

Stereotypy and variation of the mating call in the Lusitanian toadfish, *Halobatrachus didactylus*

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Abstract Signal attributes should show different degrees of variability depending on the information to be conveyed. Species identity is usually associated with stereotyped features of a signal, whereas other types of information such as individual quality and motivation are associated with signal plasticity. Lusitanian toadfish males form aggregations during the breeding season and emit a tonal advertisement call (the boatwhistle) to attract mates to their nests. We test the hypothesis that the boatwhistle can convey information both on individual identity and motivation by checking how signal parameters vary with time. We study how the physical (tide level) and social (calling alone or in a chorus) environments and male calling rate affect this advertisement signal and how all these external and internal factors (environment, social and male motivation) blend to modulate the Lusitanian toadfish's advertisement call. Boatwhistles of each male were very stereotyped in short periods of time (minutes), but intra-male signal variability greatly increased in a longer time scale (days). Nevertheless, significant differences among males could still be found even in a long time scale. Pulse period was the acoustic feature that most contributed to discriminate among males. Tide level and male calling rate modulated

boatwhistle characteristics, and there was a differential effect of tide on call attributes depending on male calling rate. Social acoustic environment only affected calling rate. These results suggest that inter-individual differences in call characteristics and call plasticity may mediate both male–male assessment and mate choice.

Keywords Acoustic communication · Individuality · Signal plasticity · Batrachoididae · Teleost fish · Tide effects

Introduction 42

Animals use acoustic signals to convey different types of information such as species identity, individual identification, condition, sexual or aggressive motivation and territorial ownership. The variability of spectral and temporal features contained in different calls or in a single call may convey different types of messages. For example, signal attributes that convey a species' identity show little variation among individuals within a species. Conversely, acoustic features that convey individual identity evidence strong stereotypy within an individual but show larger variation between individuals (Bee et al. 2001). Additionally, features such as signal repetition rate or intensity may indicate motivation or condition and thus provide fundamental information for mate choice and for male–male interactions (Bradbury and Vehrencamp 1998).

In species in which males defend territories in complex spacing systems to obtain mates, such as in male calling aggregations, advertisement acoustic signals should inform receivers about species and sex identity, sender's location, motivation and individual quality (Bradbury and Vehrencamp 1998). These different messages can be communicated in some species by large and complex

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65 acoustic repertoires while other species may exhibit more
 66 limited repertoires and rely on the plasticity of certain call
 67 features (e.g. Burmeister et al. 1999). Consequently,
 68 species with small repertoires can be excellent models to
 69 understand the interplay between variable and more
 70 stereotyped signal features not only conveying species
 71 identity but also mediating male–male competition and
 72 mate choice.

73 Many teleost fish species rely on their calls to acquire
 74 mates (e.g. Malavasi et al. 2003) and to keep intruders
 75 away from their territories (Ladich and Myrberg 2006).
 76 Teleost fish produce relatively simple sounds in small
 77 acoustic repertoires (Amorim 2006) and in a few species
 78 the vocal motor networks and the auditory systems have
 79 been well studied (Bass and Mckibben 2003). Hence,
 80 teleosts provide simple models to study the role of
 81 acoustic signals in reproductive and agonistic decisions
 82 in vertebrates.

83 Fish from the family Batrachoididae (toadfishes and
 84 midshipmen) have emerged as one of the groups where
 85 acoustic communication is best studied in this taxon (Bass
 86 and Mckibben 2003). During the breeding season, male
 87 batrachoidids produce advertisement calls (boatwhistles or
 88 hums) from their nests that are important for regulating
 89 male spacing and for mate attraction (Fish 1972; Brantley
 90 and Bass 1994). Nesting males form clusters and vocalise
 91 close together in a chorus (Bass 1996; Amorim et al. 2006).
 92 The gulf toadfish (*Opsanus beta*) changes calling rate and
 93 sound duration both during territorial intrusions and during
 94 increased crepuscular chorus activity, thus depicting a
 95 remarkable ability for teleost fishes to modulate calls
 96 according to the social context (Thorson and Fine 2002;
 97 Ramage-Healey and Bass 2005). Further, a recent study
 98 based on passive acoustics has shown that the boatwhistles
 99 of the Lusitanian toadfish *Halobatrachus didactylus* are
 100 highly stereotyped and show individual differences when
 101 considering short time periods (5–10 min) (Amorim and
 102 Vasconcelos 2008). Individuality in fish vocalisations is
 103 unusual and has only been mentioned for batrachoidids and
 104 fishes of the Mormyridae family (Crawford et al. 1997;
 105 Amorim and Vasconcelos 2008; but see also Myrberg et al.
 106 1993). Calling rate and acoustic features of batrachoidids’
 107 vocalisations are also affected by environmental factors
 108 such as water temperature and lunar cycles (Brantley and
 109 Bass 1994; Maruska and Mensinger 2009). In summary,
 110 previous studies suggest that the batrachoidid advertise-
 111 ment call may carry different messages in one relatively
 112 simple signal.

113 Male Lusitanian toadfish nest frequently in intertidal
 114 estuarine areas and are thus faced with fluctuating environ-
 115 mental parameters, such as water level and temperature,
 116 which may directly affect acoustic communication. In fact,
 117 temperature influences muscle contraction and thus may

118 affect sound production (Connaughton et al. 2000). Sound
 119 propagation is highly influenced by tide, since not only the
 120 sound attenuation increases significantly with lowering
 121 water level (Fine and Lenhardt 1983; Mann 2006) but also
 122 background noise often changes with tide. Because mate
 123 attraction in this species relies on a single call type that is
 124 broadcasted in a physically variable and acoustically
 125 complex environment, it provides an excellent model to
 126 study how environmental and social factors affect the
 127 variability of this advertisement signals. Here, we test the
 128 hypothesis that the advertisement call of the Lusitanian
 129 toadfish shows enough inter-male variability and intra-male
 130 plasticity to convey information of male identity and
 131 motivation in chorusing aggregations. We registered vocal-
 132 isations from groups of Lusitanian toadfish in an intertidal
 133 area to examine individual differences (stereotypy) among
 134 well-identified Lusitanian toadfish males in different time
 135 spans, from minutes to 1 week. We examined how
 136 environmental constraints (tide level), male calling rate
 137 and social environment modulate the Lusitanian toadfish’s
 138 advertisement call and discuss the possible role of signal
 139 stereotypy and plasticity.

140 **Material and methods**

141 **Study species**

142 The Lusitanian toadfish, *H. didactylus* (Batrachoididae) is a
 143 benthic marine fish that inhabits coastal areas and brackish
 144 environments from the Gulf of Guinea to the Tagus estuary,
 145 Portugal, appearing occasionally up to the Bay of Biscay
 146 (Roux 1986). Breeding males build nests under rocks in
 147 shallow water and attract females to spawn with long
 148 advertisement calls (boatwhistles) from May to July,
 149 forming conspicuous choruses (dos Santos et al. 2000;
 150 Amorim et al. 2006). Females show low fecundity since
 151 they lay only a few hundreds of large eggs in a single batch
 152 on the roof of a nest (Modesto and Canário 2003; Costa
 153 2004) whose survival is assured through male parental care.

154 **Sound recording and analysis**

155 We deployed 60 artificial concrete shelters every 1.5 m in
 156 rows along the shoreline in an intertidal area of the Tagus
 157 estuary (Portugal, Montijo, Air-Force Base 6; 38°42’N, 8°
 158 58’W). The shelters had a hemicylinder shape capped at one
 159 end (internal dimensions, 50 cm long, 30 cm wide and
 160 20 cm high) and were readily occupied by toadfish in the
 161 breeding season. These nests were only exposed to air
 162 during spring low tides. The water level in the nesting area
 163 varied between 0 m and 2.8 m. Three groups of six to eight
 164 males ($n=22$) that spontaneously occupied these artificial

165 concrete nests were recorded over a period of 8 days in
 166 June/July 2006 and 2007, during the peak of the reproduc-
 167 tive season. Subject males had a mean total length of
 168 42.9 cm (range, 37.9–47.7 cm) and a mean eviscerated
 169 weight of 1,207 g (857–1,612 g). Each male was recorded
 170 for an average of 35 h (11–56 h). Nests with the subject fish
 171 were placed 1.5 m apart in two rows and were at least 15 m
 172 apart from other nests that could be occupied by other
 173 males. Nests' entrances were closed with a plastic mesh that
 174 allowed prey items to enter but prevented males from
 175 abandoning the nest during recordings. The plastic mesh
 176 did not affect acoustic signals and allowed possible visual
 177 interactions. One hydrophone (High Tech 94 SSQ hydro-
 178 phone, sensitivity –165 dB re 1 V/ μ Pa, frequency response
 179 within ± 1 dB from 30 Hz to 6 kHz) was placed at about
 180 10 cm from the entrance of each subject male's nest and
 181 about 10 cm from the substrate. Simultaneous multi-channel
 182 recordings were made to a laptop connected to USB audio
 183 capture devices (Edirol UA25, Roland; 16 bit, 6 kHz
 184 acquisition rate per channel) controlled by Adobe Audition
 185 2.0 (Adobe Systems Inc., 2005). Recorded sounds could be
 186 attributed to each male due to the high acoustic attenuation
 187 observed in the simultaneous multi-channel recordings
 188 between neighbouring males. Water temperature was mea-
 189 sured every 3 h during recording periods and averaged 23°C
 190 (range, 19.5–28°C). All subject fish experienced similar
 191 water temperature variability during recordings.

192 Boatwhistles have been described in detail in Amorim
 193 and Vasconcelos (2008). This sound has a variable duration
 194 from a few hundred milliseconds up to over a second and is
 195 composed of three different segments or phases character-
 196 ized by different durations, pulse periods, relative amplitude
 197 and dominant frequencies (Amorim and Vasconcelos 2008).
 198 The tonal phase (P2) of the boatwhistle is the longest and
 199 the most characteristic of boatwhistles in the Lusitanian
 200 toadfish and in other batrachoidids (Thorson and Fine
 201 2002; Amorim and Vasconcelos 2008). We analysed boat-
 202 whistles for total sound duration (milliseconds, measured
 203 from the start of the first pulse to the end of the last pulse),
 204 pulse period of the tonal segment P2 (milliseconds, average
 205 peak to peak interval of six consecutive pulses in the
 206 middle of P2), dominant frequency of P2 (hertz, the
 207 frequency with maximum energy in P2), dominant frequen-
 208 cy modulation (the ratio of the dominant frequencies of the
 209 initial and the tonal phases) and amplitude modulation (the
 210 ratio of the mean amplitude of the initial and the tonal
 211 phases). These acoustic parameters are depicted in Fig. 1. A
 212 previous study has shown that these are the most important
 213 acoustic parameters to discriminate among individuals
 214 (Amorim and Vasconcelos 2008). Sound analysis was
 215 carried out with Adobe Audition 2.0 and Raven 1.2.1 for
 216 Windows (Bioacoustics Research Program, Cornell Labo-
 217 ratory of Ornithology, Ithaca, NY, USA).

Note that since the hydrophones were just 10 cm away
 from the calling males, any detected effects of tide level on
 acoustic parameters were not related to water level
 transmission loss dependence (see for example Fine and
 Lenhardt 1983; Mann 2006).

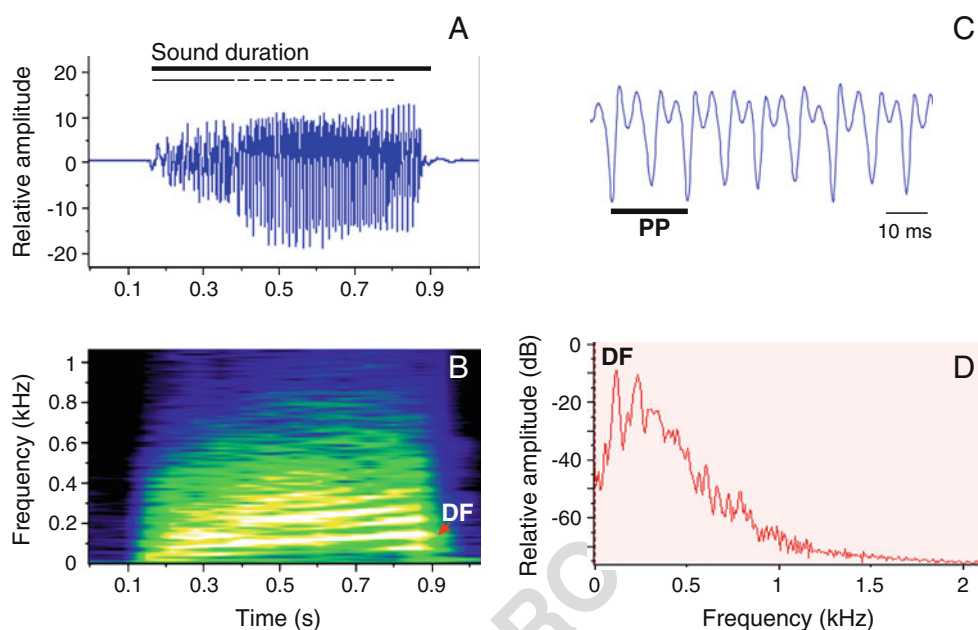
Statistical analysis

We calculated the mean and standard deviation (SD) for
 each of the five boatwhistle acoustic variables emitted by
 13 males in two time frames: short, 10 boatwhistles per
 male emitted over a time period of no longer than 10 min;
 long, mean of 41 (range, 11–95) boatwhistles per male
 produced during up to 8 days. We subsequently computed
 the overall means and SDs for each acoustic variable using
 the previously calculated mean values for each male. We
 determined the within-male variability for the five acoustic
 variables by calculating the within-male coefficient of
 variance ($CV_w = SD/mean$) for each male and subsequently
 computed the mean for all males. We also determined the
 between-male coefficient of variation (CV_b) by dividing the
 overall SD by the respective overall mean. The ratio $CV_b/$
 CV_w was then calculated to obtain a measure of relative
 between-male variability for each boatwhistle feature. When
 this ratio is larger than one, it suggests that the acoustic
 parameter is more variable between individuals relative to
 its variability within individuals and could be used as a cue
 for individual discrimination (Christie et al. 2004). Kruskal–
 Wallis tests were computed to compare differences among
 males for each acoustic feature in the two time frames.

Discriminant function analysis (DFA) was carried out as a
 multivariate tool to determine if males could be discriminated
 based on their sounds considering these five acoustic variables
 and to verify which acoustic features better distinguish males.
 DFA also gives a measure of discrimination accuracy by
 revealing the percentage of sounds correctly assigned to each
 individual (Mundry and Sommer 2007). DFA were performed
 both on the short time frame (ten boatwhistles per male
 emitted within 10 min by 14 males) and on the longer time
 frame (ten boatwhistles per male emitted during up to 8 days
 by the same males, $n=13$) data sets. Data were standardised
 $[(xi-mean)/SD]$ to remove differences of magnitude observed
 between individuals for a given acoustic parameter. We
 confirmed that DFA assumptions were met with the inspection
 of residual plots (predicted vs residuals values, normal
 probability plots), by performing Levene's and the multivariate
 Box M tests for homogeneity of variances/covariances
 and by checking tolerance levels to assess possible multi-
 collinearity among variables. In addition, to validate the
 models obtained, a cross-validation method ('leave-one-out')
 was carried out (Mundry and Sommer 2007). In this method
 each sound is classified by the discriminant functions derived
 by the $n-1$ remaining sounds.

Q1

Fig. 1 Oscillogram (a), sonogram (b) and power spectrum (d) of a boatwhistle. Sound duration (thick continuous line), the initial phase (P1, fine continuous line) and the tonal phase (P2, fine dashed line) of the boatwhistle are depicted in the oscillogram. Dominant frequency (DF) of the tonal phase is shown in the sonogram and in the power spectrum. Detail of the boatwhistle tonal phase waveform depicting the pulse period (PP) (c)



269 We also tested whether environmental and social factors
 270 and male calling rate had a significant effect in boatwhistle
 271 acoustic variables in a long time frame with multi-way
 272 analysis of covariance (ANCOVA). In this analysis, we
 273 used sounds registered in the longest possible time span per
 274 individual (up to 8 days) and during different tide levels
 275 and social environments. We considered an average of 34
 276 sounds per male (range=10–92) for 16 males. As data was
 277 unbalanced ANCOVA were based on sum of squares III.
 278 Tide level was included as a factor with three levels: 1=full
 279 tide, 2=ebb tide and 3=low tide. Rising tide was not
 280 considered because of the reduced number of males calling
 281 during this tide level, which was significantly lower than at
 282 high and ebb tide, and lower, although not significantly,
 283 than low tide (Kruskal–Wallis test, $n=190$, $H=26.11$, $p<$
 284 0.001 ; Fig. 2). Social environment was another factor with
 285 two levels: 1=calling alone and 2=calling in a chorus of at
 286 least two males. We included a third factor with two levels
 287 that represented male’s calling rate (1=low rate <9 BWmin⁻¹
 288 and 2=high rate ≥ 9 BWmin⁻¹). Nine BWmin⁻¹ was
 289 considered the cutpoint since the average calling rate for all
 290 fish was of 8.8 BWmin⁻¹ ($n=576$). This factor was included
 291 because exploratory analysis showed calling rate affected
 292 call features. We also considered calling rate as a dependent
 293 variable. In this case only tide and social factors were
 294 included in the models. Water temperature was included as a
 295 continuous variable (covariate) because it can influence
 296 acoustic parameters (Amorim et al. 2006). When the effect
 297 of this covariate was not significant, multi-way ANOVA
 298 were carried out instead. The final models complied with
 299 normality and homogeneity of variance assumptions. We
 300 also confirmed the absence of multicollinearity between the
 301 predictors (variance inflation factors were always smaller

than 5 and tolerance levels larger than 0.1; Montgomery et al. 2006) since water temperature tended to be on average 2°C higher at low tide than in other tide levels.

Non-parametric statistical tests were carried out when parametric assumptions were not met. Non-parametric statistics and ANCOVA tests were carried out with Statistica (9, Statsoft Inc., USA) and DFA were performed using SPSS (16.0, SPSS Inc., USA) for Windows.

Results

Stereotypy: short time frame

All five acoustic parameters showed significant differences between individuals (Table 1). There was a strong stereotypy in most acoustic parameters measured, and only the

Q2

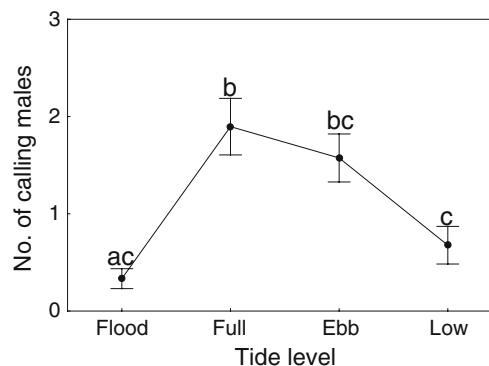


Fig. 2 Variation of the number of calling males (max=8) with tide levels. Dots and error bars are means and standard errors. Different letters indicate pairwise significant differences given by Dunn tests, i.e. factor levels with the same letter show no significant differences

Table 1 Within-male variability (CV_w) and between-male variability (CV_b) for the five acoustic variables analysed from the same 13 Lusitanian toadfish males in a short (up to 10 min) and in a long time frame (up to 8 days)

Acoustic variables	Short time frame					Long time frame				
	Mean ^a (\pm SD)	CV_w	CV_b	CV_b/CV_w	H ^b	Mean ^a (\pm SD)	CV_w	CV_b	CV_b/CV_w	H ^c
Sound duration (ms)	723.1 (\pm 161.9)	0.10	0.21	2.11	101.33	686.8 (\pm 190.2)	0.22	0.21	0.95	147.47
Pulse period P2 (ms)	18.9 (\pm 1.4)	0.02	0.07	2.92	109.9	18.5 (\pm 1.7)	0.08	0.04	0.47	111.12
Dominant frequency P2 (Hz)	143.0 (\pm 43.0)	0.11	0.27	2.54	95.08	127.6 (\pm 46.6)	0.31	0.23	0.73	166.53
Frequency modulation	0.9 (\pm 0.3)	0.18	0.21	1.20	63.11	0.8 (\pm 0.4)	0.46	0.21	0.47	78.7
Amplitude modulation	0.7 (\pm 0.1)	0.04	0.11	2.49	111.17	0.7 (\pm 0.8)	0.31	0.23	0.77	175.54

Ten boatwhistles were analysed per male for the short time frame analysis, whereas an average of 41 sounds per male (range=11–95) were analysed from the long time frame data set. Note that only 13 individuals are considered since only these are in common in the short and the long time frame analyses data sets. P2, middle tonal phase in the boatwhistle

^a Results computed for all sounds considered per analysis.

^b Results of Kruskal–Wallis tests (DF=12, $n=130$) comparing differences between males for each acoustic feature

^c Results of Kruskal–Wallis tests (DF=12, $n=534$) comparing differences between males for each acoustic feature. All comparisons are significant at $p < 0.001$

frequency modulation showed a within-male CV_w larger than 0.11 (Table 1). Consistently, with the Kruskal–Wallis results, all five acoustic variables were more variable between than within males as all CV_b/CV_w ratios were larger than one (Table 1).

A discriminant function analysis (DFA, $n=140$, Wilks' lambda=0.0004, DF=65, 580, $p < 0.001$) assigned boatwhistles to the correct male with an average success of 86% (range, 30–100%). The first two discriminant functions explained 74% of data variability with P2 pulse period weighing most heavily in explaining variation in the first function and sound duration and amplitude modulation in the second function (Table 2). After cross-validation the correct classification assigned by the DFA model was similar (80%).

Taken together, these data reveal that male Lusitanian toadfish show individual differences in the properties of boatwhistle in a time frame of a few minutes.

Variability: long time frame

Considering a period of time as long as possible for each male (up to 8 days), significant differences between males were kept for all five acoustic parameters (Table 1); however, only the pulse period of P2 showed a within-male CV smaller than 0.1 and all the CV_b/CV_w ratios were smaller than one showing that when a long time frame is considered all five acoustic variables become more variable within than between males. A discriminant function analysis ran on this long-term data set (DFA, $n=130$, Wilks' lambda=0.09, DF=60, 532, $p < 0.001$) showed that the average classification success decreased to 45% (range, 0–90%) with the first two discriminant functions explaining

72% of data variability. The percentage of correct classification after cross-validation (leave-one-out procedure) was 35%. In conclusion, significant differences among male calls in a long time scale were still found, although to a lesser extent than in a short time scale.

Sources of call variability: long time frame

Sound duration decreased significantly during low tide (Table 3; Fig. 3a) and this parameter was also significantly affected by the calling rate. The interaction between the factors calling rate and social environment was significant (Table 3) and sound duration significantly decreased in males calling at a low rate and significantly increased in

Table 2 Standardised canonical DFA coefficients, eigenvalues and cumulative percentage of variance explained by the first two discriminant functions of a DFA classifying 14 Lusitanian toadfish males by their boatwhistles' acoustic characteristics

Discriminant variables	Discriminant functions		
	First	Second	
Sound duration (ms)	0.05	0.57 ^a	t2.4
Pulse period P2 (ms)	0.69 ^a	0.00	t2.5
Dominant frequency P2 (Hz)	-0.03	-0.22	t2.6
Frequency modulation	-0.25	0.07	t2.7
Amplitude modulation	0.44	-0.55	t2.8
Eigenvalue	10.53	9.21	t2.9
Cumulative % of variance	39.6	74.3	t2.10

Boatwhistles were emitted in a period of 10 min

^a Discriminant variable with the highest pooled within-groups correlations with the standardised discriminant functions.

Table 3 Effects of tide level, social environment and male calling rate on boatwhistles' acoustic variables

Factors	Duration (ms)			Pulse period P2 (ms)			Dominant frequency P2 (Hz)			Frequency modulation			Amplitude modulation		
	DF	F	P	DF	F	P	DF	F	P	DF	F	P	DF	F	P
Tide	2,535	6.01	0.003	2,536	56.42	<0.001	2,535	2.66	0.07	2,536	0.11	0.90	2,536	0.40	0.67
Social	1,535	0.12	0.73	1,536	0.25	0.73	1,535	0.01	0.92	1,536	0.10	0.75	1,536	0.05	0.82
Call rate	1,535	5.05	0.03	1,536	78.43	<0.001	1,535	7.61	0.006	1,536	3.63	0.06	1,536	0.49	0.49
T×S	2,535	0.04	0.96	2,536	4.28	0.01	2,535	0.72	0.49	2,536	0.57	0.57	2,536	0.08	0.93
T×CR	2,535	1.99	0.14	2,536	9.98	<0.001	2,535	0.68	0.51	2,536	7.08	<0.001	2,536	0.30	0.74
S×CR	1,535	4.52	0.03	1,536	2.00	0.16	1,535	0.00	0.94	1,536	0.10	0.76	1,536	0.00	0.97
T×S×CR	2,535	0.17	0.85	2,536	1.06	0.35	2,535	0.38	0.69	2,536	0.41	0.67	2,536	0.11	0.89
T°C	1,535	15.37	<0.001	-	-	-	1,535	19.05	<0.001	-	-	-	-	-	-

Temperature was included as a covariate when its effect was significant
 T tide level, S social environment, CR calling rate, T°C temperature

males calling at a high rate with the raise of social complexity, i.e. in a chorus situation (Fig. 3b). There was a difference in sound duration between males calling at high and low rate only when the fish were singing in a chorus (Fig. 3b).

P2 pulse period decreased significantly at low tide (Table 3; Fig. 4). The interaction term between tide and calling rate was significant. The decrease in the pulse period with water level was more marked in males calling at a high rate than at a low rate. Males calling at a high rate had a shorter pulse period in their calls and significant differences between call rate levels were observed at ebb and low tide levels (Fig. 4a). The interaction between tide level and social environment was also significant. Pulse period decreased gradually with water level in males calling alone, but in males calling in groups there was only an abrupt decrease at low tide. There was, however, no significant effect of social environment on pulse period as differences for this parameter between males calling alone or in a chorus were not significant in any tide level (Fig. 4b).

P2 dominant frequency was only significantly affected by calling rate (Table 3) and males calling at a high rate showed significantly higher values for this acoustic parameter than males calling at a low rate (Fig. 5).

Only the interaction term between tide level and calling rate had a significant effect on frequency modulation and males calling at a high rate showed higher frequency modulations than less vocal males at ebb tide (Table 3; Fig. 6). There was no significant effect of any variable or interaction term in amplitude modulation (Table 3).

Social environment had an effect in the calling rate (ANCOVA, social environment: $F_{1,541}=42.17$, $p<0.001$; covariate water temperature, $F_{1,541}=6.16$, $p=0.01$). Data inspection showed that males that call alone mostly call at low rates, but males in a chorus call at all rates. There was a significant effect of the interaction between social environment and tide level (ANCOVA, interaction: $F_{2,541}=11.55$, $p<0.001$, tide level: $F_{2,541}=0.97$, $p=0.38$). Calling rate of males calling alone increased at low tide and with the exception of this tide level it was significantly lower than the calling rate for males in a chorus situation (Fig. 7).

Discussion

Stereotypy vs. variability

There was strong stereotypy in the boatwhistles produced by Lusitanian toadfish males when considering periods of 10 min consistent with the study of Amorim and Vasconcelos (2008) based on unidentified fish. Males differed significantly in all five acoustic parameters and

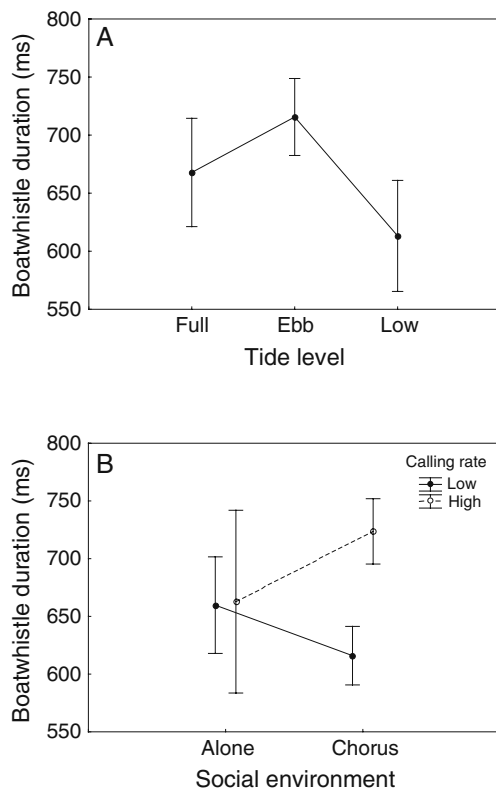


Fig. 3 Effect of tide level on boatwhistle duration (a). Effect of social environment (calling alone or in a chorus) and male calling rate (low rate, filled circle and high rate, open circle) on sound duration (b). Circles are means computed for the covariate water temperature mean and error bars are 95% confidence intervals

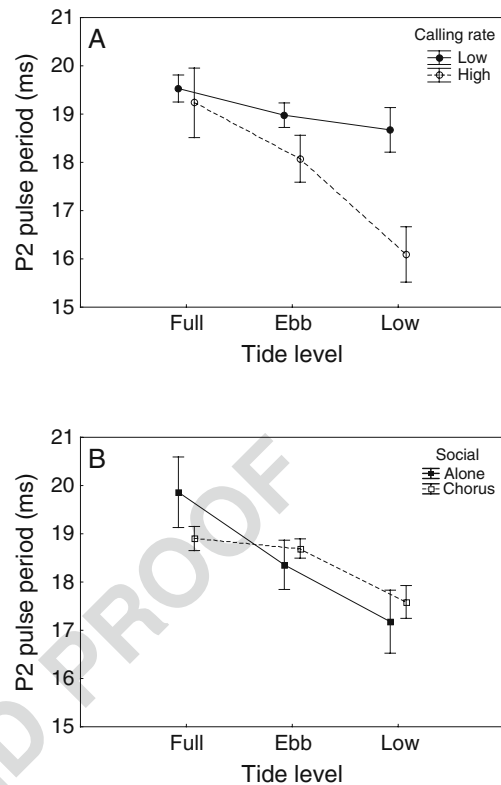


Fig. 4 Effect of tide level and male calling rate (low rate, filled circle and high rate, open circle) (a) and of tide level and social environment (alone, filled square and chorus, open square) on P2 pulse period (b). Circles and squares are means and error bars are 95% confidence intervals

407 average correct classification of individuals based on their
 408 calls was high (86%) as shown by DFA, with P2 pulse
 409 period, sound duration and amplitude modulation being the
 410 best parameters to discriminate among males. Individual
 411 differences in signals are important to mediate social
 412 interactions, namely between neighbouring territorial
 413 males (Bradbury and Vehrencamp 1998). Individual
 414 recognition is especially relevant when animals defend
 415 long-term territories, such as breeding Lusitanian toadfish
 416 males do, because territory holders can reduce aggression
 417 towards familiar neighbours, which are less likely to
 418 intrude their territories ('dear enemy effect', Temeles
 419 1994). Although this effect was not yet identified in
 420 toadfishes, examples exist in several taxa. For instance,
 421 territorial male bullfrogs (*Rana catesbiana*) show individual
 422 differences in their advertisement calls that mainly differ in
 423 the fundamental frequency (Bee and Gerhardt 2001a).
 424 Playback experiments have shown that this acoustic
 425 parameter mediates neighbour-stranger recognition in this
 426 species since they show less aggression in response to
 427 familiar calls (Bee and Gerhardt 2001b).

428 When considering a longer time frame (up to a week)
 429 there were still significant differences among Lusitanian

430 toadfish males for all five acoustic features, but these become
 431 more variable within a male (Table 1). With the exception of
 432 P2 pulse period, all other parameters showed high intra-male
 433 variability over a week. This variability of the Lusitanian
 434 advertisement calls in a longer time frame suggests
 435 that boatwhistles are being modulated by either external
 436 factors such as the physical or the social environment or by

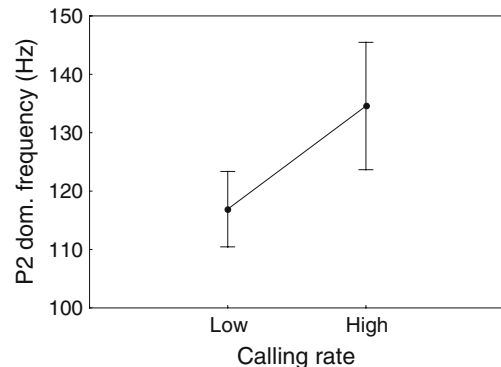


Fig. 5 Effect of male calling rate on P2 dominant frequency. Dots are means computed for the covariate water temperature mean and error bars are 95% confidence intervals

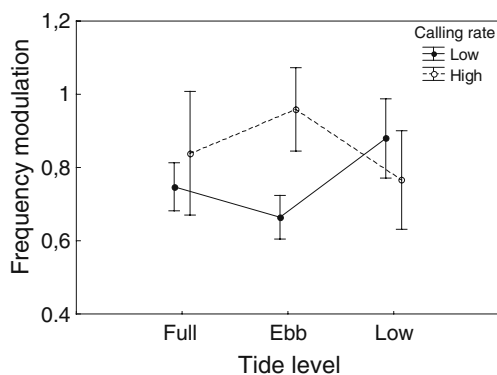


Fig. 6 Effect of tidal level and male calling rate on dominant frequency modulation. Dots and error bars are means and 95% confidence intervals

437 internal factors such as the internal physiological state of the male (Ramage-Healey and Bass 2005).
438

439 Interestingly, the P2 pulse period was the least variable
440 acoustic feature both within and between males and kept
441 the CV_w and CV_b under 0.1, regardless of the time span
442 considered. Pulse period corresponds to the sonic muscle
443 contraction period (Skoglund 1961; Fine et al. 2001), which
444 is controlled by central vocal pattern generators in
445 batrachoidids, and is stereotyped at the species level (Bass
446 and McKibben 2003); nevertheless, P2 pulse period was the
447 parameter that contributed the most to discriminating
448 among individuals in a short time scale (DFA) suggesting
449 it presents fine differences among individuals regardless of
450 the time scale considered, which might be used for
451 individual recognition among neighbouring nesting males.

452 P2 pulse period could also be indicative of male quality.
453 In a recent study, Amorim et al. (2010) found that in the
454 Lusitanian toadfish the pulse period reflects male condition
455 and males with a higher body lipid content produced
456 vocalisations with shorter pulse periods. These authors
457 suggested that males that contract the sonic muscles at a
458 very fast rate could reliably be indicating to neighbouring
459 males or females their better quality (condition) with the

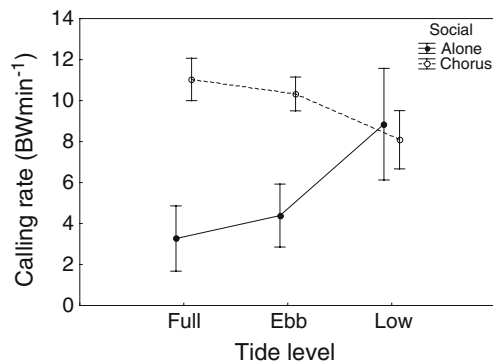


Fig. 7 Effect of tidal level and social environment on calling rate. Dots are means computed for the covariate water temperature mean and error bars are 95% confidence intervals

460 ability to sustain sonic muscle contraction close to their
461 physiological limit. Consistently, males of the nonpasserine
462 bird Brown Skuas that produce long difficult calls close to
463 their performance limit are honestly advertising a higher
464 breeding success (Janicke et al. 2008).

Sources of variability

465
466 Tide level had a significant effect in boatwhistle duration and
467 P2 pulse period, which showed lower values at low tide.
468 Consequently, vocal conspicuity was reduced at low tide as
469 the number of calling males (see “Material and methods”) and
470 sound duration decreased. A decrease of calling effort at low
471 tide is expected because low frequency sounds attenuate very
472 rapidly in low water levels and the chance of being detected
473 by distant females is reduced (Fine and Lenhardt 1983;
474 Mann 2006). Nevertheless, males that were calling at a high
475 rate showed a pronounced increase of the fundamental
476 frequency (the inverse of the pulse period) with low tide thus
477 increasing the chances of sound detection at low water levels
478 (Mann 2006). To the best of our knowledge, the effect of tide
479 levels on fish vocalisations has not been studied before, but
480 Barimo and Fine (1998) have mentioned that oyster toadfish
481 increase calling on incoming tide, suggesting that as other
482 intertidal fish, batrachoidids may have endogenous activity
483 rhythms related to tides with lowest levels of activity at low
484 tide (Gibson 1982). Future work should address the stability
485 of call rate and characteristics in Lusitanian toadfish males
486 that inhabit deeper areas and are less subject to the harsh
487 fluctuating physical environment of the intertidal area.

488 Social environment affected calling rate in the Lusitanian
489 toadfish and chorusing males called at higher rates. Accord-
490 ingly, calling rate is influenced by the vocal behaviour of
491 nearby male conspecifics in other batrachoidids (Winn 1967;
492 Fish 1972; Ramage-Healey and Bass 2005). For example,
493 Gulf toadfish males when experimentally placed in an active
494 calling environment started to vocalise within 48 h (Ramage-
495 Healey and Bass 2005). Males in a chorus probably benefit
496 from increased mate attraction, reduced assessment costs or
497 reduced predation risks, although experimental evidence
498 supporting these hypotheses is scarce (Gerhardt and Huber
499 2002). We have also observed that high-calling rate and
500 probably motivated Lusitanian toadfish males more to
501 produced longer boatwhistles than low-calling rate males
502 but only when singing in a chorus. Burmeister and
503 Wilczynski (2001) examined the influence of androgens on
504 calling behaviour in the presence and the absence of social
505 acoustic signals in male green treefrogs (*Hyla cinerea*) and
506 concluded that the influence of androgens on the motivation
507 to call depended on the social stimuli. It is likely also that in
508 the Lusitanian toadfish, androgens have a differential effect
509 in boatwhistle acoustic features, namely duration dependent
510 on social stimuli.

511 Male calling rate also had a significant effect on acoustic
 512 features of boatwhistles. High-calling rate males produced
 513 on average longer boatwhistles with higher fundamental
 514 frequencies. The higher calling rate and associated longer
 515 sound durations observed in the Lusitanian toadfish could be
 516 related to higher levels of circulating steroid hormones such
 517 as 11-ketotestosterone (11KT), which is a teleost-specific
 518 androgen (Remage-Healey and Bass 2006). Neurophysio-
 519 logical experiments have shown that steroid hormones exert a
 520 rapid and complex neuromodulatory effect in the activity of
 521 the vocal pattern generator and in the vocal behaviour of
 522 batrachoidids, modulating calling rate and call duration
 523 (Remage-Healey and Bass 2003, 2004, 2006). Also, nesting
 524 batrachoidid males have elevated 11 KT during vocal
 525 advertisement compared to non-calling periods (Knapp et
 526 al. 2001; Remage-Healey and Bass 2005). In addition,
 527 batrachoidid males presented higher calling rate, call
 528 duration and elevated levels in circulating 11KT levels after
 529 experiencing a territorial intrusion simulated by playback
 530 (Remage-Healey and Bass 2005). In this context, Lusitanian
 531 toadfish males with higher calling rates and longer sounds
 532 could reliably advertise high circulating androgen levels,
 533 which also mediates aggressive behaviour (social status) and
 534 reproduction (Remage-Healey and Bass 2006). Moreover,
 535 calling rate reflects male condition (body lipid content) in
 536 this species (Amorim et al. 2010), suggesting that calling rate
 537 and associated call features could be advertising male quality
 538 traits that are important for social status, territorial defence
 539 and possibly parental ability (Andersson 1994). From the
 540 female perspective, females that would choose males based
 541 on their vocal behaviour could both gain direct benefits (such
 542 as enhanced male parental care and better territories for the
 543 development of their offspring) and indirect (genetic)
 544 benefits (Andersson 1994).

545 In conclusion, our study has shown that the advertisement
 546 calls of the Lusitanian toadfish differ among individuals even
 547 when considering several days, but high stereotypy is found
 548 only in P2 pulse period when considering longer periods of
 549 time. Differences in this parameter could promote individual
 550 recognition and also advertise male quality if only some males
 551 can perform close the physiological limit. Our study has also
 552 produced evidence to support the acoustic plasticity hypoth-
 553 esis since calling rate seems to be one of the major factors
 554 influencing boatwhistle characteristics and could be informa-
 555 tive about male motivation and physiological condition (i.e.
 556 male quality). The social acoustic environment, on the other
 557 hand, seems to mainly influence calling rate (vocal facilita-
 558 tion). We suggest that absolute differences in calling rate and
 559 in call characteristics (mainly P2 pulse period, dominant
 560 frequency and sound duration), together with the differential
 561 way in which tide modulates boatwhistles according to male's
 562 motivation level may be used in male–male assessment and in
 563 mate choice by females.

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