

RESEARCH ARTICLE

How effective are acoustic signals in territorial defence in the Lusitanian toadfish?

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ABSTRACT

The function of fish sounds in territorial defence, in particular its influence on the intruder's behaviour during territorial invasions, is poorly known. Breeding Lusitanian toadfish males (*Halobatrachus didactylus*) use sounds (boatwhistles) to defend nests from intruders. Results from a previous study suggest that boatwhistles function as a 'keep-out signal' during territorial defence. To test this hypothesis we performed territorial intrusion experiments with muted Lusitanian toadfish. Males were muted by making a cut and deflating the swimbladder (the sound-producing apparatus) under anaesthesia. Toadfish nest-holder males reacted to intruders mainly by emitting sounds (sham-operated and control groups) and less frequently with escalated bouts of fighting. When the nest-holder produced a boatwhistle, the intruder fled more frequently than expected by chance alone. Muted males experienced a higher number of intrusions than the other groups, probably because of their inability to vocalise. Together, our results show that fish acoustic signals are effective deterrents in nest/territorial intrusions, similar to bird song.

KEY WORDS: Batrachoididae, *Halobatrachus didactylus*, 'Keep-out' signal, Muting experiments, Sound production, Teleost fish, Territorial behaviour

INTRODUCTION

An individual fish's probability of surviving and reproducing depends to a large extent on its social behaviour in which communication takes a major role. In contests for the establishment of social hierarchies and territories, differences in fighting ability between contestants influence the outcome of disputes (Parker, 1974; Arnott and Elwood, 2009). Fighting ability or resource-holding potential (Parker, 1974) is often related to size, but also to other factors such as development of weaponry, physiological state, sex and residency status (Turner and Huntingford, 1986; Enquist and Leimar, 1987; Arnott and Elwood, 2009). Hence, when a contest occurs, opponents typically start a ritualised sequence of displays that facilitate opponent assessment and when asymmetries between contestants are large the contest should be settled without the need for costly combats (Enquist and Leimar, 1983, 1987).

Empirical evidence shows that acoustic signals are often used in mutual assessment during agonistic interactions in mammals (Clutton-Brock and Albon, 1979), birds (Krebs, 1976; Krebs

et al., 1978; Searcy and Beecher, 2009), anurans (Davies and Halliday, 1978; Cocroft and Ryan, 1995) and fishes (Ladich and Myrberg, 2006), because acoustic features may signal the sender's quality. For example, lower-frequency calls usually reflect larger body size and hence better competitive ability because larger vocal organs and vocal tracts produce and radiate lower frequencies more efficiently (Bradbury and Vehrencamp, 1998). Also, other features such as calling rate or sound amplitude may be condition dependent (Clutton-Brock and Albon, 1979; Prestwich, 1994; Wyman et al., 2008; Amorim et al., 2010a).

In fish, different studies have shown that several properties of acoustic signals are related to body size. Larger fish tend to produce lower-frequency (e.g. Ladich, 1998; Myrberg et al., 1993; Lobel and Mann, 1995; Connaughton et al., 2000), louder (Ladich, 1998; Connaughton et al., 2000; Lindström and Lugli, 2000; Amorim et al., 2013) and longer sounds (Wysocki and Ladich, 2001; Amorim and Hawkins, 2005; Amorim and Neves, 2008) than smaller individuals. Also, the level of calling activity may reflect the amount of fat reserves (Amorim et al., 2010a, 2013; Pedroso et al., 2013).

Less known is how acoustic communication affects agonistic interactions in fish, but in at least a few species sounds seem to be used in mutual assessment and influence fight outcome (reviewed in Ladich and Myrberg, 2006; Raffinger and Ladich, 2009). However, studies on the function of sounds in territorial defence are scarce, in particular in its influence on the intruder's behaviour during territorial invasions by conspecifics. For example, playing back click sounds to skunk loaches *Yasuhikotakia morleti* during territorial intrusions made residents increase the number of lateral displays performed at intruders (Valinski and Rigley, 1981) whereas the playback of ratchet sounds to brown bullhead catfish *Ameiurus nebulosus* decreased the number of attacks residents made at intruders (Rigley and Muir, 1979). These experiments clearly show that sounds can have a major role in modulating the resident's territorial behaviour. However, the deterrent function of sounds on territorial intrusion has seldom been demonstrated. Playbacks of conspecific sounds in the absence of a resident male have been shown to have a deterrent effect in territorial intrusion in the bicolor damselfish *Stegastes partitus* (Myrberg, 1997) and in the painted goby *Pomatoschistus pictus* (Pereira et al., 2014), equivalent to the 'keep-out' effect of bird song (Krebs, 1976).

To experimentally test the 'keep-out signal' hypothesis, we used the vocal Lusitanian toadfish *Halobatrachus didactylus* Bloch and Schneider 1801. In the reproductive season (May to July in Portugal) males occupy rock crevices or excavate under rocks in shallow water and attract females with long tonal sounds (~800 ms) named boatwhistles (dos Santos et al., 2000; Modesto and Canário, 2003; Amorim et al., 2006). Females deposit their eggs under the roof of the nest and males guard the eggs of multiple females until the offspring is able to swim away (Ramos et al., 2012; Roux, 1986). During this period competition for nests is high (Amorim et al., 2010b) and males actively defend the nest from intruders with visual

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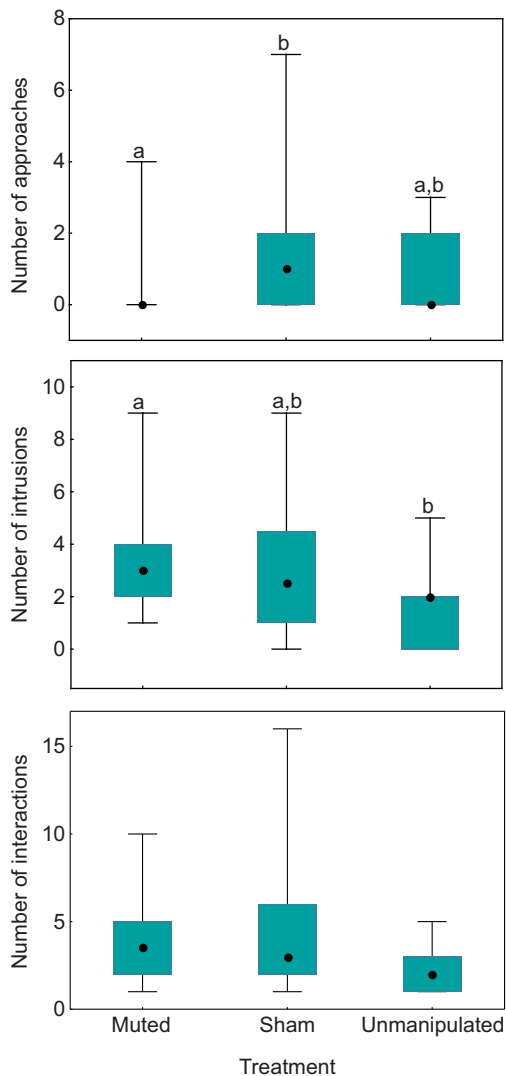


Fig. 1. Number of approaches, intrusions and interactions experienced by resident male toadfish. Dots indicate medians whereas boxes and error bars depict quartiles and range. Different letters indicate pairwise differences given by *post hoc* Kruskal–Wallis tests. In the case of approaches, differences are marginally non-significant ($P=0.06$) and for intrusions differences are significant at the level of $P<0.01$.

and acoustic behaviour (Vasconcelos et al., 2010; Ramos et al., 2012). Recently, Vasconcelos and colleagues (Vasconcelos et al., 2010) have proposed that the boatwhistle functions as a ‘keep-out’ signal and suggested that vocalising may be an effective means to avoid territorial intrusions and escalated levels of fighting in the Lusitanian toadfish. However, the study of Vasconcelos et al. (2010) cannot exclude the possibility that chemical or other cues could also be at play. In the Lusitanian toadfish, vocalisations are generated by vibration of the swimbladder caused by the contraction of intrinsic sonic muscles (dos Santos et al., 2000); muting can therefore be easily achieved by making a cut and deflating the swimbladder under anaesthesia. Males can still contract the sonic muscles but sounds become inaudible while fish behaviour appears unaltered. Here, we used muting experiments to verify whether acoustic signals (i.e. boatwhistles) are effective deterrents of territorial intrusions in this species. We compared the dynamics of territorial defence and the number of intrusions among muted and control males (sham-operated and unmanipulated residents). We

further tested whether intruders fled more frequently than expected by chance alone when the nest-holder made a boatwhistle.

RESULTS

Interaction dynamics

Intruding males readily swam towards the shelters and often approached and tried to enter them. 44% of the resident males ($N=57$) experienced approaches (range: 0–7 approaches) and 84% experienced partial or total intrusions (range: 0–9). Muted fish experienced fewer approaches (Kruskal–Wallis test: $N=57$, $H=6.78$, $P<0.05$) but a greater number of intrusions ($H=9.65$, $P<0.01$) than other groups (Fig. 1). However, the total number of interactions (approach+intrusion) did not differ among groups ($H=4.99$, $P>0.05$; Fig. 1).

The resident males responded to an intruder’s approach either by producing sounds (mainly boatwhistles) or exhibiting escalated levels of fighting (mostly bites and mouth wrestling). During intrusions, the nest-holder response was similar but the proportion of escalated fights was higher and the number of vocalisations lower than during approaches (Table 1). Also, in contrast to approaches, the production of boatwhistles could proceed to a fight if the intrusion persisted. On many occasions there was no apparent reaction from the resident (‘no reaction’).

We found an effect of treatment on the number of ‘no reactions’ (Kruskal–Wallis test: approach, $N=25$, $H=7.04$, $P<0.05$; intrusion, $N=8$, $H=10.56$, $P<0.01$) but not on escalated levels of fighting (approach, $H=2.36$, $P>0.05$; intrusion, $H=1.76$, $P>0.05$) during approaches and intrusions. Muted fish showed the highest occurrences of ‘no reaction’ (Figs 2 and 3).

The duration of interactions (one-way ANOVA, $F_{2,144}=1.22$, $P>0.05$) and of interaction sequences ($F_{2,88}=0.91$, $P>0.05$) did not differ among groups (Fig. 4). The production of boatwhistles (BW) did not affect interaction duration in any interaction type: approach, intrusion or approach followed by intrusion (two-way ANOVA, BW: $F_{1,138}=0.12$, $P>0.05$; interaction type: $F_{2,138}=19.53$, $P<0.001$; BW×interaction type $F_{2,138}=0.05$, $P>0.05$).

There were marginally non-significant differences in takeovers of muted and vocal fish nests ($\chi^2=3.25$, d.f.=1, $P=0.07$). Overall, nest takeovers occurred infrequently. From the 48 residents that experienced intrusions, 14 got replaced. A total of 23% (3 in 13), 18% (3 in 17) and 44% (8 in 18) of unmanipulated, sham-operated and muted males got replaced by intruders, respectively. We found no differences in time until nest takeover (i.e. sequence of interaction duration until nest takeover) among treatments ($F_{2,12}=0.42$, $P>0.05$; Fig. 4). In nest takeovers, intruders and residents were of similar sizes, the difference in total lengths averaging 0.9%.

Intruder response to resident’s behaviour

Intruders usually fled when they heard a boatwhistle either while approaching (85%, $N=33$) or intruding a nest (76%, $N=25$). The probability of fleeing upon hearing a boatwhistle was significantly higher than expected by chance both during approaches (binomial test, $N=33$, $P<0.001$) or intrusions (binomial test, $N=25$, $P<0.05$). When intruders received escalated agonistic behaviour, the chances of fleeing were also higher than chance (binomial test, $N=46$, $P<0.01$) and they fled 74% of the time. When intrusions were successful, the intruder either stayed in the shelter with the resident or replaced him.

DISCUSSION

Experimental approaches to investigate the functional significance of agonistic sounds in fish and other animals include sound exposure

Table 1. Reactions to intruder approaches and nest intrusions

	Treatment	NR (%)	BW (%)	EF (%)	BW+EF (%)	N
Approach	Muted	81.25	–	18.25	–	4
	Sham	39.6	56.6	3.8	0	13
	Unmanipulated	4.2	95.8	0	0	8
Intrusion	Muted	64.2	–	35.8	–	18
	Sham	32.2	37.7	20.7	9.4	17
	Unmanipulated	43.1	23.3	25.9	7.7	13

Percentages were calculated per fish and then averaged for each treatment group. *N*, Number of fish that experienced an approach or an intrusion. NR, no reaction; BW, boatwhistle sound; EF, escalated fighting; BW+EF, sound followed by escalated fighting.

through playback, exclusion of fish sounds by keeping opponents in separate tanks or by muting individuals, the use of mirrors to level visual interactions while testing the function of sound and correlative analyses (Ladich and Myrberg, 2006). Although muting procedures are more invasive than the widely used playback approach (McGregor, 1992), they avoid the concurrent presentation of acoustical and visual stimuli in playback tests, usually needed to elicit behavioural responses in fish (Ladich and Myrberg, 2006). Muting experiments have only been carried out twice (Valinski and Rigley, 1981; Ladich et al., 1992) probably because many vocal fish species have unknown sound-producing mechanisms (Ladich and Fine, 2006). Also, when the mechanism is known, its deactivation typically results in alteration or impairment of behaviour (Ladich and Myrberg, 2006). However, in fishes that use swimbladder mechanisms, such as the Lusitanian toadfish, swimbladder deflation does not impair sonic muscle contraction but results in a marked decrease of sound amplitude (Skoglund, 1961), causing the sounds to become inaudible while behaviour remains apparently unaltered. Such fish species are ideal to investigate the function of acoustical signalling in social contexts because the outcome of social interactions of mute fish can be compared with those of vocal animals. Unlike most fish species (Ladich and Myrberg, 2006), the Lusitanian toadfish has the advantage that a great component of agonistic interactions relies on acoustic signalling performed with no accompanying visual displays (Vasconcelos et al., 2010), thus avoiding the confounding effects of the interplay of different sensory channels. Here, we experimentally investigated whether sounds (boatwhistles) made by the Lusitanian toadfish have an active role in preventing territorial intrusion by comparing territorial defence between muted fish and two control groups, sham-operated and unmanipulated males.

We found a treatment effect on the number of approaches and intrusions experienced by nest-holders. Muted fish had more intrusions and fewer approaches than the remaining groups, but experienced a similar number of interactions (approach+intrusion). These results suggest that intruders initiated interactions equally with all groups but were more likely to proceed to intrusions in the nests of muted males, probably because these males were not able to make audible sounds. This is consistent with the observed high numbers of ‘no reactions’ in muted males. Muted fish probably attempted to defend their shelters by making sounds, but as this species typically emits sounds with no accompanying visual displays, attempts at sound production could not be detected. Similarly, in the grasshopper *Chorthippus biguttulus*, males muted by removing the forewings, fictively stridulated with the same frequency and movement pattern as intact animals (Kriegbaum and von Helversen, 1992).

Nest-holder Lusitanian toadfish mainly reacted to approaches and intrusions with sounds and, less often, with higher levels of fighting. There was no significant difference in the levels of escalated fighting among the three treatment groups, either as a reaction to approaches or to intrusions, suggesting that fish did not compensate the lack of ability to produce sounds with increased levels of

aggressiveness. In contrast, muted skunk loach nest-holders increase the number of visual displays, but lowered attacks, in comparison to control fish in an attempt to prevent nest intrusion (Valinski and Rigley, 1981).

Importantly, when nest-holders made boatwhistles, intruders tended to flee. In this context, unmanipulated and sham groups had a higher probability of preventing territorial intrusion than muted fish. Escalated fights also had a higher than expected chance to expel the intruder but are more costly because they can incur physical injuries and are energetically demanding. Consistent with the keep-out signal hypothesis, an average of 44% of intrusions resulted in nest takeovers in muted males, compared with 20% for vocal males. The difference in the proportion of nest takeovers seems to be caused by the ability to vocalise and not by the intruder’s size. The difference in total length between expelled nest-holders and successful intruders was ~1% for the three treatment groups, although size differences in our experiments were generally higher with a mean difference of 9%. Altogether, the present data strongly suggest that boatwhistles are effective keep-out signals, lowering the probability of territorial intrusions and therefore nest takeovers.

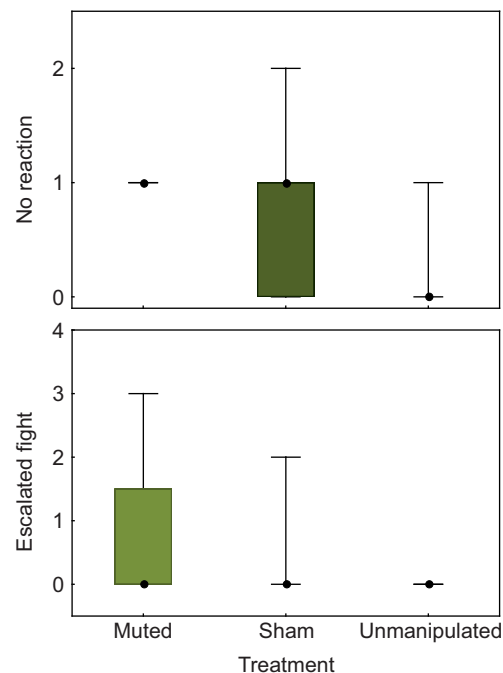


Fig. 2. Number of times resident male toadfish showed ‘no reaction’ or engaged in escalated levels of fighting when approached by intruders. Dots indicate medians and boxes and error bars depict quartiles and range. Treatment had a significant effect only on the number of ‘no reactions’ (Kruskal–Wallis test, $P < 0.05$). *Post hoc* tests indicated a marginally non-significant difference ($P = 0.07$) between muted and unmanipulated males for ‘no reaction’.

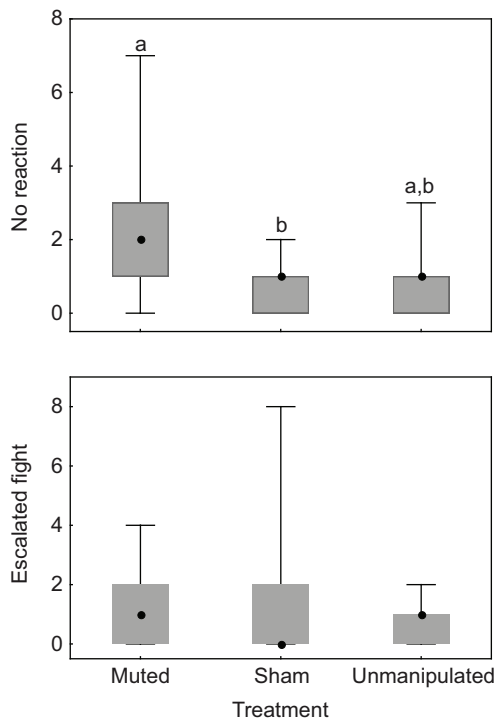


Fig. 3. Number of times toadfish male residents showed 'no reaction' or engaged in escalated levels of fighting upon intrusions. Dots indicate medians whereas boxes and error bars depict quartiles and range. Different letters denote pairwise significant differences at $P < 0.01$.

Other studies support the importance of acoustic signals in winning contests and in deterring territorial intrusion. In the croaking gourami *Trichopsis vittata*, territorial males matched in size with the opponent, had a significantly higher chance of winning the dispute when they were vocal than when muted. However, when size differences increased, larger fish tended to win the fight irrespective of the ability to vocalise (Ladich et al., 1992). Muted skunk loaches also experienced more intrusions than control fish, but differences in sizes between contestants were not mentioned (Valinski and Rigley, 1981). The deterrent effect of sounds on territorial intruders has been shown for the bicolor damselfish (Myrberg, 1997) and for the painted goby (Pereira et al., 2014) because intruders took longer to enter unoccupied territories/nests associated with conspecific sound playback than silent ones. The deterrent effect of agonistic acoustic signals on territorial intrusions has traditionally been described for birds. Muting adversely affects the ability to acquire and defend territories (e.g. McDonald, 1989) and song playback from territories after removal of owners delays occupation by intruders (e.g. Krebs et al., 1978).

Interestingly, the duration of interactions, including time to nest takeover, did not differ between muted and vocal fish. This suggests that the dynamics of mutual assessment, which involves reiteration of behaviour between opponents (Enquist and Leimar, 1983, 1987), was not altered by differences in vocal activity.

Our muting experiments did not cause alteration of the behaviour in muted fish because all groups showed similar levels of escalated fighting. Muting experiments in different taxa include examples where the subject's behaviour remains unaltered after being silenced. For example, croaking gourami males prevented from making sounds by cutting the two enhanced pectoral fin tendons involved in sound production exhibited normal swimming movements and agonistic behaviour (Ladich et al., 1992). Also, in

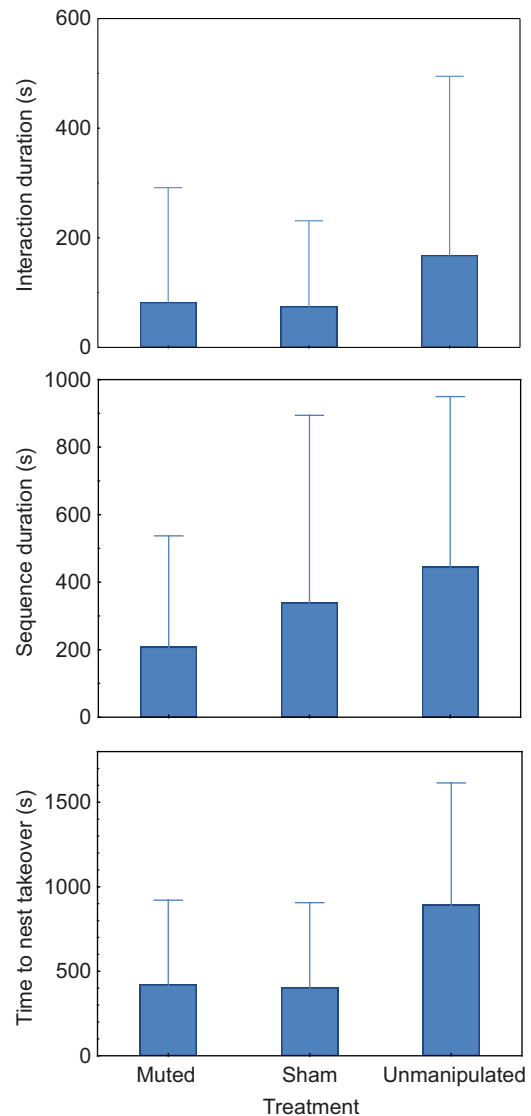


Fig. 4. Mean duration of resident–intruder interactions, sequence of interactions and sequence of interactions that lead to nest takeover.

Temporal patterns of the dynamics of territorial defence did not differ among groups (one way ANOVA, $P > 0.05$). See the Materials and methods for details of duration measurements.

the study of Davies and Halliday (1978), silencing toad (*Bufo bufo*) males did not seem to alter reproductive or agonistic behaviour.

Together, the results of this study provide experimental evidence of the deterrent function of agonistic sounds in territorial defence in fish. We show that acoustic signals play an active role in territorial defence, decreasing the probability of escalated fighting and intrusions, and thus probably reducing nest takeovers.

MATERIALS AND METHODS

Test males and maintenance

Prior to the beginning of the breeding season, 60 artificial hemicylinder concrete shelters (50 cm long, 30 cm wide and 20 cm high) were placed approximately 1.5 m apart in three rows, along an intertidal area of 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 spring tides during May to July 2011. We also used some fish caught by local fisherman. Only territorial males were used and they were identified by gently pressing their abdomen near the urogenital opening where they have accessory glands that release a dark-brown seminal fluid, unlike females and

sneaker males (Modesto and Canário, 2003). We maintained experimental males in round stock tanks (plastic swimming pools 2 m in diameter and water depth of 0.5 m) near the intertidal toadfish nesting area where males were collected. Stock and experimental tanks (similar to the stock tanks but with 2.5 m diameter) were placed on the sand just above the high tide shoreline under a shadow net cover held 170 cm high to prevent excessive solar radiation and water heating. Water temperature varied from 18 to 26°C (mean 21.4°C), within the range of the estuary water temperature variation during the same period. The water was changed every 2–3 days, by pumping water directly from the estuary. A natural light cycle was maintained because the stock tanks were outdoors.

Territorial intrusion protocol

We carried out territorial intrusion experiments with resident and intruder fish to simulate a context of male–male competition during territorial defence. Resident males were randomly assigned to three treatments: muted, sham-operated and unmanipulated males. Males were muted with a small surgical procedure after they were anaesthetised with a benzocaine solution (0.1 g l⁻¹) for few minutes. A small incision in the abdominal area was made and the swimbladder was deflated through a small cut to prevent sound production. The abdominal opening was then closed with two stitches. To control for possible effects of the surgery on toadfish territorial behaviour (apart from the ability to vocalise) a sham-operated treatment was also used. Sham-operated fish were given the same procedure as the muted group, except for the actual swimbladder cutting and deflation, and they were still able to vocalise normally. Fish were allowed to recover from anaesthesia before being placed in the experimental tanks. Resident test males from the unmanipulated group did not experience any surgical intervention and controlled for possible effects of anaesthesia and surgery procedures. The muting procedure was effective because muted males did not make sounds during trials and the number of resident–intruder interactions with sound production did not differ between vocal groups (Mann–Whitney test, $N_{\text{Sham}}=20$, $N_{\text{unmanip}}=19$, $U=154.5$, $P>0.05$; Table 1).

Two males from the same experimental group were placed in an experimental tank at least 24 h before the experiments, allowing them to become territorial and recover from possible short-term surgery effects. Each experimental tank was provided with two roof tiles as shelters (internal dimensions 44×18×10 cm) placed approximately 50 cm apart and 20 cm away from the tank's border. All subject males readily occupied the empty shelters and spent most of the time inside them, a normal territorial fish behaviour (Vasconcelos et al., 2010). We placed one hydrophone (High Tech 94 SSQ, High Tech Inc., Gulfport, MS, USA; frequency response: 30 Hz to 6 kHz ±1 dB; voltage sensitivity: -165 dB re. 1 V μPa⁻¹) in front of each nest, at about 10 cm from its entrance and from the tank bottom, attached to a wooden rod kept over the tank. Simultaneous two-channel recordings were made with a USB audio capture device (Edirol UA-25, Roland, Osaka, Japan; 16 bit, 44.1 kHz acquisition rate per channel) connected to a laptop and down-sampled to 6 kHz by Adobe Audition 3.0 (Adobe Systems, San José, CA, USA). Recorded sounds could be attributed to a particular territorial male because of the proximity of each hydrophone to one nest. Usually, only territorial males produce sounds (Vasconcelos et al., 2010). In one exceptional case (M.C.P.A., unpublished data) <CQ2>, we observed one intruder producing boatwhistles during intrusions but the resident's and the intruder's sounds could clearly be distinguished due to spectral differences.

In each trial, two intruder males (unmanipulated) were placed sequentially in the experimental tank with an interval of 30 min between intrusions and remained in the tank until the end of the trial (following Vasconcelos et al., 2010). Our experimental design resembles the natural chorusing aggregations, where territorial males nest very close together (Amorim et al., 2010b) and may attract several competitor males (Vasconcelos et al., 2012). It also aimed to increase the motivation of subject males to become territorial and the number of territorial defence interactions during trials, thus decreasing the need for a larger number of operated males. The first intruder was not removed when the second was introduced in the tank to avoid disturbing resident males. Intruders were chosen randomly from stock tanks, but in most cases, residents and intruders were matched in total length (TL) (mean total length difference resident

TL/intruder TL×100=7%; median=1%; range: -20% to 67%) with only 9 out of 57 residents experiencing size asymmetries larger than 20%. Fish were labelled with marks in the fins (i.e. a small cut between the fin rays) to identify them during trials. Marking did not cause any measurable change in behaviour. Behavioural interactions and sound produced were registered for 60 min beginning with the placement of the first intruder male. After each trial all specimens were measured for total length (TL) to the nearest mm and weighed to the nearest gramme. We used a total of 18, 20 and 19 resident males for the muted, sham-operated and unmanipulated treatments, with a mean (range) TL of 41.3 (32.4–48.0) cm, 43.9 (36.6–50.0) cm and 40.5 (26.8–47.0) cm, respectively. We used a total of 64 intruders with a mean (range) TL of 39.5 (27.0–50.0) cm.

Behavioural analysis

Behaviour of residents and intruders was assessed by direct observation, noted on paper and later tallied following Vasconcelos et al. (2010). Sound production was simultaneously monitored with headphones that were connected to the recording laptop. For residents, we registered the number of non-escalated behaviours including mouth opening with the extension of pectoral fins and opercula and escalated behaviours including chasing, bite attempts, bites and mouth–mouth fighting. The number of times residents showed no apparent reaction ('no reaction', i.e. no visible or audible behaviour) upon and intruder's approach or nest intrusion was also measured. We also tallied the duration of resident–intruder interactions and the sequence of interactions because duration of fighting is an important measurement of mutual assessment (Enquist and Leimar, 1983). An interaction was considered as a set of consecutive behaviours involving one resident and one intruder that started with the latter approaching or intruding the nest and stopped when he fled to the border of the tank or took over the nest. A sequence of interactions were a set of consecutive interactions involving the same resident and intruder that were not interrupted by an interaction with another male (usually the other intruder) and that finished with either the intruder fleeing and not further resuming the interaction or with a nest takeover. We tallied the number of sounds emitted by the resident including agonistic boatwhistles or other sound types (grunts, long grunt trains, croaks and double croaks; see Amorim et al., 2008 for a description). For the intruders, we tallied the number of approaches, intrusions in the nest (the intruder entering partially or completely) and fleeing. We defined approaches when the intruder was at least within a body length from the nest and an intrusion when the intruders managed to get at least part of the body inside the nest. Fleeing consisted of swimming away from the nest. These categories are mutually exclusive but may be performed sequentially.

Statistical analysis

Statistical tests were performed with Statistica 12.0 for Windows (StatSoft, Inc., Tulsa, OK, USA) and all data were transformed when necessary to meet assumptions of the used parametric tests. When there was no normality of the transformed data, non-parametric tests were used.

We compared the number of approaches, intrusions and total interactions (approach+intrusion) experienced by the different treatment groups with Kruskal–Wallis tests. Similarly, the responses of the residents ('no reaction' and escalated fights) were compared among treatment groups with Kruskal–Wallis tests. *Post hoc* tests available in Statistica and described in Siegel and Castellan (1988) were used for multiple comparisons between treatments.

The effect of treatment on interaction and sequence of interaction durations was tested with one-way ANOVA. We tested whether the production of boatwhistles altered interaction duration with a two-way ANOVA that included the factor interaction type (with three levels: approach, intrusion and approach+intrusion) and the factor boatwhistle production (two levels: vocal and silent). We finally compared the duration of the sequence of interactions until nest takeover among treatment groups. Interaction and sequence of interaction durations were log-transformed to meet the ANOVA assumptions.

A chi-square test of independence was performed to test whether when there was an intrusion the variable nest takeover (nest takeover versus no takeover) was independent of vocalising (vocal versus muted). The probability of the intruder fleeing after receiving a boatwhistle or an

escalated attack by the resident, when approaching or intruding its nest, was compared with what was expected to happen randomly with binomial tests.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.C.P.A. and P.J.F. were involved in conception of the study and experimental design. C.C. conducted the study. M.C.P.A. carried out statistical analyses. M.C.P.A. and C.C. drafted the article. All authors revised the article.

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