate with differences in production rate and function of the agonistic and mating boatwhistles. Toadfish advertising sounds are typically produced at relatively constant (McKibben & Bass 1998; Fine & Thorson 2008) and higher rates for longer periods of time up to several hours (personal observations in *H. didactylus*). Moreover, during confrontations fish are relatively close but for mate attraction sound waves should propagate at larger distances to attract females to the nesting areas (Amorim & Vasconcelos 2008). Higher frequencies are more easily propagated in shallow waters (Mann 2006) and more frequent acoustic signals are more easily detected and tracked from longer distances than single sounds. Differences between mating and agonistic boatwhistles are also probably associated with different costs. The costs of producing boatwhistles in an advertisement context for mate attraction are probably higher, as the signals are produced at higher rates for longer periods of time, are more complex (amplitude modulation), and are generated by higher sonic muscle contraction rates (Fine et al. 2001). Besides the likely higher physiological and metabolic costs (Mitchell et al. 2008; but see Amorim et al. 2002), the production of the conspicuous mate advertisement boatwhistles also may impose ecological costs, such as the time spent calling and not in other activities and the attraction of predators (Ryan 1988; Gannon et al. 2005). It is possible that higher calling rates, higher dominant frequencies and higher amplitude modulation are used as honest signals of male quality for mate choice by females, although this hypothesis still needs to be investigated. Therefore, we suggest that the toadfish boatwhistle functions primarily as a courtship signal although it may also serve as a less costly 'keep-out' signal during male–male competition.

### Agonistic Behavior in a Territorial Defense Context

Besides boatwhistles, resident males also uttered other agonistic vocalizations during intrusions, namely grunt trains. The occurrence of this vocalization in the male–male competition context of our intrusion experiments supported a biological role in agonistic situations as previously suggested (dos Santos et al. 2000; Amorim et al. 2006). However, the emission rate was considerably lower compared with the boatwhistles, which indicates that the grunt trains of the Lusitanian toadfish are probably more used during highly distress events such as when fish are being handheld (Vasconcelos & Ladich 2008) like

in other batrachoidids (e.g. *Opsanus tau*, Cohen & Winn 1967).

During territorial intrusions, egg-guarding resident males defended vigorously their nests also using threatening displays and attacking. These included mouth opening, erecting fins and opercula covers, chases and bites. Threatening exhibitions where territorial individuals increase their body size appearance at the shelters entrance have been reported for several fish species (e.g. Ladich 1989; Almada et al. 1996) and maybe used in assessing the fighting ability of opponents which is important to decide contests before they escalate to damaging combats. Parental toadfish males behaved more aggressively than the non-parental ones that never exhibited threatening displays or attacks towards intruders. Although parental males also emitted more sounds during agonistic interactions, no statistical significant differences were found in terms of sound production (number of total vocalizations and boatwhistles produced) in comparison with the other males. However, we noted that complex sounds composed by boatwhistles and other sound elements (grunt trains) were mostly produced by parental males, and may be linked to a higher level of aggressiveness. The increased aggression level shown by parental toadfish males towards threatening intruders, also observed in *O. tau* (Gray & Winn 1961), might be explained by the amount of energy and time already invested taking care of the offspring (Östlund-Nilsson 2002), as the eggs in the nests were mainly in advanced stages of development. The increment of aggressiveness with parental care has been broadly reported in several taxa (e.g. fish, Oliveira & Almada 1998; reptiles, Sinn et al. 2008; anuran, Townsend et al. 1984). Differences in aggression level in territorial defense between non-parental and egg-guarding fish may be facilitated by increased androgen levels in some species (e.g. goby *Lythrypnus dalli*, Rodgers et al. 2006). Although, in the batrachoidid *Porichthys notatus* a decrement in androgens was observed across the parental cycle and may play an important role inducing parental behavior (Knapp et al. 1999). Nevertheless, the influence of size and development of the clutches on parental aggression as well as hormonal changes across the parental cycle were not studied and have yet to be examined in *H. didactylus*.

In summary, we present first evidence that toadfish boatwhistles play an active role in territorial defence. Advertising and agonistic boatwhistles differ in acoustic features which are probably associated with sound production rate and broadcast demands related to the distance to the intended receiver (far

away females and close by nest intruder males). The higher calling rate kept for longer periods of time (long series) and more complex features of boatwhistles produced in a mate advertisement context suggest that this acoustic signal is mainly used as an ornament but also functions in the early phases of male–male contests by signaling territorial ownership. Whether agonistic boatwhistles give information of asymmetries between the opponents, such as size or previous fighting experience, still remains to be tested.

## Acknowledgments

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