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DOUTORAMENTO EM BIOLOGIA
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NOTA PRÉVIA

Para a elaboração da presente dissertação, e nos termos do nº1 Artigo 41, do regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no Diário da República nº 209, II Série de 30 de Outubro de 2006, foram usados integralmente seis (6) artigos científicos publicados, um (1) submetido e um (1) em preparação para publicação em revistas internacionais indexadas. Tendo os trabalhos mencionados sido efectuados em colaboração, o autor da dissertação esclarece que relativamente aos dois primeiros artigos apresentados, nomeadamente Chapter I (i) e (ii), participou na recolha e análise de dados, discussão dos resultados e redação dos manuscriptos. Relativamente aos restantes artigos, o autor esclarece que participou integralmente na sua concepção e execução, incluindo o delineamento das experiências, recolha e análise de dados, interpretação e discussão dos resultados, assim como na redação de todos os manuscriptos.

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À minha mãe, por todo o apoio, por acreditar sempre em mim.

Ao meu primo João, que nunca esquecerei...

"Fraco na caldeirada mas óptimo para a Ciência (...) Cientista portuguesa estudou um peixe do Tejo chamado xarroco e descobriu que ele usa uma linguagem sofisticada para comunicar. É exímio a ouvir e também a cantar."

- *Filomena Naves, Diário de Notícias, 9 Abril 2011.*

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RESUMO

A relação entre a emissão de sinais acústicos e o sucesso reprodutor é importante para compreender a evolução de sistemas que se baseiam fortemente na comunicação vocal e tem sido amplamente estudada em vários taxa, com excepção dos peixes. Por outro lado, a existência de padrões vocais individuais, potencialmente usados no processo de selecção sexual, tem sido pouco investigada neste taxon, apesar de peixes da família Batrachoididae revelarem diferenças vocais individuais relativamente elaboradas. No entanto, a função desta plasticidade vocal potencialmente mediadora do reconhecimento individual permanece muito pouco compreendida. Para além disso, estudos que examinam a produção de sons e a recepção auditiva, numa perspectiva comparativa entre espécies evolutivamente próximas, podem fornecer informações importantes para a compreensão da diversidade e evolução dos sistemas de comunicação.

A presente tese centrou-se no estudo do papel social da produção de sons e nas capacidades auditivas do xarroco *Halobatrachus didactylus* (Teleostei: Batrachoididae). Esta espécie baseia-se fortemente na comunicação acústica para a atracção de parceiros sexuais na época de reprodução e para a resolução de interacções agonísticas, exibindo um repertório acústico invulgarmente complexo para peixes. O xarroco foi usado como modelo de estudo para investigar os seguintes objectivos:

(1) determinar se vocalizações dos machos emitidas para atrair as fêmeas (sirenes) possibilitam o reconhecimento individual e podem ser potencialmente usadas pelas fêmeas para avaliar a qualidade do parceiro sexual; verificar a influência do comportamento vocal no sucesso reprodutor (número de ovos); testar a possível função dupla das sirenes em contextos de atracção sexual e agonísticos e verificar eventuais modificações dos seus parâmetros acústicos com o contexto social;

(2) determinar a sensibilidade auditiva através do registo dos potenciais auditivos ao nível das células sensoriais do sáculo (principal órgão auditivo em teleósteos) e verificar eventuais diferenças sazonais e inter-sexuais; avaliar a representação de sons naturais no sistema auditivo, nomeadamente vocalizações conspecíficas e estímulos ecologicamente relevantes (i.e. sons de um potencial predador e de uma espécie simpátrica);

(3) analisar o desenvolvimento da capacidade de produção de sons e da sensibilidade auditiva, i.e. do início da comunicação acústica; verificar se o desenvolvimento do sistema auditivo periférico (sensibilidade do sáculo) acompanha a diferenciação vocal.

A actividade vocal do xarroco foi registada em machos nidificantes numa zona intertidal no estuário do Tejo e, seguidamente, foram analisados os sons de atracção sexual (sirenes) relativamente a vários parâmetros acústicos. Todos os parâmetros analisados revelaram significativamente maior variabilidade inter-individual do que intra-individual. A frequência dominante e a modulação de frequência, seguidas do período entre pulsos, modulação de amplitude e duração total, foram as variáveis que melhor discriminaram diferentes machos. Estes resultados suportam a possibilidade de reconhecimento individual com base em características acústicas nesta espécie.

Posteriormente, procedeu-se à quantificação da massa dos músculos sónicos intrínsecos à bexiga gasosa (aparelho produtor de som) em vários espécimes, machos e fêmeas, bem como à medição de outros parâmetros morfométricos. A variabilidade presente na massa dos músculos sónicos foi explicada principalmente pelo comprimento total e condição em ambos os sexos. Como a massa dos músculos está relacionada fortemente com o desempenho vocal, estes resultados sugerem que as vocalizações do xarroco podem informar sobre a qualidade do emissor, informação crítica em contexto reprodutor e agonístico.

Finalmente, de forma a avaliar se o desempenho vocal condiciona o sucesso reprodutor, vários machos foram mantidos em condições semi-naturais no pico da época de reprodução em ninhos artificiais (que asseguraram a identidade dos espécimes monitorizados ao longo do estudo). A taxa máxima de canto e o esforço vocal (tempo despendido a cantar) foram os melhores preditores do sucesso reprodutor, avaliado pelo número de ovos presentes nos ninhos. Estes parâmetros vocais, por sua vez, encontraram-se correlacionados com o comprimento total e condição dos machos, indicando que o desempenho vocal condiciona fortemente o sucesso reprodutor e é informativo da qualidade do macho.

As sirenes dos batracoidídeos foram descritas até à data como sinais emitidos pelos machos para atracção das fêmeas e usados na sinalização passiva de ninhos na época reprodutora. No entanto, observações prévias apontam para a sua ocorrência fora da época de reprodução. Assim sendo, elaborou-se um desenho experimental para verificar se estes sinais são produzidos igualmente em contexto de defesa territorial, que deverá existir todo o ano. As experiências incluíram a colocação de machos residentes num tanque experimental e a libertação de machos intrusos, tendo-se procedido ao registo comportamental das interacções visuais agonísticas e dos sinais acústicos emitidos. Os machos residentes, contrariamente aos intrusos, emitiram sirenes agonísticas aquando da tentativa de ocupação dos ninhos por parte dos intrusos. As sirenes agonísticas foram emitidas em menor taxa, comparativamente com as sirenes de atracção sexual que são tipicamente emitidas em séries, com taxa mais constante e durante longos períodos de tempo. Ambos os sinais apresentaram duração e estrutura harmónica semelhantes, mas diferenças significativas na modulação de amplitude, frequência dominante e fundamental. Estas diferenças estão provavelmente relacionadas com a diferente taxa de emissão e com as exigências necessárias para a propagação dos sinais a distâncias diferentes do receptor.

Uma vez que o xarroco utiliza fortemente o canal acústico durante interacções sociais e exibe um repertório vocal complexo, é de prever que o seu sistema auditivo seja capaz de codificar características finas das suas vocalizações. No entanto, esta espécie não possui estruturas morfológicas para optimizar a audição. De forma a avaliar as capacidades auditivas do xarroco, foram registados os potenciais auditivos em populações de células sensoriais da mácula no sáculo do ouvido interno, em machos e fêmeas, dentro e fora da época de reprodução. Os audiogramas obtidos indicaram maior sensibilidade a baixas frequências inferiores a 205 Hz, onde se encontra a maior parte da energia dos sons conspecíficos. Também foi verificada a existência de elevada sensibilidade a frequências muito baixas como 15 Hz, o que sugere uma sobreposição da sensibilidade auditiva com a sensibilidade da linha lateral. Não foram encontradas diferenças auditivas sazonais e inter-sexuais. Estes resultados demonstram que o sistema auditivo periférico de fêmeas e machos do xarroco encontra-se bem adaptado para a detecção de vocalizações conspecíficas e de sons de frequências muito baixas durante todo o ano, o que certamente optimiza a detecção de predadores/presas e a presença de conspecíficos no meio natural. Contrariamente a outro batracoidídeo amplamente estudado (*Porichthys notatus*) que apenas revela actividade vocal no período reprodutor e cujas fêmeas sofrem alterações sensoriais para melhor detectarem os sons dos machos, *H. didactylus* comunica acusticamente todo o ano e possivelmente por isso a sua sensibilidade auditiva não sofre modificações sazonais.

Por outro lado, de forma a avaliar até que ponto o xarroco é capaz de integrar a complexidade das suas vocalizações, registaram-se os potenciais auditivos evocados (somatório da resposta neural sincronizada evocada por estimulação acústica) em machos e fêmeas. O sistema auditivo do xarroco demonstrou ser capaz de codificar parâmetros finos das suas vocalizações, nomeadamente de representar com elevada precisão a duração e estrutura pulsada dos sinais conspecíficos e ainda de resolver a

modulação de amplitude e o conteúdo espectral das sirenes. Adicionalmente, verificou-se que esta espécie é capaz de detectar outros estímulos ecologicamente relevantes, i.e. sons de um potencial predador como o golfinho-roaz corvineiro e sons de outro peixe simpátrico bastante vocal como a corvina.

Com base na mesma técnica electrofisiológica (somatório dos potenciais auditivos evocados), efectuou-se a medição da sensibilidade auditiva em diferentes tamanhos de juvenis e em adultos, tendo sido também gravados os seus sons agonísticos (tamborilados). A comparação dos vários audiogramas e conteúdos espectrais das vocalizações, permitiu verificar que a capacidade para comunicar acusticamente surge em etapas iniciais do desenvolvimento possivelmente quando os juvenis (> 5 cm de comprimento standard, CS) começam a ser capazes de produzir sons de maior amplitude e com frequências dominantes mais baixas. Por último, através do registo dos potenciais auditivos no sáculo do ouvido interno, foi possível verificar que o sistema auditivo periférico ainda não se encontra completamente desenvolvido em espécimens com menos de 5 cm CS e que o aumento da sensibilidade auditiva é acompanhado pela diferenciação vocal (aumento do repertório acústico) que acompanha o crescimento. Juvenis com mais de 5 cm CS, contrariamente aos mais pequenos, exibem uma sensibilidade auditiva já idêntica à dos adultos, bem como o repertório vocal completo.

Os trabalhos constituintes desta tese demonstram que o sistema comunicativo do xarroco é mais complexo do que inicialmente se encontrava descrito, sendo comparável nalguns aspectos à complexidade de sistemas vocais presente noutros taxa como anuros e aves. A demonstração da presença de assinaturas vocais, do papel fundamental do canto no sucesso reprodutor, da capacidade para codificar características complexas das vocalizações, do paralelismo entre o desenvolvimento dos sistemas auditivo e vocal num peixe teleósteo, constituiu certamente um avanço importante para a compreensão da diversidade e evolução dos sistemas de comunicação.

Palavras-chave: xarroco, comunicação acústica, produção de som, audição, ontogenia.

ABSTRACT

The existence of individual vocal signatures that can provide scope for mate choice has been scarcely investigated in fish. The relation between acoustic signalling and reproductive success has been studied in various taxa but likewise remains unclear in this taxon. Ultimately, studies that examine sound production and auditory reception, within a comparative perspective across species, can provide insights into the evolution of communication systems.

This thesis focused on the social role of acoustic signaling and hearing in the Lusitanian toadfish *Halobatrachis didactylus* (Batrachoididae), which relies heavily on acoustic communication. The goals were: (1) determine whether male advertising boatwhistles can potentially provide individual recognition and if the sonic muscle variability is related with males' quality; verify the influence of vocal behaviour in the reproductive success; test the possible function of boatwhistles in nest defence; (2) compare auditory sensitivity across seasons and between sexes; characterise the representation of vocalizations in the auditory system; and (3) analyze the development of acoustic communication.

Boatwhistles were different between males and the dominant frequency and frequency modulation were the parameters that best discriminate individuals. Sonic muscle variability was best explained by the body length and condition, suggesting that vocal output can inform about sender's quality. Reproductive success was significantly influenced by males' calling rate and calling effort. Besides mate attraction, boatwhistles were used during active territorial defence.

The inner ear saccule was well suited to detect conspecific vocalizations throughout the year and sensitivity was not seasonally plastic or sexually dimorphic. The auditory system was capable of resolving fine vocal features probably important in communication. Moreover, the ability to communicate acoustically might be absent in early developmental stages and initiates when juveniles start generating higher amplitude calls with lower dominant frequencies. Finally, the development of the peripheral auditory system seems to parallels vocal differentiation in this species.

Key-words: Lusitanian toadfish, acoustic communication, sound production, hearing, ontogeny.

CONTENT

GENERAL INTRODUCTION

1. ACOUSTIC COMMUNICATION IN FISH

The facts that fishes produce sounds and are able to detect acoustic signals are known for a long time. However, only after the second half of the XX century (Tavolga 1960) and, since the publication of Tavolga's (1964) and (1967) works, researchers started to have the appropriated equipment and to expand greatly the field of fish bioacoustics. Although more than 2000 years ago Pliny the Elder reported in the "History of the World" that fish can detect sounds, the concept that these animals can hear was only firstly established in the 1920s, with the publications of the Nobel Laureate Karl von Frisch (see reviews Ladich et al. 2006, Webb et al. 2008).

Although there is a raising number of publications on fish acoustic communication in the past years, this area remains relatively poorly studied. This contrasts to the well investigated communication in terrestrial animals, such as insects, anurans, birds and mammals (Bradbury and Vehrencamp 1998; Gerhardt and Huber 2002). The major reasons are probably related to technical problems that emerge while studying fishes in their natural environment, namely identification of the vocalizing specimens (Fay and Popper 1999; Zelick et al. 1999) and the impossibility to study the full acoustic repertoire in captivity in some species (e.g. deep-sea fishes). Due to these experimental constraints, literature has reported a limited number of vocal fish species, compared to what has been identified in anurans, for instance (Zelick et al. 1999). There are currently more than 30000 identified fish species, but only a small fraction has been investigated with reference to their ability to detect acoustic stimuli and to sound production capabilities. More than 800 species of 109 families are known to communicate acoustically, but this seems to be fairly far from the actual number (Ladich 2004).

1.1. UNDERWATER ACOUSTICS

Sound is a mechanical wave resulting from an oscillation (or periodical variation) of pressure through an elastic medium. Sound propagation velocity essentially depends on the density and elasticity of the medium, being five times higher in water than in air (1518 ms^{-1} and 343 ms^{-1}, respectively) – reviewed in Bass and Clark (2003).

Sound represents a very effective communication vehicle underwater due to its rapid and vast propagation. Moreover, communication based on acoustic signalling is especially important when visibility is impaired, which typically occurs in aquatic environments (Hawkins and Myrberg 1983). However, whether a sound can be detected depends on the source level, propagation loss, background masking noise, and the hearing threshold of the receiver (Mann 2006). Sound waves can be easily distorted due to reflection and/or refraction phenomena that typically occur in the presence of physical barriers characteristic of the natural environments. In shallow waters, such sound distortions are dramatic and the absorption coefficient rises considerably with the increasing distance between the sender and the receiver. Low frequency waves are rapidly attenuated, contrary to higher frequency signals (Fine and Lenhardt 1983; Mann 2006). Nevertheless, sound propagation of fish sounds has mostly been studied in shallow water, where many vocal species spawn and communicate acoustically. For bioacousticians, it has become easier to perform behavioural and neurophysiological studies using more accessible fish species. These studies generally found that sound pressure level falls off in the range of what would be expected for cylindrical-to-spherical spreading, but with a fair amount of variation (revised in Mann 2006).

There are several methods to detect sound, including measurements of acoustic pressure, using pressure-sensors as hydrophones, or measurements of displacement of water particles (as displacement, velocity, or acceleration), using particle motion sensors, such as geophone, laser vibrometer, anemometry, and video (revised in Higgs et al. 2005). Fishes can detect both particle acceleration and sound pressure. For detecting the latest sound component, fish need to have hearing morphological specializations (e.g. swimbladder connections with the inner ear) that function as pressure detectors converting the pressure into a particle motion signal, which can be detected by the inner ear (for details see 1.4) .

1.2. VOCAL VARIABILITY AND FUNCTION

Most vocal fishes produce low-frequency pulsed sounds that mainly vary in their temporal patterns, i.e. number of pulses, pulse intervals, repetition rate and duration (Winn 1964; Myrberg et al. 1978). Fishes are incapable of varying sound emissions based on air flow modulation, as described in birds and mammals, which restricts the sound variability to differences in amplitude and temporal patterns and limits frequency modulation (Ladich and Fine 2006; but see Amorim 2006). Although most documented fish species have restricted vocal repertoires of not more than one or two calls, some species exhibit relatively complex acoustic repertoires, with up to five different vocalizations (e.g. Crawford 1997; Amorim et al. 2008; Rice and Bass 2009). Moreover, fish appear to produce fewer calls than insects, anurans or birds, which typically produce thousands of calls per day (Zelick et al. 1999), probably due to physiological constraints of sound production underwater (Amorim et al. 2002; Thorson and Fine 2002).

Although vocal diversity found in fish may not be as rich as in other animals, acoustic information, lying mostly in the time domain, seems to provide sufficient scope for communication. Differences in vocal signalling may promote reproductive isolation between closely related sympatric species (e.g. Amorim et al. 2008); provide important information (motivation and/or quality) for mate choice (e.g. Winn 1972; Myrberg et al. 1986); be used to assess the fighting ability of opponents and to decide contests before they escalate to damaging combats (e.g. Ladich et al. 1992a; Ladich 1998); and allow identification of competitors (e.g. Myrberg and Riggio 1985). Sound production in fishes can be associated with distinct behavioural contexts, namely alarm (Hawkins 1993), agonistic interactions (Valinsky and Rigley 1981; Henglmüller and Ladich 1999; Ladich and Myrberg 2006), reproduction (Myrberg and Lugli 2006) and inter-specific (e.g. Myrberg and Spires 1972; Ladich et al. 1992b; Crawford et al. 1997; Amorim 2006) and intra-specific (e.g. Ladich et al. 1992b; Myrberg et al. 1993; Crawford et al. 1997; Thorson and Fine 2002) recognition.

Intraspecific vocal variability mostly relates to dominant frequency, although individual differences in duration, pulse period and amplitude modulation, have also been reported (see Amorim 2006). The inverse relation between dominant frequency and fish size has been described in several species that produce sounds with short repeated pulses, such as cichlids (e.g. Amorim et al. 2003), croaking gouramis (e.g. Ladich et al. 1992b), damselfish (e.g. Myrberg et al. 1993), mormyrids (e.g. Crawford et al. 1997) and sciaenids (e.g. Connaughton et al. 2000). For mate choice, the ability to assess the size of the vocalizing male is important because larger males are usually dominant, hold better territories or spawning sites, and have higher reproductive success (Oliveira et al. 1996). On the other hand, from the point of view of male-male interactions, acoustic signals may allow individuals to judge the opponent’s size and predict the fight outcome (Ladich 1998). The relationship between dominant frequency and fish size is mediated either by resonance of the swimbladder or other resonating structure associated to sound production or by scaling of the sonic muscles. Larger swimbladder or sonic muscles typically result in sounds with lower frequency (Connaughton et al. 2000).

The vocal variability described in fish is mostly associated with variation in both sound producing mechanisms and species-specific differences. Moreover, the social context, motivation level, social status, season, circadian activity, sexual dimorphism and ontogenetic development are additional sources of sound variability that may occur at the individual level (Amorim 2006).

1.2.1. Ontogenetic development of acoustic communication

As previously mentioned intra-specific variability in sound production may also result from differences in the developmental stage of an individual. Sound production is common among juvenile fishes mostly because competition for food and space may occur regardless of reproductive stage. Previous investigations showed that immature skunk loach, triglids, tigerperch, gouramis and catfishes are capable of producing sounds (Schneider 1964; Valinsky and Rigley 1981; Amorim and Hawkins 2005; Wysocki and Ladich 2001; Lechner et al. 2010).

Numerous studies have been conducted on the ontogenetic development of hearing and acoustic communication in mammals and birds (Dimitrieva and Gottlieb 1992; Dimitrieva and Gottlieb 1994; Podos et al. 1995; Ruben 1995; Reimer 1996; Moss et al. 1997; Branchi et al. 2001), but only a few have been carried out in fishes. Studies on the ontogeny of hearing in teleosts show varying results, ranging from no differences between two size groups of goldfish (Popper 1971) and various size groups of the zebrafish (Zeddies and Fay 2005), no change in absolute thresholds but expansion of the detectable frequency range in the zebrafish (Higgs et al. 2001; Higgs et al. 2003), up to an improvement of hearing abilities with size in the damselfish (Kenyon 1996), the Red Sea bream (Iwashita et al. 1999), the labyrinth fish *Trichopsis vittata* (Wysocki and Ladich 2001) and the batrachoidid midshipman fish (Sisneros and Bass 2005).

Whereas sound production clearly changes in all fish species investigated so far, no clear picture exists on whether auditory sensitivity changes during development and, more importantly, the relationship between development of hearing and sound production is almost unknown in this taxon (Wysocki and Ladich 2001). The study of ontogenetic development of hearing abilities and the relation to the development of the vocal motor output are still definitely open fields for bioacoustic researchers.

1.3. SOUND-GENERATING MECHANISMS

Fishes developed the largest diversity of sonic organs among vertebrates (Ladich and Fine 2006). Consequently, there is no general classification consensus for the different sound producing mechanisms. Most of the sound producing mechanisms have been classified as follows (Hawkins and Myrberg 1983; Hawkins 1993; Ladich 2003): (1) hydrodynamic action, which originates low frequency sounds resulting from the body movement through the aquatic environment; (2) stridulatory mechanisms, which are based on friction of skeletal elements such as teeth, fin rays and vertebrae, and generate broad-band sounds of higher dominant frequencies (> 1 kHz); and (3) contraction of specialized sonic muscles, allowing production of more complex vocalizations that can be pulsed and/or harmonic with most energy below 1 kHz.

More recently, Ladich and Fine (2006) suggested a classification based merely on sounds produced intentionally for communication, excluding those emitted during swimming, feeding, breathing, or gas exchange. The authors suggest a classification based on morphological structures that evolved exclusively for sound production. The main group comprises the swimbladder mechanisms and their several modifications. This groups is subdivided in intrinsic muscles (e.g. batrachoidids, dos Santos et al. 2000; triglids, Yabe 1985), and direct and indirect extrinsic muscles (e.g. Siluriformes, Fine and Ladich 2003; Characiformes, Ladich and Bass 2005). Sonic muscle contraction induces swimbladder vibration up to extremely high rates (50–250 Hz) (Ladich and Fine 2006). Low contraction rates generate low-frequency broad-band pulsed sounds, whereas high contraction rates result in low-frequency harmonic sounds, in which the fundamental frequency is defined by the sonic muscle contraction rate (e.g. batrachoidids, Fine et al. 2001). On the other hand, the second group comprises the pectoral mechanisms, which are extremely variable and include modifications in the pectoral fins and fin tendons. Sounds are generated through pectoral spine rubbing, pectoral tendon plucking and pectoral girdle vibrations (e.g. Siluriformes, Fine and Ladich 2003; anabantoides, Ladich et al. 1992b). According to Ladich and Fine (2006), sonic mechanisms become less clear when non-specialized morphological structures are involved, such as pharyngeal teeth or jaw teeth grating.

The motor vocal control described for fish has some similarities with that reported in amphibians, for instance (Greenfield 1994). The maintenance of vocal activity is achieved by pacemaker cells in the central nervous system responsible for controlling vocal periodicity (Bass and Baker 1990). The teleost *Porichthys notatus* (Batrachoididae) has been extensively used as a model system to investigate the neural circuitry underlying sound production (e.g. Bass and Baker 1990; Remage-Healey and Bass 2006). The sound generating apparatus in Batrachoididae consists in a pair of sonic muscles attached to the swimbladder walls. Each muscle is innervated by a single nerve formed by branches of the ventral occipital nerve roots that exit the hindbrain. The occipital nerves carry motor axons originating from two nuclei of vocal motoneurons extending along the midline of the caudal hindbrain (medulla oblongata) and rostral spinal cord. Intracellular recordings have identified vocal pacemaker neurons that are ventrolateral to the motoneurons. There is a direct relationship between the rhythmic, patterned output of a pacemaker motoneuron circuit and the physical attributes of vocalization (see Bass and Baker 1990).

1.4. SOUND DETECTION IN FISH

Acoustic communication implies that vocalizations can be detected and encoded by the receptor. Currently, only a small number of fish species have been investigated with reference to their ability to detect acoustic stimuli. However, approaches from comparative biology, through phylogenetic analysis for instance, can be used to estimate the number of fishes that are likely to be hearing specialists based on anatomical features. Namely, approximately 360 species of clupeiform fishes (e.g. herrings and shads) have an air bubble associated with the inner ear, and circa of 7800 species of otophysans (e.g. goldfish and catfishes), which represent more than two-thirds of freshwater fish species and more than 25% of all fish species, have a series of bones (Weberian ossicles) that mechanically connect the swimbladder to the inner ear (Webb et al. 2008). Moreover, literature reports that 26 other families of teleosts present hearing specializations (Braun and Grande 2008). Altogether, these evidences point to a remarkable importance of the hearing sense in this taxon.

The diversity of structure and function of sensory systems in fish is exceptional, and suggests that, along the evolutionary process, species have found ways to become more adapted and gather more information about their highly diverse environments.

This diversity is particularly evident in the octavolateralis system of fishes that include information from both systems, i.e. the inner ear and the lateral line (Braun and Grande 2008).

The initial idea for the origin of the ear in fish suggested that this structure evolved from the lateral line - acousticolateralis hypothesis (reviewed in Popper et al. 1992). Recent investigations, and particularly those using modern anatomical and physiological methods, suggest that the ear and lateral line may have shared a common ancestor but these are two completely distinct systems (Popper et al. 1992).

Few data are presently available that help us answer questions related to the evolution of the auditory system. In fact, the study of auditory systems can probably provide more information related to the evolution of sensory systems than any other vertebrate senses. There is information about evolutionary changes in the ear lying in the fossil record, which is not available for any other sensory system (e.g. Clack 1997). Also, the comparative data obtained from different levels of the vertebrate auditory system, from periphery to central nervous system, is far the richest. Moreover, the ear may have evolved multiple times (Fritzsch 1992), providing considerable comparative information to evaluate evolution of this system.

Sounds can be detected and encoding with respect to their temporal patterns, amplitude and spectral content, which are known to be represented in the auditory system of fishes (Wysocki and Ladich 2003). The frequency range of most sound energy is generally coincident with the frequency range of higher hearing sensitivity (Myrberg and Spires 1980; Stabentheiner 1988; Crawford 1997; Ladich and Yan 1998; McKibben and Bass 1999). However, a mismatch has also been documented in freshwater fishes that present hearing morphological specializations (Ladich and Yan 1998; Ladich 1999, 2000). In these studies, authors investigated freshwater species that inhabit low noise environments and suggested that the auditory skills have evolved independently of vocalizations, and the selective pressure acting on the evolution of the hearing structures and sound features probably did not evolved to optimize acoustic communication, but instead to detect predators and prey.

1.4.1. Hearing mechanisms

The inner ear of fish presents multiple functions, i.e. orientation in the gravity field, sensitivity to acceleration and sound detection. The main auditory endorgans of these animals are the otoliths present in the inner ear that are composed by chambers and canals full of endolymph. Fish possess three spatially oriented otolithic organs – utricle, saccule, and lagena, which are connected by semicircular canals (Hawkins, 1993; Popper and Fay 1999 - see Figure 1). Each of these contains a sensory epithelium (macula), composed by mechanoreceptor sensory hair-cells that convey the information to the neural afferents, and a calcareous otolith that attaches to the epithelium via a gelatinous otolitic membrane (Hawkins 1993; Lu and Popper 1998).

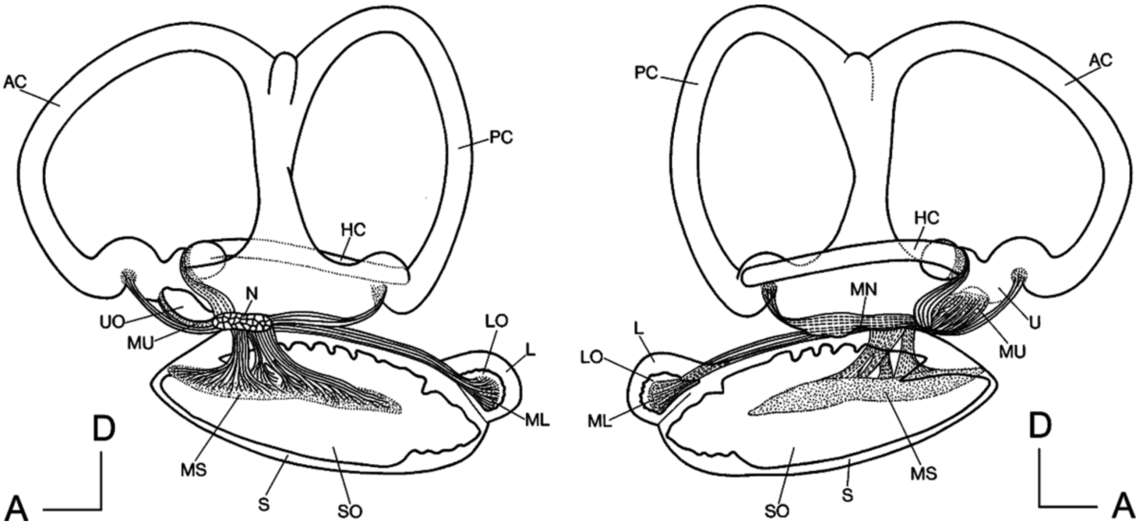

Figure 1 - Inner ear of a teleost fish (perch). Medial view on the left and lateral view on the right. AC, HC, PC, anterior, horizontal, and posterior semicircular canals; L, lagena; LO, lagena otolith; MN, macula (papilla) neglecta; MU, utricular epithelium; MS, saccular epithelium; N, eighth cranial nerve; S, saccule; SO, saccular otolith; UO, utricular otolith. (Popper and Schilt 2008)

The otolithic organs function as biological accelerometers sensitive to particle motion due to the difference in inertia between the sensory macula and the associated otolith (Popper and Fay 1999). The structural similarity of the otolithic organs suggests that all three may play a role in hearing. The saccule has been described as the most important auditory endorgan in most teleost fishes (reviewed in Wysocki 2006), although auditory sensitivity of utricle and lagena has been also reported in a few species (e.g. Popper and Fay 1999; Lu et al. 2003; Lu et al. 2004). In fact, in clupeid fishes a portion of the utricle is connected to an air-filled chamber (auditory bulla), suggesting a function in sound pressure detection (Denton et al. 1979; Blaxter et al. 1981). Moreover, Fay (1984) showed directional responses of utricular fibers in the goldfish and, more recently, Lu et al. (2004) demonstrated that the utricle as well as the lagena of a fish without hearing specializations (sleeper goby *Dormitator latifrons*) responds similar to the saccule in terms of frequency, but at stimulus levels above saturation for most saccular units. According to these authors, the utricle and lagena may serve an auditory role in directional processing (due to the horizontal orientation of the utricle and the vertical orientation of the lagena) and by extending the hearing dynamic range (Lu et al. 2004). One hypothesis, suggested by Edds-Walton and Fay (2008), is that the utricle may provide feedback for self-generated vocalizations, as saccule saturates at high amplitude sound levels.

Auditory information is transferred from the otolithic endorgans into the several hindbrain auditory nuclei and then ascends to the midbrain torus semicircularis, and then to forebrain processing regions (reviewed in McCormick 1992, 1999). While the encoding properties of auditory primary afferents are relatively well studied in many fishes (Popper and Fay 1999), less is known about how acoustic signals are encoded in higher processing centers of the fish brain (Maruska and Tricas 2009).

Fish present hearing variability mostly because of the presence of accessory hearing structures in some species. Teleost fish have been classified, according to the hearing sensitivity and the presence of such structures, in specialists, generalists and intermediates (Hawkins and Myrberg 1983; Hawkins 1993; Ladich and Popper 2004). Specialists have been characterized as presenting the morphological specializations that consist in vesicles full of gas or bone structures (Weberian ossicles) connected to anterior part of the swimbladder, which may be connected or close to the inner ear. Vibrating the wall of such cavity causes air pressure fluctuations that are rapidly transmitted to the inner ear and, consequently, hearing sensitivity is enhanced. This group of hearing-specialized fishes is sensitive to sound pressure up to several kHz (e.g. mormyrids, anabantoids, cyprinids, Crawford 1997; Ladich 1999; Scholz and Ladich 2006). On the other hand, generalists such as batrachoidids and salmonids that lack accessory hearing structures essentially respond to the kinetic component of the sound waves at low frequencies (below 1 kHz) and at high amplitudes (Fish and Offut 1972; Hawkins 1993). The third group that comprises species with intermediate auditory skills also sensitive to sound pressure may have, in turn, extensions of the swimbladder that are not connected but are close to the inner ear (e.g. gadids and pomacentrids, Chapman and Hawkins 1973; Myrberg and Spires 1980). However, investigators working in the field of fish audition have recently claimed that such classification should not be adopted as a continuous scale should rather be closer to the reality (Popper, personal communication).

In order to analyse hearing abilities in fish, most researchers have been using artificial stimuli, such as pure tones, tone bursts and clicks. Studies on fish audition have mostly focused on how the sound spectra fit to the audiograms in terms of dominant frequencies of sounds versus best hearing frequencies (e.g. Ladich and Yan 1998; Ladich 1999), but a few other works analysed different auditory aspects, such as temporal resolution abilities (e.g. Wysocki and Ladich 2002) and noise masking effects (e.g. Vasconcelos et al. 2007). Some of the acoustic stimuli used (e.g. sinusoidal amplitude-modulated tones, beats or click trains) approached the characteristics of natural sounds in some fish species (e.g. Bodnar and Bass 1997; McKibben and Bass 1998), but still do not reflect the natural acoustic signals that animals actually confront in their environments. So far, natural conspecific sounds have only been used in a very few investigations (Wysocki and Ladich 2001; Maruska and Tricas 2009).

2. LUSITANIAN TOADFISH, A MODEL SPECIES FOR STUDYING ACOUSTIC COMMUNICATION

Representatives of the family Batrachoididae (Teleostei, Actinopterygii), which include toadfishes and the plainfin midshipman fish, have emerged as one of the main study models for both behavioural and neurobiological studies in fish acoustic communication (Bass and MacKibben 2003). Vocal diversity associated with different social contexts has been described in several fish families (e.g. Amorim et al. 2004; Gerald 1971; Lobel 2001; Malavasi et al. 2008). However, Batrachoididae probably exhibits the most complex patterns of call structure so far shown for any fish family, which includes complex long tonal advertising sounds (reviewed in Amorim 2006).

The Lusitanian toadfish, *Halobatrachus didactylus* (Block & Schneider 1801) - Figure 2a, is a member of the Batrachoididae family, which has recently become a remarkable model for studying the function role of acoustic signals and the importance of acoustic communication in various social contexts. Phylogenetic analysis indicated that Lusitanian toadfish represents a basal lineage in the Batrachoididae (Rice and Bass

2009 – see Figure 2b), providing an excellent model for understanding integrated mechanisms underlying the evolution of acoustic communication in fishes.

Halobatrachus didactylus occurs in subtropical regions, along the Northeastern Atlantic and in the Mediterranean (Roux 1986). This sedentary benthic species is mostly found in estuaries and coastal shallow waters (up to 50 m depth) living in sand and mud substrates (Roux 1986). This fish species often inhabits shallow waters, where visual communication is often limited, and probably as a consequence relies heavily on acoustic signalling to interact with conspecifics throughout life and to advertise nests and attract mates in the breeding season.

During the reproductive season, from May to July in Portugal (Modesto and Canário 2003a), territorial males ("type I") build nests in aggregations in shallow waters under rocks or in crevices. Reproductive nesting males produce a long-distance advertisement call, the boatwhistle, forming conspicuous choruses to attract females to spawn (dos Santos et al. 2000). Females deposit their eggs in the roof of the nest where they attach by an adhesive disk and are guarded by the male until the offspring are free-swimming (Roux 1986; personal observations). Like other batrachoidids, this species presents sexual polymorphism with another male morphotype, "type II" (or sneaker), which is smaller, with higher gonadsomatic index but smaller sonic muscles (Modesto and Canário 2003a,b) that parasite the nests to attempt opportunistic fertilizations.

According to dos Santos et al. (2000) and Amorim et al. (2008), Lusitanian toadfish exhibits a rich vocal repertoire rare among fishes that comprises at least five different vocalizations. At least three sounds, namely grunt call, croak and double-croak, are likely associated with agonistic contexts (dos Santos et al. 2000), and the complex amplitude-modulated call (boatwhistle) is known to play an important role in mate attraction in batrachoidids (Gray and Winn 1961; Winn 1967; Fish 1972; McKibben and Bass 1998). The boatwhistle is mostly produced in the breeding season in the Lusitanian toadfish (Amorim et al. 2006) and preliminary observations suggest the existence of intra-specific variability that is probably important for assessment between males and mate choice by females. However, how such vocal variation shapes social communication remains unknown. In addition, it is still unclear whether the auditory system of this species, that lacks accessory hearing structures, is capable of perceiving such acoustic complexity.

Finally, the Lusitanian toadfish is highly tolerant to physiological experiments, displays the full acoustic repertoire in captivity and mates in constrained semi-natural experimental situations (personal observations). This important feature allowed addressing important questions in the past years that required electrophysiological recordings for long periods of time and manipulation of specimens in the field.

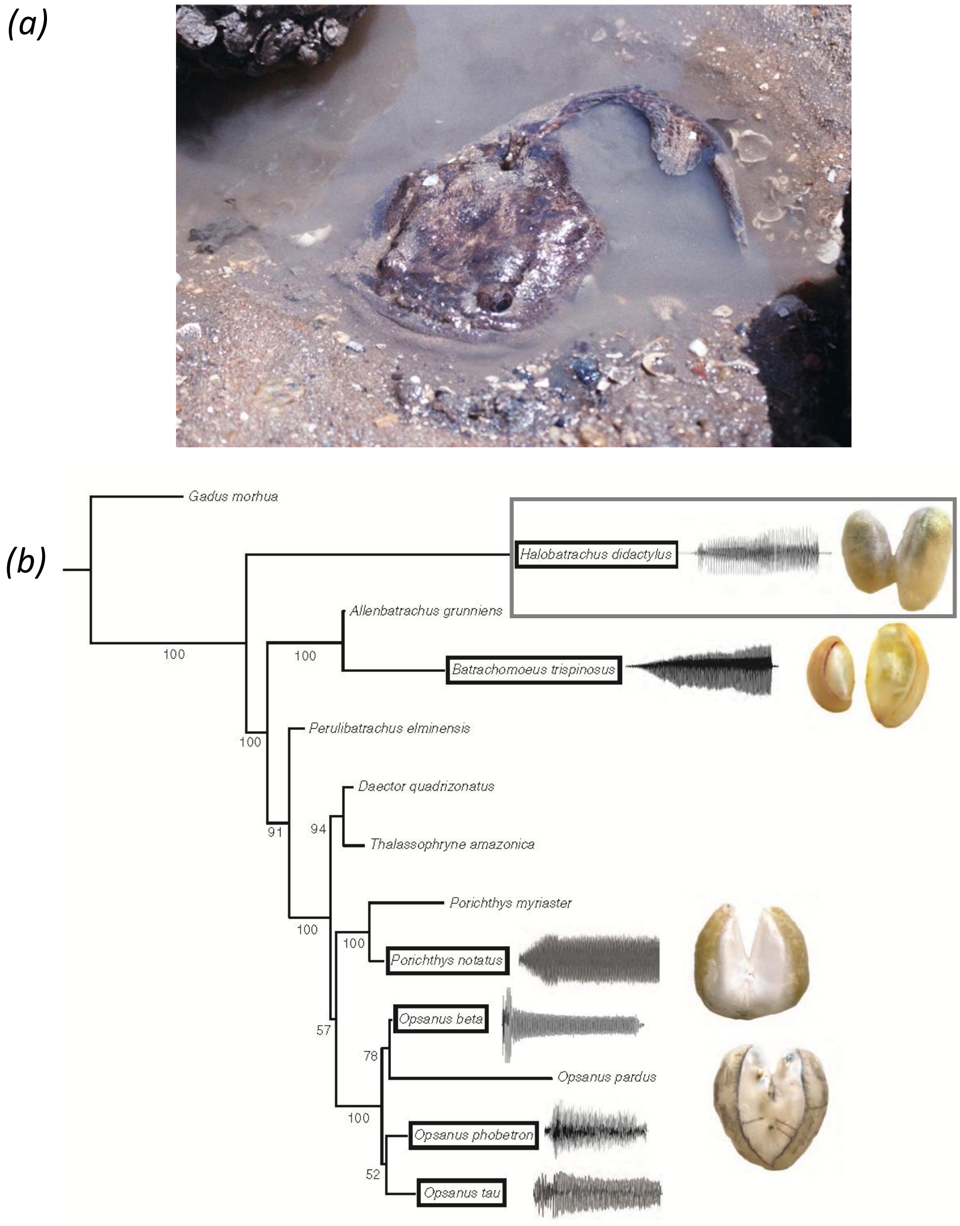

Figure 2 – (a) Lusitanian toadfish *Halobatrachus didactylus*, Tagus estuary, Montijo, Portugal; (b) Phylogenetic relationships of Batrachoididae species, generated from a maximum likelihood analysis of genes from available sequences in GenBank (16S; 28S; cytochrome oxidase subunit I, COI; cytochrome b, CytB). Branch lengths are drawn proportional to the amount of character change. Bootstrap values are shown next to nodes. The gadid, *Gadus morhua*, was used as an outgroup for the Batrachoididae. Boxes around species names indicate the most studied species in acoustic communication within this family. A representative waveform of the species' advertising call and the sound generating apparatus (swimbladder) is depicted for each taxon. Adapted from Rice and Bass (2009).

4. OBJECTIVES AND THESIS STRUCTURE

The existence of individual characteristics in vocal signals is also well known in various animals, such as anurans and birds (Bee et al. et al., 2001; Christie et al., 2004), but has been poorly studied in fishes (Amorim, 2006). The most common intraspecific variation in fish acoustic signals is an inverse relation of dominant frequency with body size (Myrberg & Riggio, 1985; Myrberg et al., 1993). More elaborated multi-featured individual differences in fish calls occur in Batrachoididae, but whether they mediate individual recognition remains unclear.

On the other hand, the relation between acoustic signalling and reproductive success is important to understand the evolution of vocal communication systems and has been well studied in several taxa (e.g. Searcy and Andersson 1986; Kroodsma and Byers 1991; Nordby et al. 1999; White et al. 2010). Teleost fishes may represent the largest group of sound-producing vertebrates that have evolved a variety of mechanisms to produce vocalizations crucial during social interactions including mate attraction (Ladich and Myrberg 2006; Myrberg and Lugli 2006). However, a link between characteristics of vocal behavior and reproductive success has never been straightly shown in fishes.

Moreover, studies that examine sound production and auditory reception, within a comparison perspective across closely-related species, can provide valuable insights into the diversity and evolution of communication systems.

In this thesis, the Lusitanian toadfish was used as a model species to investigate these topics and other more species-specific questions. Although this species exhibits an unusually large vocal repertoire for fish, the importance of such vocal plasticity remains uninvestigated. Also, whether the auditory system of this species is adapted to detect low frequencies and to encode fine features of complex acoustic signals needs to be addressed.

The present thesis focused on the social role of acoustic signaling and auditory abilities of *H. didactylus*. More specifically, the following questions were addressed:

- CHAPTER I:

I. (i) Do mate advertising calls (boatwhistles) represent individual acoustic signatures that could potentially provide individual recognition?

I. (ii) Do sonic muscles, which control the vocal output, correlate to fish quality (size, condition)?

I. (iii) Can vocal performance influence attraction of conspecifics and reproductive success?

I. (iv) Is there a dual function of the toadfish advertising call (boatwhistles), as this call type is also produced during the non-breeding season?

- CHAPTER II:

II. (i) Is this species' peripheral auditory sensitivity well adapted to detect the low frequency components of its vocal repertoire? Does the auditory sensitivity show sexual dimorphism and/or seasonal plasticity?

II. (ii) How well are the fine features of conspecific vocalizations represented in the auditory system? Is this species also adapted to detect other ecologically relevant signals from the acoustic environment (e.g. predator, sympatric species)?

- CHAPTER III:

III. (i) How does sound production and hearing sensitivity change during development? When is the onset of acoustic communication?

III. (ii) How does the vocal repertoire develop throughout ontogeny? Does the peripheral auditory sense parallel vocal differentiation?

References

Amorim, M.C.P. (2006). Diversity of sound production in fish, in: Ladich, F., Collin, S.P., Moller, P., Kapoor, B.G. (Eds.), Communication in Fishes, vol. 1. Science Publishers, Enfield, U.S.A., pp. 71-104.

Amorim, M.C.P., McCracken, M.L. and Fine, M.L. (2002). Metabolic costs of sound production in the oyster toadfish, *Opsanus tau*. Can. J. Zool. 80, 830-838.

Amorim, M.C.P., Fonseca, P.J. and Almada, V.C. (2003). Sound production during courtship and spawning of *Oreochromis mossambicus*: male-female and male-male interactions. J. Fish Biol. 62, 658-672.

Amorim, M.C.P., Knight, M.E., Stratoudakis, Y. and Turner, G.F. (2004). Differences in sounds made by courting males of three closely related Lake Malawi cichlid species. J. Fish Biol. 65, 1358-1371.

Amorim, M.C.P., Simões, J.M. and Fonseca, P.J. (2008). Acoustic communication in the Lusitanian toadfish, *Halobatrachus didactylus*: evidence for an unusual large vocal repertoire. J. Mar. Biol. Assoc. UK 88, 1069-1073.

Bass, A.H. and Baker, R. (1990). Sexual dimorphisms in the vocal control system of a teleost fish: Morphology of physiologically identified neurons. J. Neurobiol. 21, 1155-1168.

Bass, A.H. and McKibben, J.R. (2003). Neural mechanisms and behaviors for acoustic communication in teleost fish. Prog. Neurobiol. 69, 1-26.

Bass, A.H. and Clark, C. (2003). The physical acoustics of underwater sound communication, in: Simmons, A.M., Fay, R. and Popper A. (Eds.) Acoustic Communication. Springer Handbook of Auditory Research. Springer-Verlag, New York, U.S.A., pp. 15-64.

Bass, A.H. and Ladich, F. (2008). Vocal-acoustic communication: from neurons to behavior, in: Webb, J.F., Fay R.R., Popper, A.N. (Eds.), Fish bioacoustics. Springer Handbook on Auditory Research, New York, U.S.A. pp. 253-278.

Bee, M.A., Kovich, C.E., Blackwell, K.J. and Gerhardt, H.C. (2001). Individual variation in advertisement calls of territorial male green frogs, *Rana clamitans*: implications for individual discrimination. Ethology 107, 65-84.

Blaxter, J.H.S., Denton, E.J. and Gray, J.A.B. (1981). The auditory bullae swimbladder system in late stage hearing larvae. J. Mar. Biol. Assoc. UK 61, 315-326.

Bodnar, D.A. and Bass, A.H. (1997). Temporal coding of concurrent acoustic signals in the auditory midbrain. J. Neurosci. 17, 7553-7564.

Bradbury, J.W. and Vehrencamp, S.L. (1998). Principles of Animal Communication. Sinauer Associates, Massachussets., U.S.A.

Branchi, I., Santucci, D. and Alleva, E. (2001). Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. Behav. Brain Res. 125, 49-56.

Braun, C.B. and Grande, T. (2008). Evolution of peripheral mechanisms for the enhancement of sound reception., in: Popper, A.N., Fay, R.R., Webb, J.L. (Eds.). Springer-Verlag, New York, U.S.A., pp. 99-144.

Chapman, C.J. and Hawkins, A.D. (1973). A field study of hearing in the cod, *Gadus morhua*. J. Comp. Physiol. A 85, 147-167.

Christie, P.J., Mennill, D.J. and Ratcliffe, L.M. (2004). Chickadee song structure is individually distinctive over long broadcast distances. Behaviour 141, 101-124.

Clack, J.A. (1997). The Evolution of Tetrapod Ears and the Fossil Record. Brain Behav. Evol. 50, 198-212.

Connaughton, M.A., Taylor, M.H. and Fine, M.L. (2000). Effects of fish size and temperature on weakfish disturbance calls: implications for the mechanism of sound generation. J. Exp. Biol. 203, 1503-1512.

Crawford, J.D. (1997). Hearing and acoustic communication in mormyrid electric fishes. Mar. Fresh. Beh. Phy. 29, 65-86.

Crawford, J.D., Cook, A.P. and Heberlein, A.S. (1997). Bioacoustic behavior of African fishes (Mormyridae): potential cues for species and individual recognition in *Pollimyrus*. J. Acous. Soc. Am. 102, 1200-1212.

Denton, E.J., Gray, J.A.B. and Blaxter, J.H.S. (1979). The mechanics of the clupeid acousticolateralis system: frequency responses. J. Mar. Biol. Assoc. UK 59, 27-47.

Dimitrieva, L.P. and Gottlieb, G. (1992). Development of brainstem auditory pathway in mallard duck embryos and hatchlings. J. Comp. Physiol. A 61, 19-28.

Dimitrieva, L.P. and Gottlieb, G. (1994). Influence of auditory experience on the development of brain stem auditory-evoked potentials in mallard duck embryos and hatchlings. Behav. Neurol. Biol. 61, 19-28.

dos Santos, M.E., Modesto, T., Matos, R.J., Grober, M.S., Oliveira, R.F. and Canário, A. (2000). Sound production by the Lusitanian toadfish, *Halobatrachus didactylus*. Bioacoustics 10, 309-321.

Edds-Walton, P.L. and Fay, R.R. (2008). Directional and frequency response characteristics in the descending octaval nucleus of the toadfish (*Opsanus tau*). J. Comp. Physiol. A 194, 1013-1029.

Fay, R.R. (1984). The goldfish ear codes the axis of acoustic particle motion in three dimensions. Science 225, 951-954.

Fay, R.R. and Popper, A. (1999). Hearing in Fishes and Amphibians: An Introduction, in: Fay, R., Popper, A. (Eds.), Comparative Hearing: Fish and Amphibians. Springer-Verlag, New York, U.S.A., pp. 1-14.

Fine, M.L. and Lenhardt, M.L. (1983). Shallow-water propagation of the toadfish mating call. Comp. Biochem. Physiol. A 76, 225-231.

Fine, M.L., Malloy, K.L., King, C.B., Mitchell, S.L. and Cameron, T.M. (2001). Movement and sound generation by the toadfish swimbladder. J. Comp. Phys. A 187, 371-379.

Fine, M.L. and Ladich, F. (2003). Sound production, spine locking and related adaptations, in: Arratia, G., Kapoor, B.G., Chardon, M., Diogo, R. (Eds), Catfishes. Science Publ., Enfield, U.S.A., pp. 249-290.

Fish, J.F. (1972). The effect of sound playback on the toadfish, in: Winn, H.E., Olla, B. (Eds.), Behavior of Marine Animals. Plenum Press, New York, U.S.A., pp. 386-434.

Fish, J. and Offutt, C. (1972). Hearing Thresholds from Toadfish, *Opsanus tau*, measured in the laboratory and field. J. Acous. Soc. Am. 51, 1318-1321.

Fritsch, B. **(**1992). The Evolution of the Amphibian Auditory System. Wiley**,** New York.

Gerald, J.W. (1971). Sound production during courtship in six species of sunfish (Centrarchidae). Evolution 25, 75-87.

Gerhardt, C. and Huber, H. (2002). Acoustic Communication in Insects and Anurans: common problems and diverse solutions. The University of Chicago Press, Chicago and London.

Gray, G.A. and Winn, H.E. (1961). Reproductive ecology and sound production of the toadfish *Opsanus tau*. Ecology 28, 274-282.

Greenfield, M.D. (1994). Synchronous and alternating choruses in insects and anurans: common mechanisms and diverse functions. Am. Zool. 34, 605-615.

Hawkins, A.D. (1993). Underwater sound and fish behaviour, in: Pitcher, T. (Ed.), Behaviour of Teleost Fishes, Chapman and Hall, London, U.K., pp. 129-169.

Hawkins, A.D. and Myrberg, A.A. (1983). Hearing and sound communication under water, in: Lewis, B. (Ed.), Bioacoustics: A Comparative Approach. Academic Press, London, U.K., pp. 347-405.

Henglmüller, S.M. and Ladich, F. (1999). Development of agonistic behaviour and vocalization in croaking gouramis. J. Fish Biol. 54, 380-395.

Higgs, D.M., Souza, M.J., Wilkins, H.R., Presson, J.C. and Popper, A.N. (2001). Age- and size related changes in the inner ear and hearing ability of the adult zebrafish (*Danio rerio*). JARO 3, 174-184.

Higgs, D.M., Lui, Z. and Mann, D.A. (2005). Hearing and Mechanoreception, in: Evans, D.H. (Ed.) The Physiology of Fishes. CRC Press, Boca Raton, U.S.A., pp. 391-429.

Iwashita, A., Sakamoto, M., Kojima, T., Watanabe, Y. and Soeda, H. (1999). Growth effects on the auditory threshold of Red Sea bream. Nip. Suis. Gak. 65, 833-838.

Kenyon, T.N. (1996). Ontogenetic changes in the auditory sensitivity of damselfish (Pomacentridae). J. Comp. Physiol. A 179, 553-561.

Kihslinger, R.L. and Klimley, A.P. (2002). Species identity and the temporal characteristics of fish acoustic signals. J. Comp. Psychol. 116, 210-214.

Kroodsma D.E. and Byers B.E. (1991). The function(s) of bird song. Am. Zool. 31, 318-328.

Ladich, F. (1998). Sound characteristics and outcome of contests in male croaking gouramis (Teleostei). Ethology 104, 517-529.

Ladich, F. (1999). Did auditory sensitivity and vocalization evolve independently in otophysan fishes? Brain Behav. Evol. 53, 288-304.

Ladich, F. (2000). Acoustic communication and the evolution of hearing in fishes. Phil. Trans. Roy. Soc. L. B 355, 1285-1288.

Ladich, F. (2003). Sound Production and Acoustic Communication, in: Von der Emde, G., Mogdans, J., Kapoor, B. (Eds.), The Senses of Fishes. Narosa Publishing House, New Delhi, India, pp. 210-230.

Ladich, F. (2004). Sound production and acoustic communication, in: van der Emde,G., Mogdans, J., Kapoor, B.G. (Eds.), The senses of fishes: adaptations for the reception of natural stimuli. Narosa Publishing House, New Delhi, India, pp. 210–230.

Ladich, F., Brittinger, W. and Kratochvil, H. (1992a). Significance of agonistic vocalization in the croaking gourami (*Trichopsis vittatus*, Teleostei). Ethology 90, 307-314.

Ladich, F., Bischof, C., Schleinzer, G. and Fuchs, A. (1992b). Intra- and interspecific differences in agonistic vocalization in croaking gouramis (Genus: *Trichopsis*, Anabantoidei, Teleostei). Bioacoustics 4, 131-141.

Ladich, F. and Yan, H.Y. (1998). Correlation between auditory sensitivity and vocalization in anabantoid fishes. J. Comp. Phys. A 182, 737-746.

Ladich, F. and Popper, A.N. (2004). Parallel evolution in fish hearing organs, in: Manley, G., Popper, A.N., Fay, R.R. (Eds.), Evolution of the Vertebrate Auditory System. Springer-Verlag, New York, U.S.A., pp. 95-127.

Ladich, F. and Bass, A.H. (2005). Sonic/vocal motor pathways in piranhas (family Characidae) with a comparison to other teleosts. Brain Behav. Evol. 66, 167-176.

Ladich, F. and Myrberg, A.A. (2006). Agonistic behavior and acoustic communication, in: Ladich, F., Collin, S.P., Moller, P., Kapoor, B.G. (Eds.), Communication in Fishes, Vol. 1. Science Publishers, Enfield, U.S.A., pp. 121-148.

Ladich, F., Collin, S.P., Moller, P. and Kapoor, B.G. (2006). Communication in Fishes, Vol. 1. Science Publishers, Enfield, U.S.A.

Ladich, F. and Fine, M.L. (2006). Sound-generating mechanisms in fishes: a unique diversity in vertebrates, in: Ladich, F., Collin, S.P., Moller, P., Kapoor, B.G. (Eds.), Communication in Fishes, Vol. 1. Science Publishers, Enfield, U.S.A., pp. 1-43.

Lechner, W., Wysocki, L.E. and Ladich, F. (2010). Ontogenetic development of auditory sensitivity and sound production in the squeaker catfish *Synodontis schoutedeni*. BMC Biol. 8, 10.

Lobel, P.S. (2001). Acoustic behavior of cichlid fishes. J. Aquaricult. Aquat. Sci. 9, 89-108.

Lu, Z. and Popper, A.N. (1998). Morphological polarizations of sensory hair cells in the three otolithic organs of a teleost fish: fluorescent imaging of ciliary bundles. Hear. Res. 126, 47-57.

Lu, Z., Xu, Z. and Buchser, W.J. (2003). Acoustic response properties of lagenar nerve fibers in the sleeper goby, *Dormitator latifrons*. J. Comp. Physiol. A 189, 889-905.

Lu, Z., Xu, Z. and Buchser, W.J. (2004). Coding of acoustic particle motion by utricular fibers in the sleeper goby, *Dormitator latifrons*. J. Comp. Physiol. A 190, 923-938.

Malavasi, S. Collatuzzo, S. and Torricelli, P. (2008). Interspecific variation of acoustic signals in Mediterranean gobies (Perciformes, Gobiidae): comparative analysis and evolutionary outlook. Biol. J. Linn. Soc. 93, 763-778.

Mann, D.A. (2006). Propagation of fish sounds, in: Ladich, F., Collin, S.P., Moller, P., Kapoor, B.G. (Eds.), Communication in Fishes, Vol. 1. Science Publishers, Enfield, U.S.A., pp. 107-120.

Mann, D.A. and Lobel, P.S. (1998). Acoustic behavior of the damselfish *Dascyllus albisella*: behavioral and geographic variation. Environ. Biol. Fish. 51, 421-428.

Maruska, K.P. and Tricas, T.C. (2009). Encoding properties of auditory neurons in the brain of a soniferous damselfish: reponse to simple tones and complex conspecific signals. J. Comp. Physiol. A 195, 1071-1088.

McCormick, C.A. (1992). Evolution of central auditory pathways in anamniotes, in: Webster, D.B., Fay, R.R., Popper, A.N. (Eds.) The evolutionary biology of hearing. Springer-Verlag, New York, U.S.A., pp. 323-350.

McCormick, C.A. (1999). Anatomy of the central auditory pathways of fish and amphibians. in: Fay, R.R., Popper, A.N. (Eds.) Comparative hearing: fish and amphibians. Springer, New York, U.S.A., pp. 155-217.

McKibben, J.R. and Bass, A.H. (1999). Peripheral encoding of behaviorally relevant acoustic signals in a vocal fish: single tones. J. Comp. Physiol. A 184, 563-576.

Modesto, T. and Canário, A.V.M. (2003a). Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, *Halobatrachus didactylus*. Gen. Comp. End. 131, 220-231.

Modesto, T. and Canário, A.V.M. (2003b). Hormonal control of swimbladder sonic muscle dimorphism in the Lusitanian toadfish *Halobatrachus didactylus*. J. Exp. Biol. 206, 3467-3477.

Moss, C.F., Redish, D., Gounden, C. and Kunz, T.H. (1997). Ontogeny of vocal signals in the little brown bat, *Myotis lucifugus*. Anim. Behav. 54, 131-141.

Myrberg, A.A. and Spires, J.Y. (1972). Sound discrimination by the bicolor damselfish, *Eupomacentrus partitus*. J. Exp. Biol. 57, 727-735.

Myrberg, A.A., Spanier, E. and Ha, S.J. (1978). Temporal patterning in acoustic communication, in: Reese, E.S., Lighter, F.J. (Eds.) Constrasts in Behavior. John Wiley & Sons, New York, U.S.A., pp. 137-179.

Myrberg, A.A. and Spires, J.Y. (1980). Hearing in damselfishes: an analysis of signal detection among closely related species. J. Comp. Physiol. A 140, 135-144.

Myrberg, A.A. and Riggio, R.J. (1985). Acoustically mediated individual recognition by a coral reef fish (*Pomacentrus partitus*). Anim. Behav. 33, 411-416.

Myrberg, A.A., Mohler, M. and Catala, J. (1986). Sound production by males of a coral reef fish (*Pomacentrus partitus*): its significance to females. Anim. Behav. 24, 923-933.

Myrberg, A.A., Ha, S.J. and Shamblott, M.J. (1993). The sounds of the bicolor damselfish (*Pomacentrus partitus*): predictors of body size and a spectral basis for individual recognition and assessment. J. Acous. Soc. Am. 94, 3067-3070.

Myrberg, A.A. and Lugli, M. (2006). Reproductive Behavior and Acoustical Interactions, in: Ladich, F., Collin, S.P., Moller, P., Kapoor, B.G. (Eds.), Communication in Fishes, vol. 1. Science Publishers, Enfield, U.S.A., pp. 149-176.

Nordby, J.C., Campbell, S.E. and Beecher, M.D. (1999). Ecological correlates of song learning in song sparrows. Behav. Ecol. 10, 287-297.

Oliveira, R.F. and Almada, V.C. (1996). Dominance hierarchies and social structure in captive groups of the Mozambique tilapia *Oreochromis mossambicus* (Teleostei: Cichlidae). Ethol. Ecol. Evol. 8, 39-55.

Parmentier, E., Lagardere, J.P., Vandewalle, P. and Fine, M.L. (2005). Geographical variation in sound production in the anemonefish *Amphiprion akallopisos*. Proc. R. Soc. B 272, 1697-1703.

Podos, J., Sherer, J.K., Peters, S. and Nowicki, S. (1995). Ontogeny of vocal tract movements during song production in song sparrows. Anim. Behav. 50, 1287-1296.

Popper, A.N. (1971). The effects of size on auditory capacities of the goldfish. J. Aud. Res. 11, 239-247.

Popper, A.N., Platt, C. and Edds, P. (1992). Evolution of the vertebrate inner ear: an overview of ideas, in: Webster, D.B., Fay, R.R., Popper, A.N. (Eds.) Comparative evolutionary biology of hearing. Springer, New York, U.S.A., pp. 49-57.

Popper, A.N. and Fay, R. (1999). The auditory periphery in fishes, in: Fay, R., Popper, A.N. (Eds.), Comparative Hearing: Fish and Amphibians. Springer-Verlag, New York, U.S.A., pp. 43-55.

Popper, A.N. and Schilt, C.R. (2008). Hearing and acoustic behavior (basic and applied). in: Webb, J.F., Fay, R.R., Popper, A.N. (Eds.). Fish Bioacoustics. Springer, New York, U.S.A., pp. 17-48.

Price, J.J. and Lanyon, S.M. (2002). Reconstructing the evolution of complex bird song in the oropendolas. Evolution 56, 1514-1529.

Reimer, K. (1996). Ontogeny of hearing in the marsupial, *Monodelphis domestica*, as revealed by brainstem auditory evoked potentials. Hear. Res. 92, 143-150.

Rémage-Healey, L. and Bass, A.H. (2006). From social behavior to neural circuitry: Steroid hormones rapidly modulate advertisement calling via a vocal pattern generator. Horm. Behav. 50, 432-441.

Rice A.N. and Bass, A.H. (2009) Novel vocal repertoire and paired swimbladders of the three-spined toadfish, *Batrachomoeus trispinosus*: insights into the diversity of the Batrachoididae. J. Exp. Biol. 212, 1377-1391.

Roux, C. (1986). Batrachoididae, in: Whitehead, P.J.P., Bauchot, M.L., Hureau, J.C., Nielsen, J., Tortonese, E. (Eds.), Fishes of the North-Eastern Atlantic and Mediterranean. Unesco, Paris, France, pp.1360-1361.

Ruben, R.J. (1995). The ontogeny of human hearing. Int. J. Ped. Otor. 32, 199-204.

Ryan, M.J. (1986). Factors influencing the evolution of acoustic communication: biological constraints. Brain Behav. Evol. 28, 70-82.

Schneider, H. (1964). Physiologische und morphologische Untersuchungen zur Bioakustik der Tigerfische (Pisces, Theraponidae). Z. Vergl. Physiol. 47, 493-558.

Scholz, K. and Ladich, F. (2006). Sound production, hearing and possible interception under ambient noise conditions in the topmouth minnow *Pseudorasbora parva*. J. Fish Biol. 69, 892-906

Searcy, W.A. and Andersson, M. (1986). Sexual selection and the evolution of song. Annu. Rev. Ecol. Syst. 17, 507-533.

Sisneros, J.A. and Bass, A.H. (2005). Ontogenetic changes in the response properties of individual, primary auditory afferents in the vocal plainfin midshipman fish *Porichthys notatus* Girard. J. Exp. Biol. 208, 3121-3131.

Stabentheiner, A. (1988). Correlations between hearing and sound production in piranhas. J. Comp. Physiol. A 162, 67-76.

Tavolga, W. (1960). Sound Production and Underwater Communication in Fishes, in: Lanyon, W., Tavolga, W. (Eds.), Animal Sound and Communication. Intelligencer Printing Company, Washington, U.S.A., pp. 93-136.

Tavolga, W. (1964). Marine Bio-Acoustics. Pergamon Press. Oxford, U.K.

Tavolga, W. (1967). Marine Bio-Acoustics II. Pergamon Press. Oxford, U.K.

Tavolga, W. (1971). Sound production and detection, in: Hoar, W.S., Randall, D.J. (Eds.). Fish Physiology, Vol. V. Academic Press, New York, U.S.A., pp. 135-205.

Thorson, R.F. and Fine, M.L. (2002). Crepuscular changes in emission rate and parameters if the boat whistle advertisement call of the gulf toadfish, *Opsanus beta*. Environ. Biol. Fishes 63, 321-331.

Valinski, W. and Rigley, L. (1981). Function of Sound Production by the Skunk Loach *Botia horae* (Pisces Cobitidae). Zeit. Tierpsy. 55, 161-172.

Vasconcelos, R.O., Amorim, M.C.P. and Ladich, F. (2007). Effects of ship noise on the detectability of communication signals in the Lusitanian toadfish. J. Exp. Biol. 210, 2104-2112.

Webb, F., Popper, A.N. (2008). Fish Bioacoustics. Springer, New York, U.S.A.

White, D.J., King, A.P., West, M.J., Gros-Louis, J. and Tuttled, E.M. (2010). Effects of singing on copulation success and egg production in brown-headed cowbirds, *Molothrus ater*. Behav. Ecol. 21, 211-218.

Winn, H. (1967). Vocal facilitation and the biological significance of toadfish sounds, in: Tavolga, W. (Ed.) Marine Bio-Acoustics 2, Pergamon Press, Oxford, U.K. pp. 281-304.

Winn, H. (1972). Acoustic discrimination by the toadfish with comments on signal systems, in: Winn, H.E., Olla, B.L. (Eds.). Behavior of Marine Animals Vol. 2. Plenum Press, New York, U.S.A., pp. 361-385.

Winn, H.E., Marshall, J.A. and Hazlett, B. (1964). Behavior, diel activities, and stimuli that elicit sound production and reactions to sounds in the longspine squirrelfish. Copeia 1964, 413-425.

Wysocki, L.E. (2006). Detection of Communication Sounds, in: Ladich, F., Collin, S.P., Moller, P., Kapoor, B.G. (Eds.), Communication in Fishes. Science Publishers, Enfield, U.S.A., pp. 177-205.

Wysocki, L.E. and Ladich, F. (2001). The ontogenetic development of auditory sensitivity, vocalisation and acoustic communication in the labyrinth fish *Trichopsis vittata*. J. Comp. Physiol. A 187, 177-187.

Wysocki, L.E. and Ladich, F. (2002). Can fishes resolve temporal characteristics of sounds? New insights using auditory brainstem response. Hear. Res. 169, 36-46.

Wysocki, L.E. and Ladich, F. (2003). The representation of conspecific sounds in the auditory brainstem of teleost fishes. J. Exp. Biol. 206, 2229-2240.

Yabe, M. (1985). Comparative osteology and myology of the subfamily Cottoidea (Pisces: Scorpaeniformes), and its phylogenetic classification. Memoirs of the Faculty of Fisheries, Hokkaido University 32, 1-130.

Zeddies, D.G. and Fay, R.R. (2005). Development of the acoustically evoked behavioral response in zebrafish to pure tones. J. Exp. Biol. 208, 1363-1372.

Zelick, R., Mann, D. and Popper, A. (1999). Acoustic Communication in Fishes and Frogs, in: Fay, R., Popper, A. (Eds.), Comparative Hearing: Fish and Amphibians. Springer-Verlag, New York, U.S.A., pp. 363-411.

CHAPTER I

VOCAL BEHAVIOUR AND FUNCTION OF ACOUSTIC SIGNALS

(i) **Variability in the mating calls of the Lusitanian toadfish *Halobatrachus didactylus*: cues for potential individual recognition**

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Variability in the mating calls of the Lusitanian toadfish *Halobatrachus didactylus*: cues for potential individual recognition

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The mating sounds (boatwhistles) of nesting batrachoidid *Halobatrachus didactylus* males were recorded in the Tagus Estuary from piers. Thirteen males with 16 boatwhistles per fish were analysed for 20 acoustic features. All variables showed larger between-male than within-male variation and differed significantly among individuals. Discriminant function analyses (DFA) considering seven of these variables assigned 90–100% of boatwhistles to the correct individual, depending on the number of males and number of sounds per male included in the model. The acoustic features that consistently best discriminated individuals were the dominant frequency of the middle tonal segment of the boatwhistle (P_2) and dominant frequency modulation, followed by P_2 pulse period, amplitude modulation and sound duration. These results suggest the possibility of individual recognition based on acoustic cues.

Key words: acoustic communication; Batrachoididae; individuality; signal variability; sound production.

INTRODUCTION

The ability to discriminate between individuals or groups of individuals is important for the establishment of social relations and implies individual distinctiveness (Bradbury & Vehrencamp, 1998). Individuality in acoustic signalling (vocal signatures) arises when the within-individual variation is smaller than the variation between individuals in one or more acoustic characteristics or when individuals differ in the presence or absence of particular vocal features (Beecher, 1989; Bee *et al.*, 2001). Individual identification through vocal signatures can mediate kin recognition (Jouventin *et al.*, 1999), territorial neighbour recognition (Bee & Gerhardt, 2001), mate-pair recognition (Speirs & Davis, 1991) and true individual recognition (Sayigh *et al.*, 1999).

The existence of individual characteristics in vocal signals is well known in various groups of animals including mammals, birds and amphibians (Bee

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*Author to whom correspondence should be addressed. Tel.: +351 218811700; fax: +351 218860954; email: amorim@ispa.pt *et al.*, 2001; Christie *et al.*, 2004) but has been poorly studied in fishes (Amorim, 2006). The most common intraspecific variation in fish sounds is an inverse relation of dominant frequency with fish size that may mediate individual recognition based on size information (Myrberg & Riggio, 1985; Myrberg *et al.*, 1993). More elaborate (multi-featured) individual differences in fish sounds occur in Batrachoididae (toadfishes; Barimo & Fine, 1998; Edds-Walton *et al.*, 2002; Thorson & Fine, 2002*a*, *b*; Fine & Thorson, 2008) and Mormyridae (weakly electric fishes; Crawford *et al.*, 1997; Lamml & Kramer, 2006). In both families, territorial males rely on their advertisement calls to attract females in turbid waters or at night (Winn, 1967; Crawford *et al.*, 1997). Additionally, these calls are involved in male–male competition (Winn, 1967; Remage-Healy & Bass, 2005). It has been suggested that mating calls may promote individual recognition in these animals. For example, differences in waveform, sound duration and distribution of energy in different harmonic bands allow clear identification of different male toadfishes [*Opsanus tau* (L., 1766) and *Opsanus beta* (Goode & Bean, 1880)] recorded through passive acoustics (Edds-Walton *et al.*, 2002; Thorson & Fine, 2002*a*).

Despite the clear indication of the existence of complex acoustic signals in batrachoidids and mormyrids that may involve individual recognition, there is to date no detailed statistical analysis of individual variation in fish sounds. The goal of the present study is to describe in detail the boatwhistles of nesting Lusitanian toadfish *Halobatrachus didactylus* (Bloch & Schneider) males and to determine which signal properties may potentially mediate individual recognition. A comparison of the intra- with inter-male variability in 20 acoustic features was made. Multivariate statistics were used to identify the best variables to discriminate between individuals. A preliminary study has shown that different nesting *H. didactylus* males can be recognized by ear and easily identified through inspection of the spectrogram and oscillogram of their mating sounds (Amorim *et al.*, 2006). Moreover, this species has an unusual large acoustic repertoire for fishes, consisting of at least five distinct sound types (dos Santos *et al.*, 2000; Amorim *et al.*, 2008), suggesting it has a complex acoustic communication system. This study provides a basis for future playback experiments in order to test for individual recognition among nesting males and support the use of acoustic cues in mate attraction and choice.

MATERIALS AND METHODS

STUDY SPECIES

Halobatrachus didactylus is an eastern Atlantic member of the Batrachoididae that occurs in estuaries and coastal lagoons (Roux, 1986). During the reproductive season, that lasts in Portugal from May to July (Modesto & Canário, 2003), breeding males defend nests under rocks in shallow water. Nesting males use an advertisement call (the boatwhistle) to attract females that results from the contraction of sonic muscles attached to the swimbladder (dos Santos *et al.*, 2000). Spawning females attach their eggs to the roof of a nest and leave the area, while the resident males provide parental care until the young are free-swimming (Roux, 1986; dos Santos *et al.*, 2000). As in other batrachoidids, a second type of male with different morphometric and endocrine characteristics is thought to use a sneaking strategy for mating (Brantley & Bass, 1994).

These type II males have larger testes, smaller sonic muscle mass and lower levels of 11-ketotesterone than nesting males (Modesto & Canário, 2003).

Since territorial males nest close together, boatwhistles are emitted in choruses resulting in a very conspicuous acoustic output (dos Santos *et al.*, 2000). In the peak of the breeding season, a small aggregation of males vocalizing close to the hydrophone can reach an average of 30 boatwhistles min^{-1} (Amorim *et al.*, 2006).

The boatwhistle is a tonal multi-harmonic sound lasting *c.* 800 ms (Amorim *et al.*, 2006). The fundamental frequency is *c.* 60 Hz [H1; Fig. 1(b)] and the dominant frequency is typically either the second or the fourth harmonic (Fig. 1) (Amorim *et al.*, 2006).

RECORDING AND ACOUSTIC ANALYSIS OF BOATWHISTLES

Several recording sessions lasting from 5 to 10 min were carried out during the mating season in July 2001 and July 2002 in two areas within the Tagus Estuary, Portugal: Montijo (38°42′ N; 8°58′ W) and Barreiro (38°39′ N; 9°04′ W). These areas had been previously identified as *H. didactylus* breeding areas (Amorim *et al.*, 2006). Moreover, nesting males were also observed to call in nests exposed at low spring tides at these locations (pers. obs.). During recording periods, water temperature ranged between 21–22° C. A hydrophone [High Tech 94 SSQ (High Tech Inc., Gulfport, MS, U.S.A.), with a sensitivity of -165 dB re 1 V μPa^{-1}, flat frequency response from 30

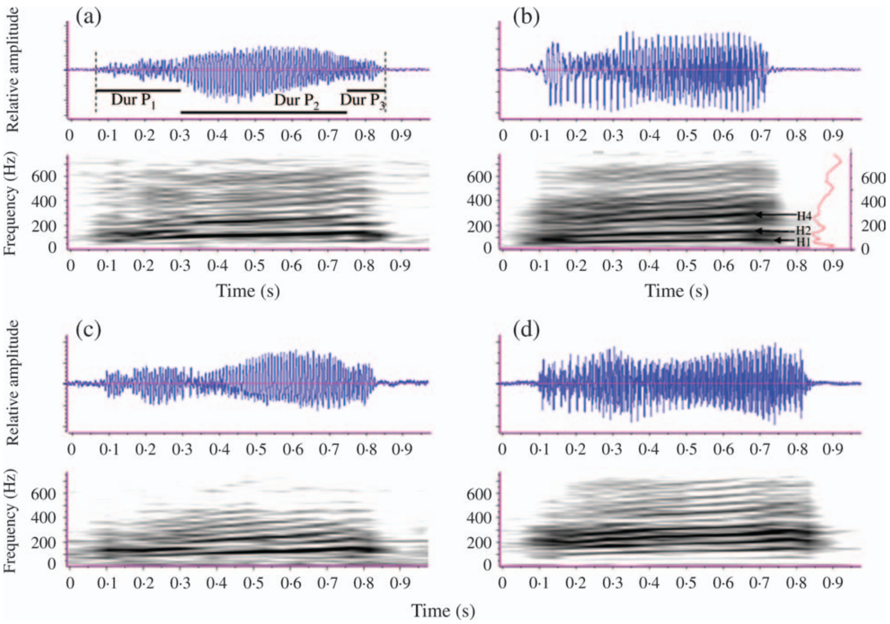

FIG. 1. (a), (b), (c) and (d) Oscillograms and sonograms of boatwhistles emitted by four nesting *Halobatrachus didactylus* males. The middle tonal phase of a boatwhistle (P_2) dominant frequency coincides with the second harmonic (H2) in (a), (b) and (c), and with the fourth harmonic (H4) in male (d), which are multiples of the fundamental frequency (H1). A power spectrum of phase 2 is given for male (b). Dur P_1, Dur P_2 and Dur P_3 duration of phases 1, 2 and 3 of the boatwhistle. The dotted line depicts total boatwhistle duration. Sampling frequency 44 kHz; FFT size 8192 points; Hamming window.

Hz to 6 kHz ± 1 dB] was lowered from piers in these two locations in several sites where acoustic activity was evident. The hydrophone was *c.* 150–200 mm above the substratum. Recording sites within the same pier were at least 4 m apart and each recording session was made from a different site. Water depth varied approximately between 2 and 5 m depending on tide.

Sounds were recorded on tape (Sony TCD-D8, 44 kHz, 16 bit resolution; Sony, Tokyo, Japan) and the analogue output of the recorder was digitized with a similar sampling frequency and resolution to a computer with a sound capture device (Edirol UA-5; Roland, Osaka, Japan). Sound files were analysed with Raven 1.2.1 for Windows (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Cornell, NY, U.S.A.). A total of seven recording sessions were considered. Sound analysis was restricted to the males calling close to the hydrophone that presented a high signal to noise ratio (SNR) (mean = 29 dB; the minimum SNR considered was of 18 dB) and a maximum of three individuals was considered per recording session. Only one recording session was considered per male. Distinction of different individuals between years was assured by considering males from different location, *i.e.* Montijo in 2001 ($n = 7$) and from Barreiro in 2002 ($n = 6$). Distinction of different males in the same recording session was based on differences in waveform envelope and relative sound amplitude that reflected the distance of the calling male to the hydrophone (an example is depicted in Fig. 2). As expected, relative sound amplitude of a particular male remained constant throughout a recording session since nesting males are stationary for long periods (dos Santos *et al.*, 2000), especially in the peak of the breeding season when territories are already established and males call to attract females while caring for their young (Barimo & Fine, 1998; Knapp *et al.*, 1999). To ensure that the distance between recording sites (minimum of 4 m) sufficed to prevent considering a male twice, boatwhistles were played back at the recording locations and recorded simultaneously at different distances from the speaker with similar gains. The playback audio chain consisted of a laptop computer, an amplifier (Phoenix Gold QX 4040, Portland, OR, U.S.A.) and a speaker (Electrovoice UW-30; Lubell Labs Inc. Columbus, OH, U.S.A.) placed 150 mm above the substratum. Played-back sounds were recorded with a second laptop computer, a sound capture device (Edirol UA25; Roland) and three hydrophones (High Tech 94 SSQ) placed *c.* 150–200 mm above the substratum and at 0·5, 1·5 and 4 m from the speaker. Amplitude of sound playback was determined by recording a male in a closed nest with the same recording settings as during recordings of sound playback. The nest was naturally occupied by the male and its entrance was closed with a plastic mesh that allowed prey items to enter the nest but prevented the subject male from abandoning the nest during recordings. The acoustic energy fell off very rapidly (21 dB loss from 0·5 m to 4 m from the speaker) and at 4 m away from the speaker, the boatwhistle could hardly be distinguished from the background noise (Fig. 3). The marked sound attenuation observed in the study sites, typical of shallow waters (Fine & Lenhardt, 1983), strongly suggests that the males considered

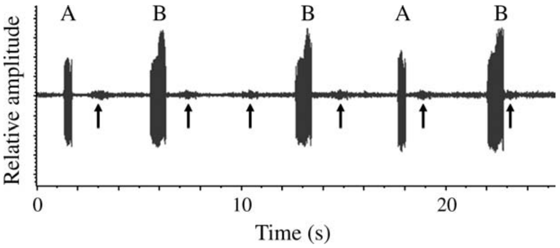

FIG. 2. Example of boatwhistles produced by two male *Halobatrachus didactylus* (A and B) that can be distinguished by the waveform envelope and relative sound amplitude, *i.e.* distance from the hydrophone. Arrows point at background boatwhistles.

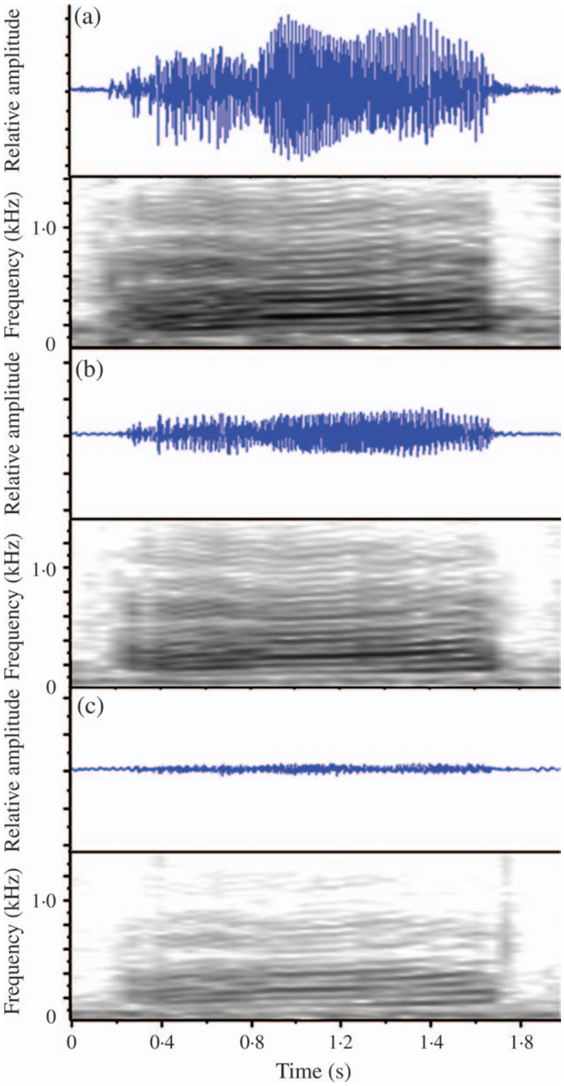

FIG. 3. Oscillograms and sonograms of a *Halobatrachus didactylus* boatwhistle played back by an underwater speaker recorded at (a) 0·5, (b) 1·5 and (c) 4 m away from the source. The acoustic energy of boatwhistles suffered an average attenuation of 21 dB from 0·5 to 4 m from the speaker and at 4 m could hardly be distinguished from the background noise. Sampling frequency 44 kHz; fast fourier transform (FFT) size 8192 points; Hamming window.

in the present study are distinct individuals. Other studies have used similar criteria to the present study to identify unseen toadfishes in sound recordings (Edds-Walton *et al.*, 2002; Thorson & Fine, 2002*a*, *b*) and, in one occasion, male identity was confirmed through diving (Barimo & Fine, 1998).

A total of 13 males with 16 boatwhistles per fish was analysed for 20 acoustic features. The classification used by dos Santos *et al.* (2000) that considers three distinct phases in the boatwhistle [beginning (P_1), middle (P_2) and end (P_3)] was adopted. These three phases differ in pulse period and dominant frequency (dos Santos *et al.*, 2000), with the pulse period typically decreasing and the dominant frequency increasing from phases 1–3 (see Table I). The identification of these phases was also based on differences in sound amplitude (Fig. 1) and fine waveform structure (Fig. 4). The following acoustic variables were measured: sound duration (ms), measured from the start of the first pulse (when acoustic energy appears above the background noise) to the end of the last pulse [Fig. 1 (a)]; duration of the segments P_1, P_2 and of P_3 (ms) [Fig. 1 (a)]. Relative P_2 duration was calculated by dividing the duration of P_2 by the total sound duration and was expressed as a percentage; pulse period in P_1, P_2 and P_3 (ms), calculated as the average peak-to-peak interval between six consecutive pulse units in the middle of each segment, except in P_3 that considered the whole segment (Fig. 4); number of pulses in P_1, P_2, P_3 and the total number of pulses in the whole sound; dominant frequency (Hz), *i.e.* the frequency with maximum energy, was determined in P_1, P_2, P_3 and in the entire sound. Fundamental frequency was calculated as the inverse of the average pulse period measured in P_1 and P_2. In batrachoidids, the fundamental frequency of the mating signals is determined by the rate of contraction of the sonic muscles attached to the swimbladder (Skoglund, 1961; Fine *et al.*, 2001). These measurements were confirmed with the power spectra [Fig. 1(b)] and were preferred to measuring the fundamental frequency directly because in many fish this frequency band had little energy. Dominant frequency modulation was calculated by dividing P_1 by P_2 dominant frequencies and fundamental frequency modulation was calculated in a similar way; amplitude modulation was similarly calculated by dividing the mean amplitude (RMS) measured for the P_1 segment by the one measured for the P_2 segment; RMS amplitude is a measurement native to Raven software. Time to maximum amplitude was measured from the start of the first pulse to the sound peak amplitude; this is also a measurement native to Raven software.

Temporal variables were measured from oscillograms and the dominant frequencies from power spectra [fast fourier transform (FFT) size 8192 points; Hamming window].

STATISTICAL ANALYSIS

Mean ± S.D. values were calculated for the above 20 acoustic features for all males. Overall means, S.D. and range values were subsequently calculated using each male mean values for each variable. In order to compare between-male with within-male variability for each acoustic feature the within-male coefficient of variance (C.V._{w} = S.D.:mean) was calculated and compared with the between-male coefficient of variation (C.V._{b}). The C.V._{b} was obtained by dividing the overall S.D. by the respective overall mean. The ratio $\text{C.V.}_{\text{b}}:\text{C.V.}_{\text{w}}$ was calculated to obtain a measure of relative between-male variability for each boatwhistle feature. When this ratio assumes values larger than one, it suggests that an acoustic feature could be used as a cue for individual recognition (Bee *et al.*, 2001; Christie *et al.*, 2004). Kruskal–Wallis analysis was used to test for differences between males for each acoustic variable. Non-parametric statistics were preferred to parametric ANOVAs due to the lack of homoscedasticity of variances.

Discriminant function analysis (DFA) was carried out using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, U.S.A.) as a multivariate tool to determine which acoustic features best discriminate between males. DFA also gives a measure of discrimination accuracy by revealing the percentage of sounds assigned to the correct individual. Only seven of the 20 acoustic variable were considered for the DFA: total sound duration, relative P_2 duration, P_2 pulse period, P_2 dominant frequency, dominant frequency modulation, fundamental frequency modulation and amplitude modulation. These variables were chosen because they were uncorrelated, had a $\text{C.V.}_{\text{b}}:\text{C.V.}_{\text{w}}$ ratio larger than one and presented a low C.V._{w} ($\leq 0{\cdot}1$; Table I). To assess the predictive accuracy of the models obtained, a cross-validation method ('leave-one-out') was carried out. In this method each sound is classified by the discriminant functions derived by the $n - 1$ remaining sounds. Because the H. didactylus emits boatwhistles in aggregations of different sizes, further DFA were performed to explore the variation of classification success with fish

TABLE I. Means, ± S.D., range, within-male variability (C.V.$_w$) and between-male variability (C.V.$_b$) for the 20 acoustic features analysed from 13 *Halobatrachus didactylus* males with 16 sounds each. P_1, P_2 and P_3 are the initial, middle and end segments in the boatwhistle (after dos Santos *et al.*, 2000)

Acoustic variables	Overall mean ± S.D. (range)	C.V.$_w$ (mean)	C.V.$_w$ (range)	C.V.$_b$	C.V.$_b$:C.V.$_w$	H[a]
Sound duration (ms)	767·2 ± 168·9 (458·0–1052·4)	0·10	0·05–0·20	0·22	2·23	155·61
P_1 duration (ms)	268·1 ± 39·3 (201·8–346·8)	0·09	0·04–0·16	0·15	1·71	149·99
P_2 duration (ms)	431·7 ± 118·0 (243·9–670·8)	0·17	0·06–0·33	0·27	1·62	140·06
P_3 duration (ms)	67·5 ± 42·0 (0·0–147·1)	0·59	0·10–4·00	0·62	1·05	160·55
Relative P_2 duration (%)	55·6 ± 5·0 (44·3–65·0)	0·09	0·03–0·15	0·09	1·03	96·17
Number of pulses P_1	12·6 ± 3·0 (8·6–18·4)	0·11	0·05–0·18	0·24	2·11	167·44
Number of pulses P_2	26·5 ± 6·6 (15·1–37·6)	0·17	0·07–0·33	0·25	1·51	139·80
Number of pulses P_3	4·6 ± 3·2 (0·0–11·4)	0·67	0·20–4·00	0·70	1·04	158·03
Total number of pulses	43·6 ± 10·4 (24·4–61·1)	0·11	0·06–0·24	0·24	2·09	158·28
Pulse period P_1 (ms)	20·6 ± 1·3 (18·6–22·2)	0·03	0·01–0·06	0·06	1·98	161·25
Pulse period P_2 (ms)	16·3 ± 0·9 (14·8–17·9)	0·03	0·01–0·05	0·05	1·79	148·97
Pulse period P_3 (ms)	13·0 ± 4·2 (4·3–22·0)	0·17	0·03–0·39	0·33	1·88	91·34[b]
Dominant frequency P_1 (Hz)	169·3 ± 56·5 (70·0–259·8)	0·07	0·00–0·17	0·33	4·83	189·16
Dominant frequency P_2 (Hz)	185·9 ± 67·1 (113·0–264·7)	0·08	0·00–0·37	0·36	4·67	170·93
Dominant frequency P_3 (Hz)	197·0 ± 66·9 (123·8–307·9)	0·14	0·04–0·44	0·34	2·47	133·68[b]
Dominant frequency boatwhistle (Hz)	182·8 ± 67·6 (91·1–265·0)	0·10	0·00–0·40	0·37	3·55	161·58
Dominant frequency modulation	0·96 ± 0·20 (0·65–1·20)	0·12	0·02–0·38	0·21	1·72	147·34
Fundamental frequency modulation	0·79 ± 0·00 (0·74–0·85)	0·04	0·02–0·07	0·04	1·10	118·83
Amplitude modulation	0·62 ± 0·20 (0·37–0·86)	0·09	0·04–0·16	0·24	2·70	172·96
Time to maximum amplitude (ms)	528·9 ± 205·6 (208·7–948·0)	0·21	0·11–0·57	0·39	1·82	154·58

[a] Results of Kruskal–Wallis tests (d.f. = 12, $n = 207$) comparing differences between males for each acoustic feature. All comparisons are significant at $P < 0·001$.

[b] The statistical analysis excluded two males with no P_3 segment in the boatwhistle.

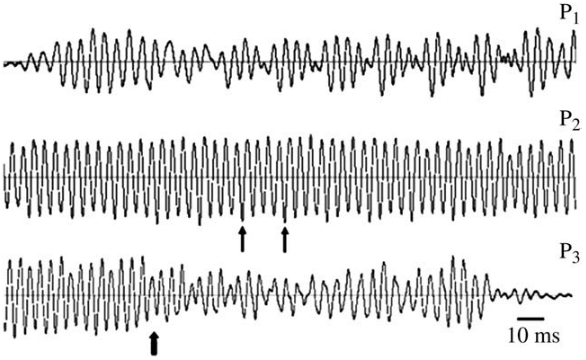

FIG. 4. Oscillograms of the initial (P_1), the middle tonal place (P_2) and the end phase (P_3) of a *Halobatrachus didactylus* boatwhistle. Thin arrows indicate the peak amplitude of two consecutive pulses, *i.e.* the pulse period. The thick arrow depicts the start of P_3. Note the differences in the fine waveform structure among the boatwhistle phases.

group size. Ten groups of males were considered. Each group consisted of randomly chosen males from the initial data set in various sample sizes: three, five, eight and 11 males. Five and 10 boatwhistles randomly chosen per male for each male group were used to further verify the change in classification accuracy with the number of sounds considered in the analysis.

RESULTS

BOATWHISTLE STRUCTURE

The mating sounds of the *H. didactylus* varied considerably in duration ranging from 317 to 1290 ms ($n = 207$ sounds analysed from all males), with average values of 767 ms (Table I). The fundamental frequency (H1) and the harmonics (multiples of H1) showed a slight frequency modulation that was more obvious in the higher harmonics (Fig. 1). H1 was the dominant frequency in only one male that exhibited eight of the 16 sounds analysed with dominant frequencies in the H1 and the remaining in the H2 (see male 5 in Fig. 5). H4 was the most common dominant frequency (51·2%) followed by H2 (44·9%).

The three segments (P_1, P_2 and P_3) that make up the boatwhistle (dos Santos *et al.*, 2000) were characterized by different durations, pulse periods, relative amplitude and dominant frequencies (Table I). The tonal phase of the boatwhistle (P_2) was the longest segment, lasting on average 56% of the sound, and exhibited an intermediate pulse period and dominant frequency to P_1 and P_3. The boatwhistle dominant frequency typically corresponded to P_2 dominant frequency. Pulses in P_1 and P_3 were of a more irregular shape and had clear starts and ends (Fig. 4), while pulses in P_2, the tonal segment, were more regular and fused together sometimes resembling a sinusoidal wave (Fig. 4). The third boatwhistle segment was more variable in duration, pulse number, pulse period and dominant frequency than the two previous segments (Fig. 4 and Table I) and was not present in all males. Two males never exhibited

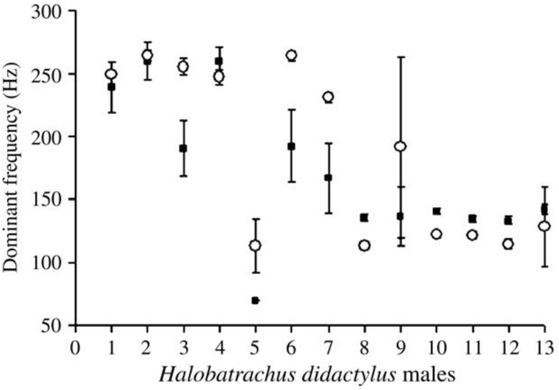

FIG. 5. Mean ± S.D. dominant frequencies of *Halobatrachus didactylus* boatwhistle segments initial phase (P_1) (■) and middle tonal phase (P_2) (○) in the 13 males analysed.

the segment P_3 in their boatwhistles and in another male it was present only in some of the calls analysed.

INDIVIDUALITY

Boatwhistles were distinct between individuals in terms of waveform (amplitude modulation) and spectral characteristics (Fig. 1). Detailed waveform patterns were also distinctive among calling males (Fig. 6). There was a strong stereotypy in most acoustic variables measured, with half of these features showing within-male C.V.s ≤ 0·10 (Table I). All the 20 features analysed had C.V._b:C.V._w ratios >1, showing that they were more variable among than within males. Consistently, the Kruskal–Wallis analyses demonstrated significant differences among males for all features (Table I), indicating that these acoustic variables can potentially provide recognition cues to identify calling males. The larger relative between-male variability (larger C.V._b:C.V._w ratios) corresponded to the dominant frequencies of P_1 and P_2 and of the whole signal (Table I and Fig. 5). Most males presented dominant frequencies of P_2 and of the whole boatwhistle either in the H2 or in the H4 and showed little within-male variation (Fig. 5). Three males exhibited, however, higher within-male variability in this feature (males 5, 9 and 13 in Fig. 5) because the dominant frequency in different sounds corresponded to different harmonic bands. Figure 6 also illustrates that approximately half of the males had lower dominant frequencies in P_1 than in P_2, whereas the remaining males showed an opposite trend.

A discriminant function analysis using only seven uncorrelated acoustic features generated a significant model (DFA, $n = 207$, d.f. = 84, 1159, $P < 0{\cdot}001$). The three first discriminant functions explained almost all data variability (91%; Table II). The sound features which weighted most heavily in explaining variation in the first three discriminant functions were P_2 dominant frequency followed by dominant frequency modulation for the first function, P_2 pulse period followed by amplitude modulation for the second function and total

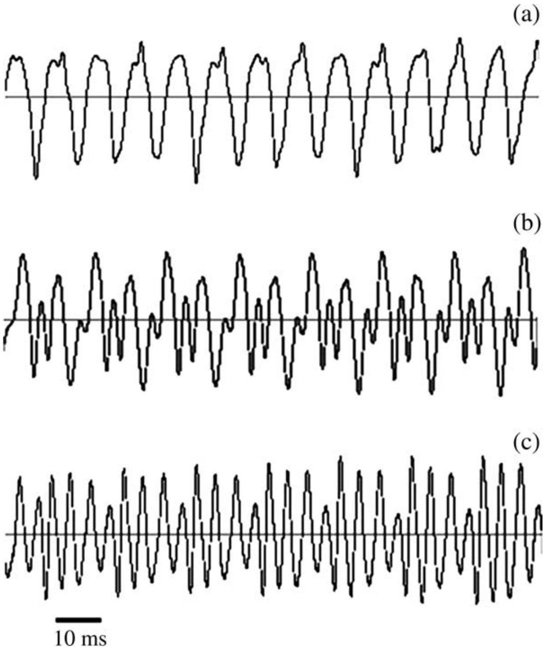

FIG. 6. (a), (b) and (c) Boatwhistles from different *Halobatrachus didactylus* males show differences in the waveform details in the tonal phase (P_2).

duration for the third function (Table II). The highest correlations between the discriminant variables and these discriminant functions were P_2 dominant frequency for the first, amplitude modulation for the second and total sound duration for the third discriminant functions. Classification success averaged 90·9% [± S.D. (range) = ± 10·4% (68·8–100%)] and was significantly greater than the classification expected by chance (*a priori* probability range = 0·072–0·077; Wilcoxon test, $n = 13$, $P < 0{\cdot}01$). A clear separation between individuals in the two-dimensional space defined by the first two discriminant functions is depicted in Fig. 7. After cross-validation, the correct classification decreased to a mean ± S.D. of 85·6 ± 16·8% with values ranging from 56·3–100%.

Subsequent discriminant analyses, including the same seven acoustic features, explored variation of classification success with fish group size (three, five, eight and 11 males) and number of sounds (five and 10) per male. The mean percentage of correct classification increased in groups of fewer males from *c.* 90% (11 males) to 100% (three males) of boatwhistles assigned to the correct individual (Fig. 8). There was no difference in the classification success between the analyses that included 10 boatwhistles per male and those that included only five boatwhistles, except in the sample size of eight males where mean correct classification values were 3·5% higher in the five boatwhistle analysis (Fig. 8; 95% CI). Classification success was thus consistently high even when considering few calls per individual in relatively large groups. For example, the analysis that included 10 random groups of 11 males with five randomly assigned

TABLE II. Standardized canonical discriminant function analysis (DFA) coefficients, eigenvalues and cumulative percentage of variance explained by the first three discriminant functions of a DFA classifying *Halobatrachus didactylus* males ($n = 13$) by their boatwhistle ($n = 16$) characteristics

	Discriminant functions		
Discriminant variables	First	Second	Third
Sound duration	0·34	0·35	−1·22[a]
Relative P_2 duration (%)	−0·39	0·04	0·78
P_2 pulse period	−0·44	0·83	−0·04
P_2 dominant frequency	1·55[a]	0·30	0·56
Dominant frequency modulation	1·26	0·57	0·58
Fundamental frequency modulation	0·26	−0·60	0·15
Amplitude modulation	0·05	−0·77[a]	−0·41
Eigenvalue	22·68	11·56	6·10
Cumulative % of variance	51·1	77·1	90·8

P_2, middle tonal segment of the boatwhistle.

[a] Discriminant variable with the highest pooled within-groups correlations with the standardized discriminant functions.

sounds, revealed a mean correct classification of 92·5%, which is well above the classification expected by chance alone. As with the initial DFA, which considered the whole data set, the acoustic features that loaded more heavily in the first two discriminant functions of these analyses were P_2 dominant frequency and dominant frequency modulation (typically in the first discriminant function) as well as P_2 pulse period, amplitude modulation and sound duration.

Predictive accuracy of the above models (calculated by the cross-validation leave-one-out procedure) considering different group sizes of randomly selected

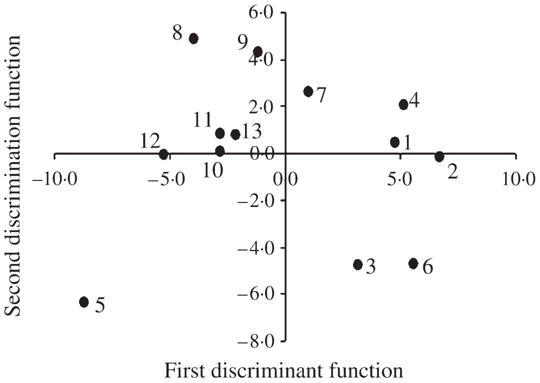

FIG. 7. Representation of the 13 *Halobatrachus didactylus* males (boatwhistle group centroids) in the bi-dimensional space defined by the first two discriminant functions of a discriminant function analysis considering seven acoustic features. Middle tonal phase (P_2) dominant frequency correlates with the first discriminant function and amplitude modulation with the second.

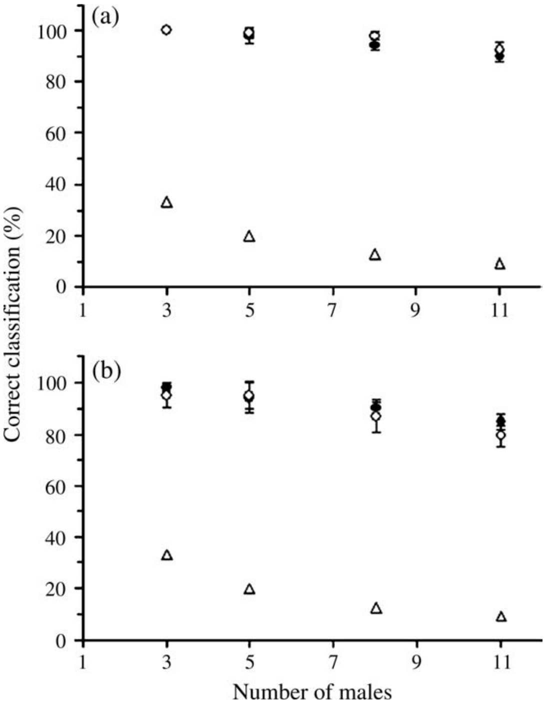

FIG. 8. (a) Variation of mean ± 95% CI classification success with fish group size (10 groups of three, five, eight and 11 randomly chosen *Halobatrachus didactylus* males) and number of sounds per individual: 5 (○) and 10 (●) boatwhistles. (b) Similar percentages of classification success obtained after cross-validation (△, the classification success expected from randomly assigning calls to the different male).

males also yielded high estimates of correct classifications. When 10 sounds were considered per male, the percentage of correct classification varied from an average of 98–85% in groups of three to 11 males, respectively (Fig. 8). Similar results were obtained when considering five boatwhistles per male with classification success decreasing from 95% in groups of three males to 79% in groups of 11 males (Fig. 8). In conclusion, after cross-validation these analyses still assigned high percentages of sounds to the correct males and considerably more than expected by chance.

DISCUSSION

The boatwhistles emitted by the *H. didactylus* consisted of a relatively long series of rapidly repeated pulses with average duration around 770 ms. These sounds exhibited the fundamental frequencies at *c.* 60 Hz with typical dominant frequencies represented by the second or the fourth harmonic bands. The boatwhistle of *H. didactylus* was very similar to the one of *O. tau*, which starts with a wide-frequency non-harmonic grunt-like phase caused by slower and more irregular sonic muscle contractions, followed by a longer tonal segment (Fine, 1978). Boatwhistles of the latter species are, however, shorter (200–500 ms) and have higher fundamental frequencies (*c.* 200 Hz) than the boatwhistles of the *H. didactylus* (Fine, 1978; Barimo & Fine, 1998; Edds-Walton *et al.*, 2002). Other well-studied batrachoidids produce more divergent calls. *Opsanus beta* emits a more complex courtship sound with fundamental frequencies around 270–280 Hz that starts with zero to three grunts followed by a long tonal ('boop') note and up to three shorter boops lasting over a second (Thorson & Fine, 2002*a*). Nesting *Porichthys notatus* Girard, males emit remarkably long courtship sounds ('hums') that last from seconds to over an hour, with fundamental frequencies around 100 Hz (Ibara *et al.*, 1983; Brantley & Bass, 1994).

Clear differences were found among boatwhistles attributed to different males that can potentially be used in individual recognition. All variables were significantly more variable between than within males and thus could all potentially provide cues to identify individuals. A DFA using a sub-set of the initial acoustic features assigned boatwhistles to the correct male in 91% of cases, and in 86% of cases after cross-validation, showing a high predictive accuracy. Classification success of boatwhistles varied with sample size (number of males and number of sounds per male) but remained high even when considering few calls per male in large groups. In accordance with the observed C.V._b:C.V._w ratios, the most important variables to allow male identification were P_2 dominant frequency followed by dominant frequency modulation (the ratio between P_1 and P_2 dominant frequencies). P_2 pulse period, amplitude modulation and total boatwhistle duration were also consistently important to discriminate among individuals in the various DFAs.

In the field, males can probably only detect a maximum of eight males at a time (maximum size of a chorus; unpubl. data) and call often in duets or in trios, thus potentially making the task of individual recognition simpler than the 13 males considered in the present study. Moreover, because males call often at rates of *c.* 10 boatwhistles min^{-1} (pers. obs.) they will easily experience more than the 16 calls from a neighbour having more opportunity to access distinct features from stationary nesting conspecifics.

In order for the above five features to be good candidates for individual identification, they should propagate well through the environment and should also be recognized by the central nervous system of the receiver. Sound propagation in shallow water can result in signal degradation over short distances, including sound pressure level and frequency attenuation, and temporal patterning loss (Mann, 2006; also see Fig. 3). Boatwhistles are thought to function both to announce territorial ownership and position to other males and to attract females as prospective mates (Winn, 1967). Because males can nest <0·5 m apart (pers. obs.) environmental degradation of call properties should not impose a major restriction between male neighbours. The effect of attenuation and signal degradation, however, should be important for female attraction. This problem has probably been overcome by the increased acoustic output resulting from *H. didactylus* male choruses. If there is mate choice based on acoustic signals, it probably takes place when females are already in close range to males with access to minimally degraded signals.

Differences among males in frequency attributes should be perceived by the *H. didactylus* although batrachoidids are hearing generalists, *i.e.* they lack morphological specializations that enhance the detection of the sound pressure component of the acoustic signals (Fay & Simmons, 1999). According to Vasconcelos *et al.* (2007), dominant and fundamental frequencies of boatwhistles match the best hearing range of the species. P_2 dominant frequency differs among individuals between >10 and 100% (Fig. 5), thus falling within the range of frequency discrimination ability of hearing generalists, which is generally slightly >10% difference (Fay & Simmons, 1999). Differences in frequency modulation should also be detected because disparities between P_1 and P_2 dominant frequencies are in the majority of the studied males >10% (Fig. 5). This variable shows high interindividual variability and dominant frequency can be modulated upward or downward (Table I and Fig. 5). The large differences in signal duration found in the present study (but not pulse period) should also fall into the hearing discrimination abilities of *H. didactylus* since other batrachoidids can detect small differences in signal duration (McKibben & Bass, 1998).

Acoustic recognition systems have arisen in situations where crowding, noisy backgrounds (such as in dense colonies of birds) or darkness reduce the roles of olfactory and visual cues or increase the risk of confusion (Beecher, 1989; Sayigh *et al.*, 1999). Likewise, acoustic recognition is also beneficial when vocal animals defend long-term territories. In this context, individual recognition is adaptive because animals can direct less aggression to familiar neighbours, which are less likely to intrude into their territories. This phenomenon, known as the 'dear enemy effect' (Fischer, 1954), has been described in several animals (Temeles, 1994). In fishes, acoustic recognition has only been demonstrated in a coral reef species that breed in dense colonies. Myrberg & Riggio (1985) tested the 'dear enemy effect' with the bicolour damselfish *Stegastes partitus* (Poey) and verified that males can recognize territorial neighbours based on acoustic cues, probably the dominant frequency that decreased pronouncedly with male size. Likewise, *H. didactylus* males establish long-term territories forming dense breeding aggregations. In addition, they live in turbid environments where vision is impaired. Consequently, being able to discriminate among different individuals would be beneficial in this species. A comparable social system where individual recognition has been demonstrated is found in anurans. Frogs and toads also form breeding choruses and establish long-term territories during the reproductive season and may show vocal individual recognition. For example, male bullfrogs *Rana catesbeiana*, Shaw can learn about individually distinct acoustic features of neighbours' calls and a neighbour's position by repeatedly hearing the call from a particular location (Bee & Gerhardt, 2001).

This study was based on short periods of recordings from unseen fish. Although the identity of the sound producers cannot be completely ascertained, the present results suggest that there is enough information in the mating calls of the *H. didactylus* to promote individual recognition. Future work carried out with fully identified males will need to address whether boatwhistle characteristics are constant over longer periods of time and whether they are related to male features.

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References

Amorim, M. C. P. (2006). Diversity of sound production in fish. In *Communication in Fishes*, Vol. I (Ladich, F., Collin, S. P., Moller, P. & Kapoor, B. G., eds), pp. 71–104. Enfield: Science Publishers.

Amorim, M. C. P., Vasconcelos, R. O., Marques, J. F. & Almada, F. (2006). Seasonal variation of sound production in the Lusitanian toadfish, *Halobatrachus didactylus*. *Journal of Fish Biology* **69,** 1892–1899. doi: 10.1111/j.1095-8649.2006.01247.x

Amorim, M. C. P., Simões, J. M. & Fonseca, P. J. (2008). Acoustic communication in the Lusitanian toadfish. *Halobatrachus didactylus*: evidence for an unusual large vocal repertoire. *Journal of Marine Biological Association of the United Kingdom* **88,** 1069–1073.

Barimo, J. F. & Fine, M. L. (1998). Relationship of swim-bladder shape to the directionality pattern of underwater sound in the oyster toadfish. *Canadian Journal of Zoology* **76,** 134–143.

Bee, M. A. & Gerhardt, H. C. (2001). Neighbour–stranger discrimination by territorial male bullfrogs (*Rana catesbeiana*): II. Perceptual basis. *Animal Behaviour* **62,** 1141–1150.

Bee, M. A., Kovich, C. E., Blackwell, K. J. & Gerhardt, H. C. (2001). Individual variation in advertisement calls of territorial male green frogs, *Rana clamitans*: implications for individual discrimination. *Ethology* **107,** 65–84. doi: 10.1046/j.1439-0310.2001.00640.x

Beecher, M. D. (1989). Signalling systems for individual recognition: an information theory approach. *Animal Behaviour* **38,** 248–261.

Bradbury, J. W. & Vehrencamp, S. L. (1998). *Principles of Animal Communication*. Sunderland: Sinauer Associates.

Brantley, R. K. & Bass, A. H. (1994). Alternative male spawning tactics and acoustic signals in the plainfin midshipman fish, *Porichthys notatus* (Teleostei, Batrachoididae). *Ethology* **96,** 213–232.

Christie, P. J., Mennill, D. J. & Ratcliffe, L. M. (2004). Chickadee song structure is individually distinctive over long broadcast distances. *Behaviour* **141,** 101–124.

Crawford, J. D., Cook, A. P. & Heberlein, A. S. (1997). Bioacoustic behavior of African fishes (Mormyridae): potential cues for species and individual recognition in *Pollimyrus*. *The Journal of the Acoustical Society of America* **102,** 1200–1212.

Edds-Walton, P. L., Mangiamele, L. A. & Rome, L. C. (2002). Variation of pulse repetition rate in boatwhistle sounds from oyster toadfish *Opsanus tau* around Waquoit bay, Massachusetts. *Bioacoustics* **13,** 153–173.

Fay, R. R. & Simmons, A. M. (1999). The sense of hearing in fishes and amphibians. In *Comparative Hearing: Fish and Amphibians* (Fay, R. R. & Popper, A. N., eds), pp. 269–318. New York: Springer.

Fine, M. L. (1978). Seasonal and geographical variation of the mating call of the oyster toadfish *Opsanus tau* L. *Oecologia* **36,** 45–57.

Fine, M. L. & Lenhardt, M. L. (1983). Shallow-water propagation of the toadfish mating call. *Comparative Biochemistry and Physiology A* **76,** 225–231.

Fine, M. L. & Thorson, R. F. (2008). Effects of passive acoustics for assessing behavioral interactions in individual toadfish. *Transactions of the American Fisheries Society* **137,** 627–637.

Fine, M. L., Malloy, K. L., King, C., Mitchell, S. L. & Cameron, T. M. (2001). Movement and sound generation by the toadfish swimbladder. *Journal of Comparative Physiology A* **187,** 371–379.

Fischer, J. B. (1954). Evolution and bird sociality. In *Evolution as a Process* (Huxley, J., Hardy, A. C. & Ford, E. B., eds), pp. 71–83. London: Allen and Unwin.

Ibara, R. M., Penny, L. T., Ebeling, A. W., van Dykhuizen, G. & Caillet, G. (1983). The mating call of the plainfin midshipman, *Porichthys notatus*. In *Predators and Prey* (Noakes, D. G. L., Lindquist, D. G., Helfman, G. S. & Ward, J. A., eds), pp. 205–212. The Hague: Junk Press.

Jouventin, P., Aubin, T. & Lengagne, T. (1999). Finding a parent in a king penguin colony: the acoustic system of individual recognition. *Animal Behaviour* **57,** 1175–1183.

Knapp, R., Wingfield, J. C. & Bass, A. H. (1999). Steroid hormones and paternal care in the plainfin midshipman fish (*Porichthys notatus*). *Hormones and Behavior* **35,** 81–89.

Lamml, M. & Kramer, B. (2006). Differentiation of courtship songs in parapatric sibling species of dwarf stonebashers from southern Africa (Mormyridae, Teleostei). *Behaviour* **143,** 783–810.

Mann, D. A. (2006). Propagation of fish sounds. In *Communication in Fishes*, Vol. I (Ladich, F., Collin, S. P., Moller, P. & Kapoor, B. G., eds), pp. 107–120. Enfield: Science Publishers.

McKibben, J. R. & Bass, A. H. (1998). Behavioral assessment of acoustic parameters relevant to signal recognition and preference in a vocal fish. *The Journal of the Acoustical Society of America* **104,** 3520–3533.

Modesto, T. & Canário, A. V. M. (2003). Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, *Halobatrachus didactylus*. *General and Comparative Endocrinology* **131,** 220–231.

Myrberg, A. A. Jr & Riggio, R. J. (1985). Acoustically mediated individual recognition by a coral reef fish (*Pomacentrus partitus*). *Animal Behaviour* **33,** 411–416.

Myrberg, A. A. Jr, Ha, S. J. & Shamblott, H. (1993). The sounds of bicolor damselfish (*Pomacentrus partitus*): predictors of body size and a spectral basis for individual recognition and assessment. *The Journal of the Acoustical Society of America* **94,** 3067–3070.

Remage-Healy, L. & Bass, A. H. (2005). Rapid elevations in both steroid hormones and vocal signalling during playback challenge: a field experiment in gulf toadfish. *Hormones and Behavior* **47,** 297–305.

Roux, C. (1986). Batrachoididae. In *Fishes of the North-eastern Atlantic and the Mediterranean*, Vol. III (Whitehead, P. J. P., Bauchot, M.-L., Hureau, J. C., Nielsen, J. & Tortonese, E., eds), pp. 1360–1361. Paris: Unesco.

dos Santos, M., Modesto, T., Matos, R. J., Grober, M. S., Oliveira, R. F. & Canario, A. (2000). Sound production by the Lusitanian toadfish, *Halobatrachus didactylus*. *Bioacoustics* **10,** 309–321.

Sayigh, L. S., Tyack, P. L., Wells, R. S., Solows, A. R., Scott, M. D. & Irvine, A. B. (1999). Individual recognition in wild bottlenose dolphins: a field test using playback experiments. *Animal Behaviour* **57,** 41–50.

Skoglund, C. R. (1961). Functional analysis of swimbladder muscles engaged in sound production in the toadfish. *Journal of Biophysical and Biochemical Cytology* **10,** 187–200.

Speirs, A. H. & Davis, L. S. (1991). Discrimination by the Adélie penguins, *Pygoscelis adeliae*, between the loud mutual calls of mates, neighbours and strangers. *Animal Behaviour* **41,** 937–944.

Temeles, E. J. (1994). The role of neighbours in territorial systems: when are they 'dear enemies'? *Animal Behaviour* **47,** 339–350.

Thorson, R. F. & Fine, M. L. (2002*a*). Crepuscular changes in emission rate and parameters of the boatwhistle advertisement call of the gulf toadfish, *Opsanus beta*. *Environmental Biology of Fishes* **63,** 321–331.

Thorson, R. F. & Fine, M. L. (2002*b*). Acoustic competition in the gulf toadfish *Opsanus beta*: acoustic tagging. *The Journal of the Acoustical Society of America* **111,** 2302–2307.

Vasconcelos, R. O., Amorim, M. C. P. & Ladich, F. (2007). Effects of ship noise on the auditory sensitivity and acoustic communication in the Lusitanian toadfish. *The Journal of Experimental Biology* **210,** 2104–2112.

Winn, H. E. (1967). Vocal facilitation and the biological significance of toadfish sounds. In *Marine Bio-Acoustics*, Vol. 2 (Tavolga, W. N., ed.), pp. 283–304. Oxford: Pergamon Press.

(ii) Variability in the sonic muscles of the Lusitanian toadfish (*Halobatrachus didactylus*): acoustic signals may reflect individual quality

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Variability in the sonic muscles of the Lusitanian toadfish (*Halobatrachus didactylus*): acoustic signals may reflect individual quality

M.C.P. Amorim, R.O. Vasconcelos, and B. Parreira

Abstract: Animal vocalizations are good examples of signals that have been shaped by sexual selection and often contribute to resolve contests or the choice of mates. We relate the mass of the sound-producing muscles of a highly vocal fish species, the Lusitanian toadfish (*Halobatrachus didactylus* (Bloch and Schneider, 1801)), with the sender's physical features, such as body size, and reproductive and body condition. In this species, both sexes are known to emit sounds during agonistic interactions and males rely on their mate attraction vocalizations to reproduce. Sonic muscles were highly variable among males (CV = 40%) and females (CV = 33%) and showed sexual dimorphism. Regression analysis showed that variability in the sonic muscles was best explained by total length and fish condition in males and females. Liver mass in both genders, and the mass of the testes accessory glands, also explained sonic muscle variability. These variables explained 96% and 91% of the sonic muscle mass variability in males and females, respectively. As in teleost fishes sonic muscle mass correlates to particular sound acoustic features, we propose that in the Lusitanian toadfish sounds can inform the receiver about the sender's quality, such as body size and condition, which are critical information in contests and mate choice.

Résumé : Les vocalisations animales sont de bons exemples de signaux qui ont été façonnés par la sélection sexuelle et qui servent souvent à déterminer l'issue des joutes ou le choix de partenaires. Nous mettons en relation la masse des muscles producteurs de sons d'un poisson à vocalisations fréquentes, le crapaud lusitanien (*Halobatrachus didactylus* (Bloch et Schneider, 1801)), avec les caractéristiques physiques de l'émetteur, telles que la taille du corps et les conditions reproductive et corporelle. Chez cette espèce, les deux sexes sont reconnus pour émettre des sons durant les interactions agressives et les mâles dépendent des vocalises d'attraction de leur partenaire pour la reproduction. Les muscles du son sont très variables chez les mâles (CV = 40 %) et les femelles (CV = 33 %) et affichent un dimorphisme sexuel. Une analyse de régression montre que la variabilité des muscles du son s'explique le mieux par la longueur totale et la condition des poissons mâles et femelles. La masse du foie des deux sexes et la masse des glandes accessoires des testicules sont aussi des variables explicatives de la variabilité des muscles du son. Ces variables expliquent respectivement 96 % et 91 % de la variabilité de la masse des muscles du son chez les mâles et les femelles. Comme chez les téléostéens la masse des muscles du son est en corrélation avec certaines caractéristiques acoustiques particulières, nous croyons que chez le crapaud lusitanien, les sons peuvent renseigner l'auditeur sur la qualité de l'émetteur, en particulier sur la taille du corps et sur la condition corporelle, qui sont des informations essentielles dans les joutes et le choix des partenaires.

[Traduit par la Rédaction]

Introduction

Exaggerated sexual secondary male traits have evolved under sexual selective pressure through male–male competition, mate choice, or both (Andersson 1994). Animal vocalizations are good examples of such traits (Andersson 1994; Bradbury and Vehrencamp 1998), and empirical evidence has shown that acoustic signals may influence the outcome of male contests or be subject to female preference in various taxa (e.g., Davies and Halliday 1978; Hasselquist et al. 1996; Márquez et al. 2008).

In order for communication to be adaptive, signals should convey honest information. Signals are expected to be reliable if they are costly, and costly signals are likely to impose even more constraints to animals in poor condition (Zahavi 1975; Grafen 1990). Alternatively, honest signals can be relatively cost-free if signallers and receivers share a common interest (in this case any signaller can do it), or if physical or physiological constraints determine the quality of the signal (indices of quality) (reviewed in Maynard Smith and Harper 2003). Vocal displays by ectothermic vertebrates are thought to be one of the most energetically costly activities (Taigen and Wells 1985; Prestwich 1994; but see Amorim et al. 2002), and costs are determined mostly by duration, amplitude, and rate of calling (Prestwich 1994). Hence, these features can potentially be honest indicators of the sender's quality. Further, some acoustic features of vocal displays are dependent on male's characteristics, such as size, being a reliable predictor of fighting ability or mating success (e.g., Bee et al. 1999; Márquez et al. 2008).

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Many species of teleost fish use acoustic signals during male–male competition and mate attraction (Ladich 2004; Amorim 2006), and are thereby expected to be subject to sexual selection pressure (Andersson 1994). Although fish sounds do not seem energetically expensive (Amorim et al. 2002), calling activity seems limited by physiological constraints such as fatigue resistance (Mitchell et al. 2008), making a high rate of sound production physiologically challenging. Some acoustic parameters are also intimately related to increased fish size, such as lower sound dominant frequency, higher sound amplitude, and increased pulse duration observed in larger fish (Myrberg et al. 1993; Connaughton et al. 2000). This evidence further suggests that calling rate and particular acoustic parameters can honestly signal the sender's quality in male contests and in courtship.

A major mechanism of sound production in fish is the rhythmical vibration of the swim bladder by the action of specialized rapid sonic muscles (Ladich and Fine 2006). In many species, sonic muscles show sexual dimorphism and hypertrophy during the mating season, with males showing heavier sonic muscles, with higher number of muscle fibres, and differences in the fine structure of muscle fibres (Fine et al. 1990; Brantley et al. 1993*a*; Connaughton et al. 2000; Modesto and Canário 2003*a*). Seasonal hypertrophy and sexual dimorphism of sonic muscles are mediated by androgens (Fine and Pennypacker 1986; Brantley et al. 1993*b*; Connaughton et al. 2000), which also modulate courtship behaviour (Knapp et al. 1999). Differences in the mass of sonic muscles, and concomitant morphological changes, thus seem to parallel the increase in vocal output by males during the breeding season (Amorim et al. 2006).

In this study we examine the possibility that fish acoustic signals can indicate the sender's quality by relating the mass of the sound-producing muscle of a highly vocal fish species with its physical features. In a first step we investigated if certain external features, such as fin size and mouth width, that are associated with agonistic displays (Vasconcelos and Ladich 2008) show sexual dimorphism. This part of the study was carried out under the premise that these could potentially be sexually selected traits and could give information on the quality of an individual (e.g., Engen and Folstad 1999). Secondly we explored the relation between sonic muscle mass and several traits, such as any dimorphic external feature, body size, reproductive status, and body condition. We use the Lusitanian toadfish (*Halobatrachus didactylus* (Bloch and Schneider, 1801)) (Batrachoididae) as a model because they are versatile and prolific sound producers, and males show a pronounced increase in the sonic muscles mass and sonic activity during the breeding season (Modesto and Canário 2003*a*; Amorim et al. 2006; Amorim et al. 2008). Indeed, batrachoidids, including the Lusitanian toadfish, have been models for studies of acoustic communication, as they show prolonged bouts of vocal activity, males nest in shallow water and are relatively easy to access, react to playback experiments, and have been subject to a large body of neurobiological studies (Cohen and Winn 1967; Barimo and Fine 1998; Bass and McKibben 2003; Modesto and Canário 2003*a*, 2003*b*; Remage-Healey and Bass 2005). Furthermore, batrachoidids present intra- and inter-sexual dimorphism in the brain, sonic muscle, and vocal behaviour, with territorial type I males showing reproductive singing behaviour. whereas type II males (sneakers) and females only produce agonistic calls (Bass and McKibben 2003).

Material and methods

Study species

During the breeding season (May–July), male Lusitanian toadfish emit advertisement calls (boatwhistles) to attract females to the nests that they defend in estuarine shallow waters (dos Santos et al. 2000; Amorim et al. 2006). Males mate with several females, and care for the fertilized eggs attached to the nest's ceiling until the young are free-swimming (dos Santos et al. 2000). Besides the boatwhistle, three other sound types are commonly produced by nesting males: grunt trains, long grunt trains, and double croaks, as well as other less frequent sound emissions such as croaks and mixed croak–grunt calls (Amorim et al. 2008). Similar to other batrachoidids, this species presents sexual polymorphism with two male morphotypes that differ in morphometric and endocrine characteristics, as well as in vocal behaviour. Nest-guarding males (type I) differ from sneaking males (type II) by having smaller testes (sevenfold), larger accessory glands (threefold; the accessory glands are part of the male reproductive apparatus, secrete mucosubstances, and are connected to the spermatic duct), and higher (sixfold) 11-ketotestosterone levels (Modesto and Canário 2003*a*, 2003*b*). Females and sneaker males are only known to emit grunt trains and females show lighter sonic muscles than males, with type II males presenting intermediate sonic muscle mass to females and type I males (Modesto and Canário 2003*a*). Sonic muscles of type I males, but not of type II males or of females, experience hypertrophy during the breeding season (Modesto and Canário 2003*a*), mirroring an increase in vocal activity (Amorim et al. 2006).

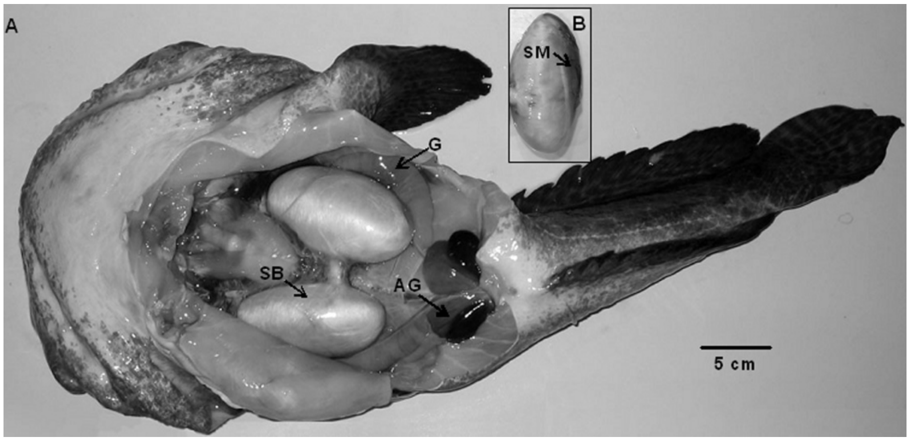
Specimen and sample collection

Fish samples were collected by trawling, angling, and hand capture by local fishermen during the months of June, August, and September in 2003 and from April to September in 2004 from Tagus estuary, areas of Montijo (38°42′N, 8°58′W) and Barreiro (38°39′N, 9°04′W). Specimens were kept frozen in the laboratory until measured. This sample included both reproductive (40.5% of males and 76.5% of females) and nonreproductive specimens, since the breeding season typically lasts from May to July (Modesto and Canário 2003*a*).

To investigate the existence of sexual dimorphism in external morphological traits involved in agonistic displays, we took the following measurements: mouth width (MW, maximum width of the lower lip); pectoral fin length (PL, base of the pectoral fin to the tip of its largest ray); ventral fin length (VL, base of the ventral fin to the tip of its largest ray); and dorsal fin length (DL, length of the largest fin ray of the first dorsal fin). All mouth and fin measurements were made to the nearest millimetre with callipers.

Gonad (M_G; Fig. 1A), accessory glands (in males, M_{AG}; Fig. 1A), and liver (M_L) mass were tallied to the nearest milligram. Both sonic muscles, which are embedded in the sides of the swim bladder (Figs. 1A, 1B), were gently cut from the swim-bladder wall with a pair of fine dissection scissors and were also weighed (M_{SM}) to the nearest milligram. We also obtained total length (TL), measured to the

Fig. 1. (A) Dissected type I male Lusitanian toadfish (*Halobatrachus didactylus*) showing the swim bladder (SB), the gonads (G), and the accessory glands (AG). (B) Dorsal view of one lobe of the swim bladder depicting the embedded sonic muscle (SM).



nearest millimetre, and eviscerated body mass (M_E), measured to the nearest gram.

Males ($n = 79$) used in this study were 33.04 ± 5.9 cm TL (mean ± SD; range 17.4–44.5 cm TL) and weighed 629 ± 303.1 g (range 84–1421 g) in eviscerated mass, whereas females ($n = 34$) were 27.02 ± 3.5 cm TL (range 18.7–33.8 cm TL) and weighed 304 ± 109.0 g (range 107–547 g) in eviscerated mass. All males were likely type I males. Type II males were not considered in this analyses because they were captured in very small quantities ($n = 2$).

Statistical analysis

We ran analysis of covariance (ANCOVA) to explore the existence of differences between sexes for fins (PF, VF, and DF) and mouth width (MW) variables, controlling for body size (TL). Initial analyses included an interaction term, which was subsequently removed because it was not significant in all cases. We $\log_{10}$-transformed the dependent variables (fin length and mouth width) and TL to meet the assumptions of the models. Kolmogorov–Smirnov tests confirmed that the assumption of normality was met in all analyses.

To quantify sonic muscle mass variability we calculated the mean ± SD (range), as well as the coefficient of variations (CV = (SD/mean) × 100), for males and females. Sexual dimorphism in sonic muscle mass was tested with ANCOVA with $\log M_{SM}$ as the response variable, sex as the factor, and $\log M_E$ as the covariate. As above, the interaction term was not included in the final model because it was not significant.

We fitted a multiple regression model with a stepwise procedure to explain variation in sonic muscle mass (dependent variable). We considered VL and DL in the initial model, as they were sexually dimorphic (see results). We included TL in the model as a metric of body size. We also considered the mass of gonads, accessory glands (in males), and liver as independent variables. We controlled for the influence of body size on M_G by using residuals of the simple linear regression of M_G on M_E (RM_G) in the multiple regression model. We considered the eviscerated mass (M_E) to represent body mass because it is independent of M_G, M_{AG}, and M_L. Likewise, we controlled for the influence of size on variations of M_{AG} (males only) and M_L by regressing these variables on M_E (RM_{AG} and RM_L, respectively). Similarly, we used the residuals of the regression of M_E on TL (COND) as a metric of condition. Positive residuals indicate that males are heavier than predicted and have good body condition, whereas negative residuals represent animals with poor condition. We $\log_{10}$-transformed TL and mass data both in the simple and in the multiple linear regressions to meet the assumptions of normality and to linearize allometric relationships. Separate models were fitted for males and females. All model assumptions were met for both male and female models. All model residuals were normally distributed. Additional residual analysis was performed using Durbin–Watson statistics (males = 2.08 and females = 1.83), residuals autocorrelation plots, and multicolinearity tests between all used variables.

All statistical analyses were performed using R version 2.8.0 (R Foundation for Statistical Computing, Vienna, Austria) and SPSS version 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

Results

External morphological sexual dimorphism

Mouth width and pectoral fin length did not differ between males and females (ANCOVA; MW: $F_{[1,110]} = 2.10$, $P > 0.05$; PF: $F_{[1,110]} = 2.59$, $P > 0.05$), but the ventral and the dorsal fins were longer in females, controlling for body length (ANCOVA; VF: $F_{[1,110]} = 14.32$, $P < 0.001$; DF: $F_{[1,76]} = 5.74$, $P < 0.05$) (Fig. 2). The covariate TL had a significant effect on mouth width and fin length, which increased with body length (ANCOVA; MW: $F_{[1,110]} = 834.65$; PF: $F_{[1,110]} = 494.76$; VF: $F_{[1,110]} = 315.28$; DF: $F_{[1,76]} = 106.24$, $P < 0.001$; Fig. 2).

Sonic muscle variability

Sonic muscle mass showed considerable variation among males (11.09 ± 4.43 g, range 1.68–20.93 g) and among females (5.26 ± 1.75 g, range 1.74–8.71 g). Coefficient of variation for this parameter was 39.9% in males and 33.2% in females. Moreover, sonic muscle mass showed significant dimorphism between sexes (Fig. 3), controlling for body mass (ANCOVA; sex: $F_{[1,110]} = 21.37$, $P < 0.001$; M_E: $F_{[1,110]} = 1549.19$, $P < 0.001$).

Table 1. Results of the multiple regression analyses of sonic muscle mass (log M_{SM}) on total length (log TL), condition (COND), residual accessory gland mass (RM_{AG}; males only), and residual liver mass (RM_L) for male and female Lusitanian toadfish (*Halobatrachus didactylus*).

Gender	Coefficients	Estimate	SE	*t*	*P*
Males (*n* = 79)	Intercept	6.76	0.22	–30.72	<0.001
	log TL	2.60	0.06	40.75	<0.001
	COND	0.12	0.01	9.21	<0.001
	RM_{AG}	0.06	0.01	4.59	<0.001
	RM_L	0.03	0.01	2.25	<0.05
Females (*n* = 34)	Intercept	–6.47	0.48	–13.59	<0.001
	log TL	2.45	0.14	16.97	<0.001
	COND	0.08	0.02	4.14	<0.001
	RM_L	0.06	0.02	2.88	<0.01

Fig. 2. Relation between fin length (VF, ventral fin; DF, dorsal fin; PF, pectoral fin) and total length (TL), and between mouth width (MW) and TL, in male (●) and female (○) Lusitanian toadfish (*Halobatrachus didactylus*).

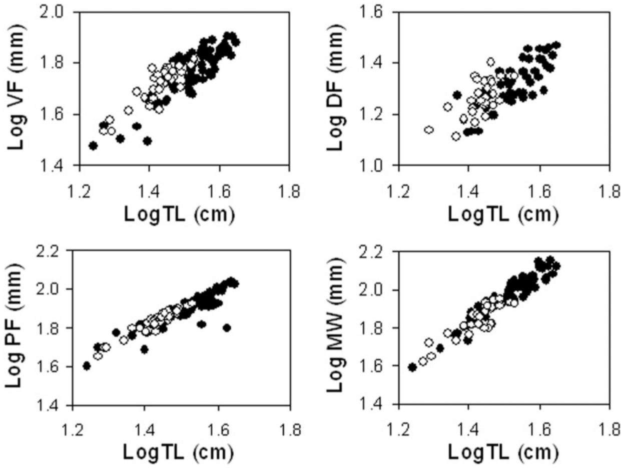
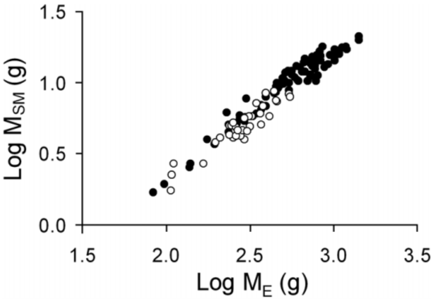


Fig. 3. Relation between sonic muscle mass (M_{SM}) and body eviscerated mass (M_E) in male (●) and female (○) Lusitanian toadfish (*Halobatrachus didactylus*).



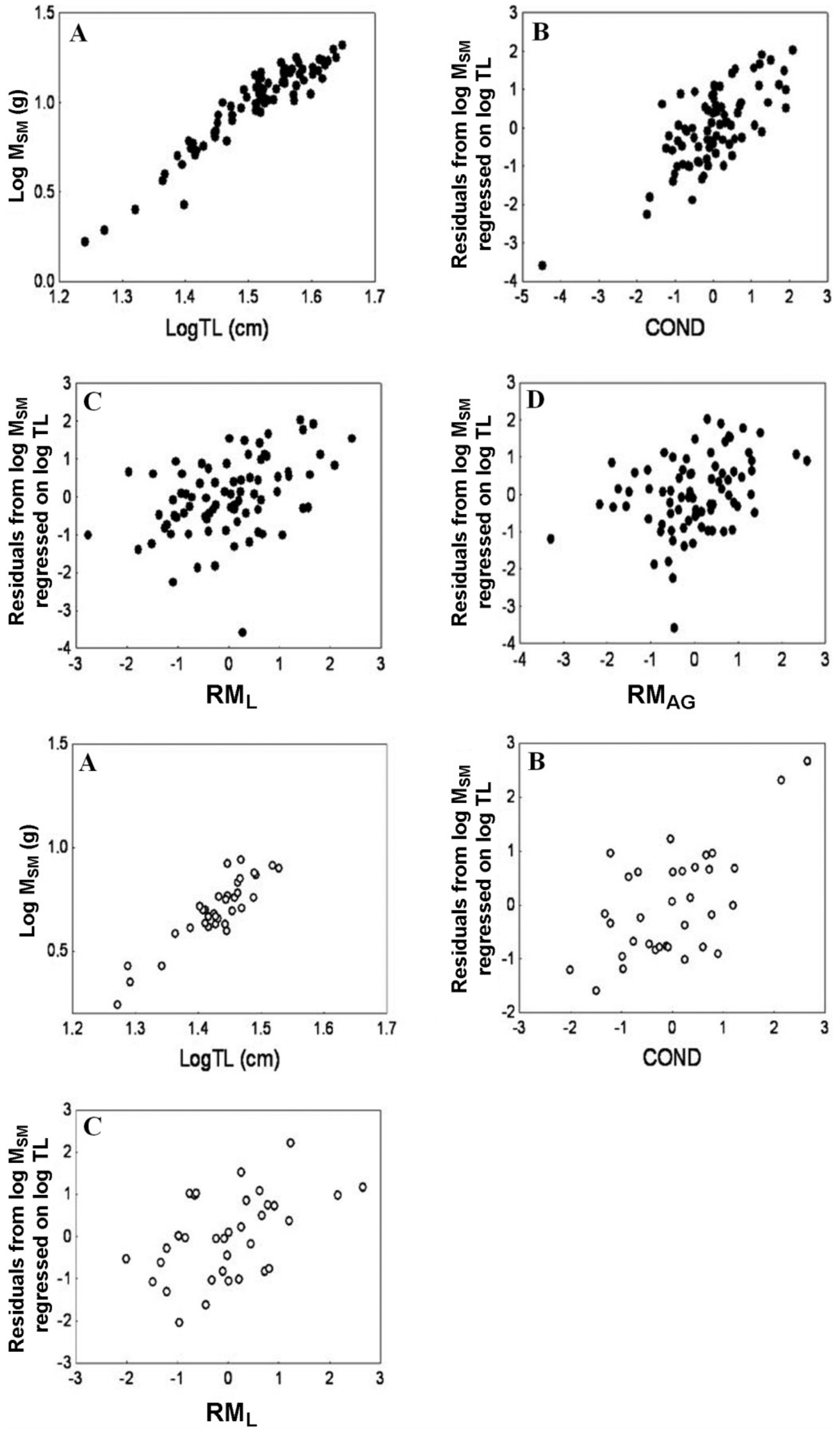
The best multiple regression model fitted with a stepwise procedure (males: $F_{[4,74]} = 447.4$, $P < 0.001$, $r^2 = 0.96$; females: $F_{[3,30]} = 105.6$, $P < 0.001$, $r^2 = 0.91$) included TL, condition, and liver mass (RM_L) as explanatory variables in both male and female models (Table 1). The residual accessory gland mass (RM_{AG}) was also included as a significant independent variable in the final model for males. Body length was the first variable included in the models and explained most of the variance of sonic muscle mass both for males (88.7%) and females (81.9%) (Fig. 4A). Condition (COND) explained an additional 6% of its variance in males and 7.1% in females (Fig. 4B). The remaining variability explained by the final models was accounted by accessory gland and liver mass in males (1.4%; Figs. 4C, 4D) and by the liver mass (2.4%) in females (Fig. 4C).

Discussion

Sexual dimorphism in external morphological traits and in sonic muscle mass

We examined the existence of sexual dimorphism in external morphological traits and in sonic muscle mass, which are associated with visual and acoustic signals used during social interactions. In the breeding season, male Lusitanian toadfish defend territories centred in the nest by displaying erected fins (dorsals and pectorals), the mouth wide open, and the body raised on the pelvic fins (Vasconcelos and Ladich 2008). Territorial defence also includes chasing the intruder, biting, and mouth locking (M.C.P. Amorim and R.O. Vasconcelos, personal observation). Because the reproductive success of males depend on their ability to hold good territories, it is plausible to hypothesize that sexual dimorphism in fin and mouth size could have evolved through sexual selection favouring males with traits that are advantageous in agonistic displays (Andersson 1994), such as found in other teleosts (e.g., Oliveira and Almada 1995). Likewise, acoustic displays seem to play a major role in both agonistic and courtship contexts in the Lusitanian toadfish (dos Santos et al. 2000; Amorim et al. 2006), and males with heavier sonic muscles should also be favoured. We found differences between gender in dorsal and ventral fins but not in the pectoral fin or in mouth width. Curiously, females had longer dorsal and ventral fins than males, contrary to the expected if these fins would have an important role in the outcome of agonistic interactions. There is, however, a moderate inter- and intra-sexual size dimorphism in body size with only type I males being found at larger sizes (Modesto and Canário 2003*b*; Fig. 2 in this study), suggesting that a large body size is an advantage for nesting males during territorial defence. We also observed sexual dimorphism in the sonic muscles, with males having significantly heavier sonic muscles than females at a given length (approximately 25% for mean TL of 30 cm), in agreement with the findings of Modesto and Canário (2003*a*). Consistently, external sexual

Fig. 4. Relation between the sonic muscle mass (M_{SM}) and the independent variables used in the regression model for male (●) and female (○) Lusitanian toadfish (*Halobatrachus didactylus*): (A) total length (TL), (B) condition (COND, the residuals of eviscerated body mass on total length), (C) residuals of liver mass on eviscerated body mass (RM_L), and (D) residuals of accessory gland mass on eviscerated body mass ($RW_{AG,}$ for males only). In (B), (C), and (D), the *y* axis represents the residuals from M_{SM} regressed on TL, i.e., sonic muscle mass with the effect of TL removed.



dimorphism in other batrachoidids is restricted to differences in body size and to the shape of the urogenital papilla (Brantley and Bass 1994), but differences in sonic muscle mass between sexes can amount to 600% in plainfin midshipman (*Porichthys notatus* Girard, 1854), owing to the different acoustic activity shown by different gender and male morphotypes (Brantley and Bass 1994). This evidence suggests that in the Lusitanian toadfish and in other batrachoidids, acoustic signals and body size may reveal information about individual quality during contests and mate choice. In many species, body size and sexual secondary male traits such as acoustic signals can directly affect the outcome of male–male contests and mating success (e.g., Davies and Halliday 1978; Castellano et al. 2000).

The sexual dimorphism in sonic muscle mass found in this study is consistent with the findings of Modesto and Canário (2003*a*), which reported that swim-bladder mass (swim bladder plus embedded sonic muscles) shows sexual polymorphism, i.e., it is larger in type I males, intermediate in type II males, and smaller in females. Sexual dimorphism in the swim bladder, sonic muscle fibres, and neural circuitry of sound production is typical among batrachoidids (Fine et al. 1984, 1990; Modesto and Canário 2003*b*; see review in Bass and McKibben 2003) and suggests that acoustic communication plays a prevalent role over other channels of communication and is essential for reproduction in the Lusitanian toadfish as in other batrachoidids (Bass and McKibben 2003). Curiously, we found that the CV for sonic muscle mass in males was only 7% higher than in females. Considering that our samples included nonspawners and that only males experience sonic muscle hypertrophy associated with the breeding season (Modesto and Canário 2003*a*), it was expected that females would show less variability than males in the mass of sonic muscles. The lack of a sharper difference between genders could reflect the relatively low sonic muscle sexual dimorphism and also the possibility that females have a higher vocal activity than traditionally described.

Traits affecting sonic muscle variability

This study showed that there is considerable variation in sonic muscle mass both in males (CV = 40%) and in females (CV = 33%). Multiple regression analysis revealed that body length and condition were good indicators of sonic muscle mass in both gender. These results are consistent with those of Modesto and Canário (2003*a*), which showed that swim-bladder mass increases with body size (eviscerated body mass) in mature and immature specimens of this species. Accordingly, swim bladder and associated sonic muscles show continuous growth in other batrachoidids (Fine et al. 1990; Brantley et al. 1993*a*). Sonic muscle mass of spawning cod has also been reported to be positively associated not only with body size and condition but also with fertilization potential (Rowe and Hutchings 2004), suggesting that acoustic features associated with sonic muscle mass also reveal information about individual quality in this vocal species. Likewise, in other taxa, acoustic cues such as sound frequency or acoustic repertoire size are related to body mass and (or) condition (e.g., Davies and Halliday 1978; Clutton-Brock and Albon 1979; Mager et al. 2007). For example, male common loons (*Gavia immer* (Brünnich, 1764)) in better condition and of larger body mass produce lower frequency sounds (yodels) during territorial defence (Mager et al. 2007). Mager and colleagues have shown with a playback experiment that lower frequency calls elicit stronger reactions from receivers, suggesting that dominant frequency of the yodel may honestly communicate fighting ability (Mager et al. 2007).

Residuals of liver mass gave a minor (1%–2%) but significant contribution to explain sonic muscle mass variability in both genders of Lusitanian toadfish. Both condition and hepatosomatic indices (analogous to the residuals of eviscerated body mass and liver mass used in the present study) are thought to represent good measures of condition in fish (Chellappa et al. 1995) and show minimal values at the end of the breeding season in the Lusitanian toadfish, probably associated to gamete production in females and to the increased metabolic needs of territorial defence and vocal activity in males (Modesto and Canário 2003*b*). Likewise the residuals of accessory gland mass contributed to sonic muscle mass in males. These glands are responsible for the exocrine production of mucosubstances, which seems to be a common feature of batrachoidids and of many other teleost species, and are thought to embed sperm and create sperm trails to reduce sperm dispersion (Barni et al. 2001). This would increase the chances of fertilizing females eggs in the nest by type I males, who have considerable larger accessory glands than type II males (Modesto and Canário 2003*b*). Nesting males with larger accessory glands may therefore gain higher chances of providing parental care to a higher percentage of own offspring than males with smaller accessory glands. Interestingly, the residuals of gonad mass did not enter in the final model, although it shows a similar seasonal variation as sonic muscle and accessory gland mass (Modesto and Canário 2003*a*).

Acoustic signals have a relevant role in the mating system of different taxa, allowing animals to convey information about their quality as mates, competitors, or both (Andersson 1994). Although fish are probably the largest group of sound-producing vertebrate, having evolved an outstanding variety of sonic organs, the functional role of their signals remains largely unknown, especially when comparing with the wealth of knowledge existent for other taxa (Ladich 2004; Ladich and Fine 2006). The present study shows that the variability of the sonic muscle mass could indicate individual quality in the Lusitanian toadfish, namely larger body size and better condition (somatic and liver) in both males and females and larger accessory glands in males. In batrachoidids, larger males with heavier sonic muscles show higher calling capabilities and emit sounds with higher amplitude (Fine et al. 2001; Vasconcelos and Ladich 2008). Frequency of sound is imparted by sonic muscle contraction rather than swim-bladder resonance, and it does not vary with fish size in the oyster toadfish (*Opsanus tau* (L., 1766)) (Fine et al. 2001), although grunt dominant frequency decreases with fish size in the Lusitanian toadfish (Vasconcelos and Ladich 2008). Females approaching a Lusitanian toadfish chorus could thus potentially select a better quality male based on acoustic cues, such as calling rate or call amplitude. Likewise males could judge their opponents based on grunt amplitude and dominant frequency. Future work is needed to associate calling rate and acoustic characteristics of sounds with sonic muscle mass and reproductive success to further support our conclusions.

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References

Amorim, M.C.P. 2006. Diversity of sound production in fish. *In* Communication in fishes. *Edited by* F. Ladich, S.P. Collin, P. Moller, and B.G. Kapoor. Science Publishers, Enfield, N.H. pp. 71–104.

Amorim, M.C.P., McCracken, M.L., and Fine, M.L. 2002. Metabolic costs of sound production in the oyster toadfish, *Opsanus tau*. Can. J. Zool. **80**(5): 830–838. doi:10.1139/z02-054.

Amorim, M.C.P., Vasconcelos, R.O., Marques, J.F., and Almada, F. 2006. Seasonal variation of sound production in the Lusitanian toadfish, *Halobatrachus didactylus*. J. Fish Biol. **69**(6): 1892–1899. doi:10.1111/j.1095-8649.2006.01247.x.

Amorim, M.C.P., Simões, J.M., and Fonseca, P.J. 2008. Acoustic communication in the Lusitanian toadfish, *Halobatrachus didactylus*: evidence for an unusual large vocal repertoire. J. Mar. Biol. Assoc. U.K. **88**(05): 1069–1073. doi:10.1017/S0025315408001677.

Andersson, M. 1994. Sexual selection. Princeton University Press, Princeton, N.J.

Barimo, J.F., and Fine, M.L. 1998. Relationship of swim-bladder shape to the directionality pattern of underwater sound in the oyster toadfish. Can. J. Zool. **76**(1): 134–143. doi:10.1139/cjz-76-1-134.

Barni, A., Mazzoldi, C., and Rasotto, M.B. 2001. Reproductive apparatus and male accessory structures in two batrachoid species (Teleostei, Batrachoididae). J. Fish Biol. **58**(6): 1557–1569. doi:10.1111/j.1095-8649.2001.tb02312.x.

Bass, A.H., and McKibben, J.R. 2003. Neural mechanisms and behaviors for acoustic communication in teleost fish. Prog. Neurobiol. **69**(1): 1–26. doi:10.1016/S0301-0082(03)00004-2. PMID:12637170.

Bee, M.A., Perrill, S.A., and Owen, P.C. 1999. Size assessment in simulated territorial encounters between male green frogs (*Rana clamitans*). Behav. Ecol. Sociobiol. **45**(3-4): 177–184. doi:10.1007/s002650050551.

Bradbury, J.W., and Vehrencamp, S.L. 1998. Principles of animal communication. Sinauer Associates, Inc., Sunderland, Mass.

Brantley, R.K., and Bass, A.H. 1994. Alternative male spawning tactics and acoustic signals in the plainfin midshipman fish *Porichthys notatus* Girard (Teleostei, Batrachoididae). Ethology, **96**: 213–232.

Brantley, R.K., Tseng, J., and Bass, A.H. 1993*a*. The ontogeny of inter- and intrasexual vocal muscle dimorphisms in a sound-producing fish. Brain Behav. Evol. **42**(6): 336–349. doi:10.1159/000114170. PMID:8275300.

Brantley, R.K., Marchaterre, M.A., and Bass, A.H. 1993*b*. Androgen effects on vocal muscle structure in a teleost fish with inter- and intra-sexual dimorphism. J. Morphol. **216**(3): 305–318. doi:10.1002/jmor.1052160306. PMID:8315650.

Castellano, S., Rosso, A., Laoretti, F., Doglio, S., and Giacoma, C. 2000. Call intensity and female preferences in the European green toad. Ethology, **106**(12): 1129–1141. doi:10.1046/j.1439-0310.2000.00639.x.

Chellappa, S., Huntingford, F.A., Strang, R.H.C., and Thomson, R.Y. 1995. Condition factor and hepatosomatic index as estimates of energy status in male three-spined stickleback. J. Fish Biol. **47**(5): 775–787. doi:10.1111/j.1095-8649.1995.tb06002.x.

Clutton-Brock, T.H., and Albon, S.D. 1979. The roaring of red deer and the evolution of honest advertisement. Behaviour, **69**(3): 145–170. doi:10.1163/156853979X00449.

Cohen, M.J., and Winn, H.E. 1967. Electrophysiological observations on hearing and sound production in the fish, *Porichthys notatus*. J. Exp. Zool. **165**(3): 355–369. doi:10.1002/jez.1401650305. PMID:6076901.

Connaughton, M.A., Taylor, M.H., and Fine, M.L. 2000. Effects of fish size and temperature on weakfish disturbance calls: implications for the mechanism of sound generation. J. Exp. Biol. **203**(9): 1503–1512. PMID:10751166.

Davies, N.B., and Halliday, T.R. 1978. Deep croaks and fighting assessment in toads *Bufo bufo*. Nature (London), **274**(5672): 683–685. doi:10.1038/274683a0.

dos Santos, M.E., Modesto, T., Matos, R.J., Grober, M.S., Oliveira, R.F., and Canário, A. 2000. Sound production by the Lusitanian toadfish, *Halobatrachus didactylus*. Bioacoustics, **10**: 309–321.

Engen, F., and Folstad, I. 1999. Cod courtship song: a song at the expense of dance? Can. J. Zool. **77**(4): 542–550. doi:10.1139/cjz-77-4-542.

Fine, M.L., and Pennypacker, K.R. 1986. Hormonal basis for sexual dimorphism of the sound-producing apparatus of the oyster toadfish. Exp. Neurol. **92**(2): 289–298. doi:10.1016/0014-4886(86)90081-6. PMID:3956662.

Fine, M.L., Economos, D., Radtke, R., and McClung, J.R. 1984. Ontogeny and sexual dimorphism of the sonic motor nucleus in the oyster toadfish. J. Comp. Neurol. **225**(1): 105–110. doi:10.1002/cne.902250111. PMID:6725634.

Fine, M.L., Burns, N.M., and Harris, T.M. 1990. Ontogeny and sexual dimorphism of sonic muscle in the oyster toadfish. Can. J. Zool. **68**(7): 1374–1381. doi:10.1139/z90-205.

Fine, M.L., Malloy, K.L., King, C.B., Mitchell, S.L., and Cameron, T.M. 2001. Movement and sound generation by the toadfish swimbladder. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol., **187**(5): 371–379. doi:10.1007/s003590100209. PMID:11529481.

Grafen, A. 1990. Sexual selection unhandicapped by the Fisher process. J. Theor. Biol. **144**(4): 473–516. doi:10.1016/S0022-5193(05)80087-6. PMID:2402152.

Hasselquist, D.S., Bensch, S., and von Schantz, T. 1996. Correlation between male song repertoire, extra-pair paternity and offspring survival in the great reed warbler. Nature (London), **381**(6579): 229–232. doi:10.1038/381229a0.

Knapp, R., Wingfield, J.C., and Bass, A.H. 1999. Steroid hormones and paternal care in the plainfin midshipman fish (*Porichthys notatus*). Horm. Behav. **35**(1): 81–89. doi:10.1006/hbeh.1998.1499. PMID:10049606.

Ladich, F. 2004. Sound production and acoustic communication. *In* The senses of fish: adaptations for the reception of natural stimuli. *Edited by* G. von der Emde, J. Mogdans, and B.G. Kapoor. Narosa Publishing House, New Delhi, India. pp. 210–230.

Ladich, F., and Fine, M.L. 2006. Sound-generating mechanisms in fishes: a unique diversity in vertebrates. *In* Communication in fishes. *Edited by* F. Ladich, S.P. Collin, P. Moller, and B.G. Kapoor. Science Publishers, Enfield, N.H. pp. 1–43.

Mager, J.N., III, Walcott, C., and Piper, W.H. 2007. Male common loons, *Gavia immer*, communicate body mass and condition through dominant frequencies of territorial yodels. Anim. Behav. **73**(4): 683–690. doi:10.1016/j.anbehav.2006.10.009.

Márquez, R., Bosch, J., and Eekhout, X. 2008. Intensity of female preference quantified through playback setpoints: call frequency versus call rate in midwife toads. Anim. Behav. **75**(1): 159–166. doi:10.1016/j.anbehav.2007.05.003.

Maynard Smith, J., and Harper, D. 2003. Animal signals. Oxford University Press, Oxford.

Mitchell, S., Poland, J., and Fine, M.L. 2008. Does muscle fatigue limit advertisement calling in the oyster toadfish *Opsanus tau*? Anim. Behav. **76**(3): 1011–1016. doi:10.1016/j.anbehav.2008.03.024.

Modesto, T., and Canário, A.V.M. 2003*a*. Hormonal control of swimbladder sonic muscle dimorphism in the Lusitanian toadfish *Halobatrachus didactylus*. J. Exp. Biol. **206**(19): 3467–3477. doi:10.1242/jeb.00581. PMID:12939377.

Modesto, T., and Canário, A.V.M. 2003*b*. Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, *Halobatrachus didactylus*. Gen. Comp. Endocrinol. **131**(3): 220–231. doi:10.1016/S0016-6480(03)00027-3. PMID:12714003.

Myrberg, A., Ha, S., and Shamblott, M. 1993. The sounds of bicolor damselfish (*Pomacentrus partitus*): predictors of body size and a spectral basis for individual recognition and assessment. J. Acoust. Soc. Am. **94**(6): 3067–3070. doi:10.1121/1.407267.

Oliveira, R.F., and Almada, V.C. 1995. Sexual dimorphism and allometry of external morphology in *Oreochromis mossambicus*. J. Fish Biol. **46**: 1055–1064.

Prestwich, K.N. 1994. Energy and constraints to acoustic communication in insects and anurans. Am. Zool. **94**: 625–643.

Remage-Healey, L., and Bass, A.H. 2005. Rapid elevations in both steroid hormones and vocal signaling during playback challenge: a field experiment in Gulf toadfish. Horm. Behav. **47**(3): 297–305. doi:10.1016/j.yhbeh.2004.11.017. PMID:15708758.

Rowe, S., and Hutchings, J.A. 2004. The function of sound production by Atlantic cod as inferred from patterns of variation in drumming muscle mass. Can. J. Zool. **82**(9): 1391–1398. doi:10.1139/z04-119.

Taigen, T.L., and Wells, K.D. 1985. Energetics of vocalization by an anuran amphibian (*Hyla versicolor*). J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. **155**: 163–170.

Vasconcelos, R.O., and Ladich, F. 2008. Development of vocalization, auditory sensitivity and acoustic communication in the Lusitanian toadfish *Halobatrachus didactylus*. J. Exp. Biol. **211**(4): 502–509. doi:10.1242/jeb.008474. PMID:18245626.

Zahavi, A. 1975. Mate selection — a selection for a handicap. J. Theor. Biol. **53**(1): 205–214. doi:10.1016/0022-5193(75)90111-3. PMID:1195756.

CHAPTER I

VOCAL BEHAVIOUR AND FUNCTION OF ACOUSTIC SIGNALS

(iii) **Vocal behaviour predicts reproductive success in a teleost fish**

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VOCAL BEHAVIOUR PREDICTS REPRODUCTIVE SUCCESS IN A TELEOST FISH

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SUMMARY

The relation between acoustic signalling and reproductive success is important to understand the evolution of vocal communication systems and has been well studied in several taxa but never clearly shown in fish. This study aims to investigate whether vocal behaviour affects the reproductive success in the Lusitanian toadfish (*Halobatrachus didactylus*) that relies on acoustic communication to attract mates. We recorded 56 nest-holding (type I) males during the breeding season and analyzed the calling performance and acoustic features of mate advertising sounds (boatwhistles) exhibited over circa two weeks. Hormonal levels of the subjects and the number of eggs (reproductive success) present in the respective nests were quantified. Nesting males attracted both females and other males, namely smaller type I males with significantly lower total length, body condition, sonic muscle mass, gonad mass and accessory glands mass. Calling rate, calling effort (% time spent calling) and sound dominant frequency were significantly higher in nesting males with clutches than in those without clutches. Sex steroids, i.e. 11-ketotestosterone and testosterone, were not correlated with vocal parameters or number of eggs. Maximum calling rate and calling effort were the best predictors of the number of eggs. In addition, these vocal variables were best explained by male's total length, condition and sonic muscle mass. We provide first evidence that vocal behaviour significantly determines reproductive success in a teleost fish and show that constant acoustic signaling at higher rates can operate as an indicator of the male's size and quality and probably of elevated motivation/readiness for reproduction.

Key words: acoustic communication, reproduction, mate attraction, fitness, Batrachoididae.

INTRODUCTION

Many studies on communication systems have centered on the relationship between signals and reproductive success. Determining the characteristics of signals that lead to enhanced mating success may help understanding how a communication system have evolved and how sexual selection may have shaped signaling (Andersson 1994; Bradbury and Vehrencamp 1998).

Acoustic signals are well known examples of sexually selected traits typically used by females of several taxa to identify, locate and select between potential mates (Andersson 1994; Bradbury and Vehrencamp 1998). The effect of acoustic signaling on mate attraction has been broadly investigated mostly in insects, anurans and birds. These studies reported that features of males' calling, such song quality and complexity, repertoire size, amplitude, singing effort and conspecific acoustic interactions, can be indicators of males' resources, health conditions, learning ability, developmental resilience to stress, attentiveness to females, or social skills (e.g. Searcy and Andersson 1986; Kroodsma and Byers 1991; Nordby et al. 1999; White et al. 2010).

Although teleost fishes may represent the largest group of sound-producing vertebrates that have evolved a variety of mechanisms to produce vocalizations crucial to social interactions including mate attraction (Ladich and Myrberg 2006; Myrberg and Lugli 2006), a link between vocal behavior and reproductive success has never been straightly shown. Few studies, however, suggest the role of certain acoustic signals in mate choice and relate sound features with male quality. For example, females of the bicolour damselfish *Stegastus partitus* (Pomacentridae) prefer males that not only produce courtship chirps of lower frequency that indicate a larger body size (Myrberg et al. 1986) but that also exhibit higher courtship rates (Knapp and Kovach 1991). Male´s courtship rate correlates positively mating success and subsequent egg survival, suggesting that chirp rate may also relate to reproductive success (Knapp and Kovach 1991). Moreover, male vocal features such as dominant frequency may operate as an indicator of body size (e.g. bicolor damselfish, Myrberg et al. 1993; croaking gouramis, Ladich et al. 1992; mormyrids, Crawford 1997).

Representatives of the family Batrachoididae, which include toadfishes and the plainfin midshipman fish, have emerged as one of the main study models for both behavioural and neurobiological studies in fish acoustic communication (Bass and McKibben 2003). Winn (1972) reported in *Opsanus tau* female phonotaxis towards male´s advertising boatwhistles emitted at higher calling rates. Females of the midshipman *Porichthys notatus* also showed phonotaxis towards hum-like (mate attraction) sounds, namely longer, higher amplitude and higher fundamental frequency tone stimuli (McKibben and Bass 1988). Recently, Amorim et al. (2010) reported that male vocal activity and mating call (boatwhistle) features reflect several aspects of male quality of another batrachoidid, the Lusitanian toadfish males (*Halobatrachus didactylus*). Males that contracted the sonic muscles at faster rates, as shown by the shorter boatwhistle pulse periods, were in better condition (increased body lipid and relative higher liver mass) and boatwhistle amplitude modulation reflected the degree of sonic muscle hypertrophy. Besides, this study also suggested that Lusitanian toadfish males advertise their quality (male condition) based on boatwhistle calling rate and calling effort, which mainly reflected male condition.

The major goal of this work was to verify whether vocal behaviour (calling rate, calling effort and signal features) can predict reproductive success, given by the number of eggs, in a vocal teleost. For this purpose, we tested the Lusitanian toadfish *H. didactylus*, which strongly depends on acoustic communication for mating. We also investigated possible sex steroids influence, namely of 11-ketotestosterone and testosterone that typically peak in the breeding season (Modesto and Canário 2003a), in the vocal performance and reproductive success. Moreover, male’s morphometric traits were also related with vocal parameters and number of eggs.

We used the Lusitanian toadfish as the study species for various reasons. This species relies heavily on acoustic communication to find mates in the breeding season and exhibits a rich vocal repertoire rare among fishes which comprises at least five different vocalizations (Amorim et al. 2008), including complex amplitude-modulated calls used in mate advertising (Amorim and Vasconcelos 2008). Phylogenetic analysis indicated that Lusitanian toadfish represents a basal lineage in the Batrachoididae, providing an excellent model for understanding integrated mechanisms underlying the evolution of acoustic communication in fishes (Rice and Bass 2009). Moreover, the Lusitanian toadfish is highly tolerant to experimental manipulations, displays the full acoustic repertoire and mates in semi-natural experimental situations (Amorim et al. 2010; Vasconcelos et al. 2010).

METHODS

Study species

The Lusitanian toadfish, *H. didactylus* (Batrachoididae) is a benthic marine fish that inhabits estuaries and coastal zones of the Eastern Atlantic and the Mediterranean (Roux 1986). During the reproductive season, from May to July in Portugal, territorial males ("type I") build nests under rocks in aggregations in shallow waters and attract females to spawn by emitting long advertisement calls (boatwhistles), forming conspicuous choruses (Amorim et al. 2006; Amorim and Vasconcelos 2008). Females deposit the eggs in the roof of the nest where they attach by an adhesive disk and are guarded by the male until the offspring are free-swimming (dos Santos et al. 2000; personal observations). Like other batrachoidids, this species presents sexual polymorphism with another male morphotype - sneaker ("type II"), which is smaller, with higher gonadsomatic index but smaller sonic muscles (Modesto and Canário 2003a,b) that attempt opportunistic fertilizations by parasiting the nests.

Test subjects

Prior to the onset of the breeding season, 60 concrete nests were placed along an intertidal area of the Tagus River estuary (Military Air Force Base, Montijo, Portugal) in order to create an aggregation of artificial shelters for Lusitanian toadfishes easily accessible in spring low tides during the whole breeding season, from May to July. These hemicylinder shaped nests (internal dimensions: 50 cm long, 30 cm wide and 20 cm height) were placed along the shore approx. 1.5 m apart in two rows.

We used a group of these nests to confine type I toadfish males that spontaneously occupied these shelters and record their vocal activity. In total, we recorded 56 males (34–49.5 cm TL, total length; 627-2097 g ME, eviscerated body mass). Tested type I males did not differ in body mass and body condition (One-way ANOVA: $F_{1,77}=0.44\text{-}0.75$; $P > 0.05$), after finishing the recording protocol, from the other territorial type I males found in the nests along the shore in the study area. The male morphotype was easily firstly identified on the basis of size and secretion of their larger accessory glands when gently pressed near the anus (Modesto and Canário 2003a). All animals used in this study, including conspecifics attracted to the experimental nests, were dissected after being sacrificed with an excessive dosage of

MS222 (tricaine methane sulphonate; Pharmaq, Norway) in the end of the study. Each subject was measured to the nearest mm for total length (TL), and to the nearest g for eviscerated body mass (ME). The gonads (M_G), the male accessory glands (M_{AG}) and the liver (M_L) mass were tallied to the nearest mg. Sonic muscles, which are embedded in the swimbladder walls, were gently cut and weighed to the nearest mg (M_{SM}). All experimental procedures comply with Portuguese animal welfare laws, guidelines and policies.

Experimental setup

We recorded simultaneously the vocal activity of nine groups of six-seven type I males, over a period of 6 hours per day (centered in the full tide) during circa two weeks. These recordings were performed throughout the peak of the toadfish breeding season (May-July) in 2008 and 2010.

We used concrete nests to confine the specimens that had spontaneously occupied the shelters in the intertidal area to record their vocal activity, ensuring male´s identity (see Vasconcelos et al. 2010). Nests were involved with a plastic net to prevent vocal males from escaping and to ensure individual identity throughout the recordings. A small opening (10 cm wide, 5 height cm) was created at the entrance of each of these nests to allow both females and eventually small type I or type II males to enter. These are typically smaller (generally TL < 30 cm, body mass < 500 g) than the tested type I males (Amorim et al. 2009). Plastic nets did not affect propagation of 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.

One hydrophone (High Tech 94 SSQ, Gulfport, MS, USA; frequency range: 30 Hz–6 kHz, ±1 dB; voltage sensitivity: –165 dB re. 1 V/μPa) was placed at about 10 cm from the entrance of each experimental nest and from the substrate. The recording chain also included audio capture devices Edirol UA-25 (Roland, 16 bit, 6 kHz acquisition rate per channel) connected to a laptop to perform simultaneous multi-channel recordings, which were controlled with Adobe Audition 2.0 (Adobe Systems Inc., 2005). Sounds captured from each hydrophone were stored in approx. 60 min duration wave files and therefore about 6 recording sessions were acquired per day for each fish.

Estuary water temperature during the recording period ranged between 19.5ºC to 24ºC and the water level varied from air exposure in the lower spring tides up to 2.8 m.

Every two weeks, when the tide was low enough to access the nesting experimental area (spring tides), any fish found parasiting the subject males' nests (ie. partially under or on the side near the entrance) were identified (sex and male type), euthanized and dissected in the laboratory. This procedure was only possible in the first breeding cycle (2008) that presented more specimens spawning in the study area.

Recorded Type I males were also removed, anesthetised in a MS222 bath and blood samples were collected within 5 min of handling the specimen. After finishing the recordings and removing the type I males from the respective nest, a photograph was taken whenever there were eggs attached to the roof of the nest. The number of eggs was counted using the software Image J (Wayne Rasband, NIH, USA).

Sound analysis

All test fish used showed vocal activity that included several vocalizations, but we only quantified and analyzed the boatwhistles (Amorim and Vasconcelos 2008). Advertising sounds were identified based on a constant emission rate during long periods of time that typically differ from the irregular emission of agonistic boatwhistles (Vasconcelos et al. 2010). Acoustic analysis was performed using Raven 1.2 for Windows (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, NY, USA). Recorded sounds 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, exceeding circa 21 dB between occupied nests.

The following parameters were calculated for each individual: CR_{mean}, mean calling rate, as the averaged number of sounds emitted per hour (i.e. per recording session); $CR_{max,}$ maximum calling rate, as the maximum mean calling rate per hour; CR_{active}, averaged number of sounds emitted per hour excluding sessions without subject's vocal activity; and CE, calling effort, percentage of time spent calling, i.e. number of 15 min intervals with calling activity divided by the total number of recorded 15 min intervals multiplied by 100. CRs were tallied on a minute basis.

Moreover, in order to relate sound features with the males' vocal performance and reproductive success, we analyzed 15 boatwhistles per male (with high signal-to-noise ratio) selected randomly from all 56 fish. For the acoustic analysis, we adopted the classification used by Amorim and 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 (of P2), as the highest energy component within the sound power spectrum (sampling frequency 8 kHz, Hamming window, filter bandwidth 10 Hz); fundamental frequency (of P2), calculated as the inverse of the average pulse period measured in the tonal phase.

Hormone assays

In order to determine whether sex steroid levels were affecting the vocal performance and reproductive success of recorded toadfish, blood samples were collected from the caudal vein in heparinised syringes. This procedure was performed after finishing the recording protocol for each test group recorded in 2010.

Plasma was separated by centrifugation (5000 rpm for 5 min) and stored at -4ºC. Plasma samples (50 µl) were diluted in phosphate buffer (450 µl) containing 0.5 g/L of gelatin (pH 7.6) and denatured at 80°C for 60 min. After cooling samples were processed. Steroids, T (17β-hydroxyandrost-4-ene-3-one), 11-KT (17β-hydroxyandrost-4-ene-3,11-dione) and cortisol (11b,17,21-trihydroxy-pregn-4-ene-3,20-dione) were measured by radioimmunoassay (RIAs). Details of the RIAs methodology have been published elsewhere (Scott et al. 1984). RIAs were performed using duplicate amounts (100 µl) of denatured samples. Cross reactions of antisera used in RIAs for T, 11-KT and cortisol were described previously in Kime and Manning (1982), Scott et al. (1984) and Rotllant et al. (2005), respectively. For each hormone, circulating plasma levels from all animals were measured within the same assay. Average intra-assay and inter-assay coefficient of variations for RIAs were 1.0 and 5.2% for T, 1.3 and 5.5% for 11-KT, 6.4 and 10.3%, for cortisol, respectively.

Statistical analysis

Similarly to Amorim (2010), we used residuals of the simple linear regression of sonic muscle mass on eviscerated body mass (RM_{SM}) as a metric of sonic muscle hypertrophy. Likewise, we used the residuals of the simple linear regressions of gonads, accessory glands and liver mass on eviscerated body mass (RM_G, RM_{AG}, RM_L, respectively) as metrics of these parameters controlled for the influence of body size. This metric gives a measure of an observed organ mass relative to a mean expected value (given by the regression model) for a given body size. Moreover, we used the residuals of ME on TL (COND) as a metric of body condition. We only used the total body mass to calculate COND when comparing recorded males and free-swimming territorial type I males, as the latter were not sacrificed and ME was not determined. We $\log_{10}$-transformed TL and ME to meet the assumptions of normality and to linearise allometric relationships.

Comparisons of morphological traits ($\log_{10}$TL, COND, RM_G, RM_{SM},) between recorded type I males and nest-parasite conspecifics were performed with M-W, Mann-Whitney, U tests. Comparisons of vocal parameters (CR_{mean}, $CR_{max,}$ CR_{active} and CE) and the morphological traits ($\log_{10}$TL, COND, RM_G, RM_{SM}) between males with eggs and males without eggs were also achieved with M-W U tests. These tests were also adopted to compare boatwhistle features between males between males with and without eggs, as well as to compare hormonal levels between tested males and free-swimming males from the same study area.

We examined general relationships among the variables across all individuals, including morphological traits, calling parameters (including boatwhistle features) and steroid levels, by performing Spearman correlations. As multiple tests were carried out, we adopted p-values to be significant if $P < 0.01$ (see Chandler 1995). We then considered 9 potential predictors of reproductive success (number of eggs), including morphological traits and calling parameters (log10TL, COND, RM_G, RM_{SM}, RM_L, CR_{mean}, CR_{max}, CR_{active}, and CE). We used multiple regression analysis to assess the statistical significance of each variable as a predictor of number of eggs with a stepwise selection procedure ($P \leq 0.05$ to add and $P \geq 0.10$ to remove). Our final regression modes complied with all assumptions of multiple linear regression. All model residuals were normally distributed. Further residual analysis was performed using Durbin–

Watson statistics, residual plots as well as multicollinearity tests (variance inflation factors, VIF).

Parametric tests were only performed when data were normally distributed and variances were homogeneous. Statistical analyses were performed using SPSS for Windows (16.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Vocal behaviour of nesting males and attraction of conspecifics

Most of the nesting toadfish males started to vocalize, predominantly with boatwhistles, within 24h-48h of confinement and interacted acoustically in a chorus, similarly to free-swimming toadfish. From the total of 56 toadfish males confined in the artificial nests, 51 toadfish (91.1%) showed vocal activity and 16 vocally active specimens (28.6%) presented clutches in their nest indicating that they successfully attracted mates. From the 28 males recorded in the first breeding season, 11 vocally active specimens (39.3%) attracted other conspecific males, which were outside the nests, close to the nest´s entrance, and partially buried in the substrate (Figure 1). These fish, which were in a position typically occupied by sneakers (type II males), were mostly type I males (n=10, 91%), but with significantly lower TL (M-W U test: U = 13.5, $N_{\text{nesting fish}} = 11$, $N_{\text{nest parasite fish}} = 7$, $P = 0.023$, Figure 2a) and COND (U-test: U = 2, $N_{\text{nesting fish}} = 11$, $N_{\text{nest parasite fish}} = 7$, $P < 0.001$, Figure 2b) relative to the vocalizing nesting type I males. Moreover, these specimens found outside the nest also presented significantly lower M_G (U-test: U = 16, $N_{\text{nesting fish}} = 11$, $N_{\text{nest parasite fish}} = 7$, $P = 0.042$, Figure 2c), M_{SM} (U-test: U = 0-12, $N_{\text{nesting fish}} = 11$, $N_{\text{nest parasite fish}} = 7$, $P < 0.001$, Figure 2d) and M_{AG} (U-test: U = 0-12, $N_{\text{nesting fish}} = 11$, $N_{\text{nest parasite fish}} = 7$, $P = 0.016$). Only one type II male was found outside the nest, among the 11 collected males. Within the same study period, also three gravid females were found inside the nests during low tides (see Figure 1).

The mean and maximum calling rates varied considerably between specimens, namely between 0 and 2.81 bw min^{-1} (0.34 ± 0.64 bw min^{-1}, mean ± SD, standard deviation) and 0-20.46 bw min^{-1} (3.60 ± 5.55 bw min^{-1}), respectively. The calling effort also differed greatly between males, i.e. 0-46% (CE, 15 ± 13%).

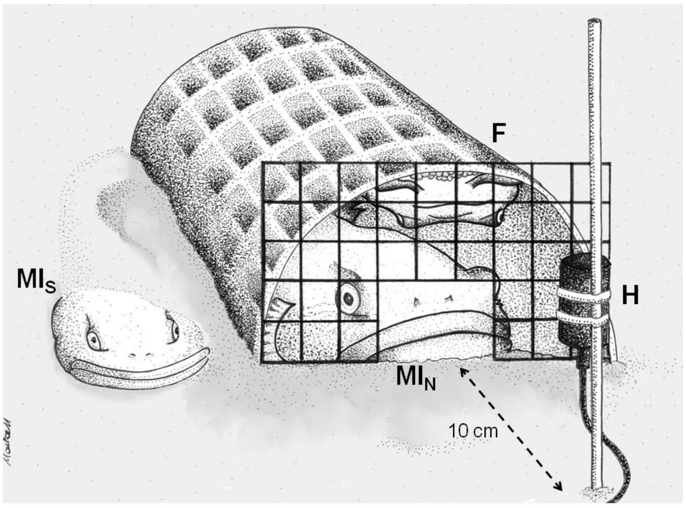

Figure 1 – Experimental setup showing a vocal type I toadfish male (MI_N) confined inside the artificial nest, female (F) inside the nest laying eggs and a satellite type I toadfish male (MI_S) that was often found outside the nest close to the entrance. A hydrophone (H) was placed in front of the nest entrance in order to record male´s vocal activity. Illustration by Marta Bolgan.

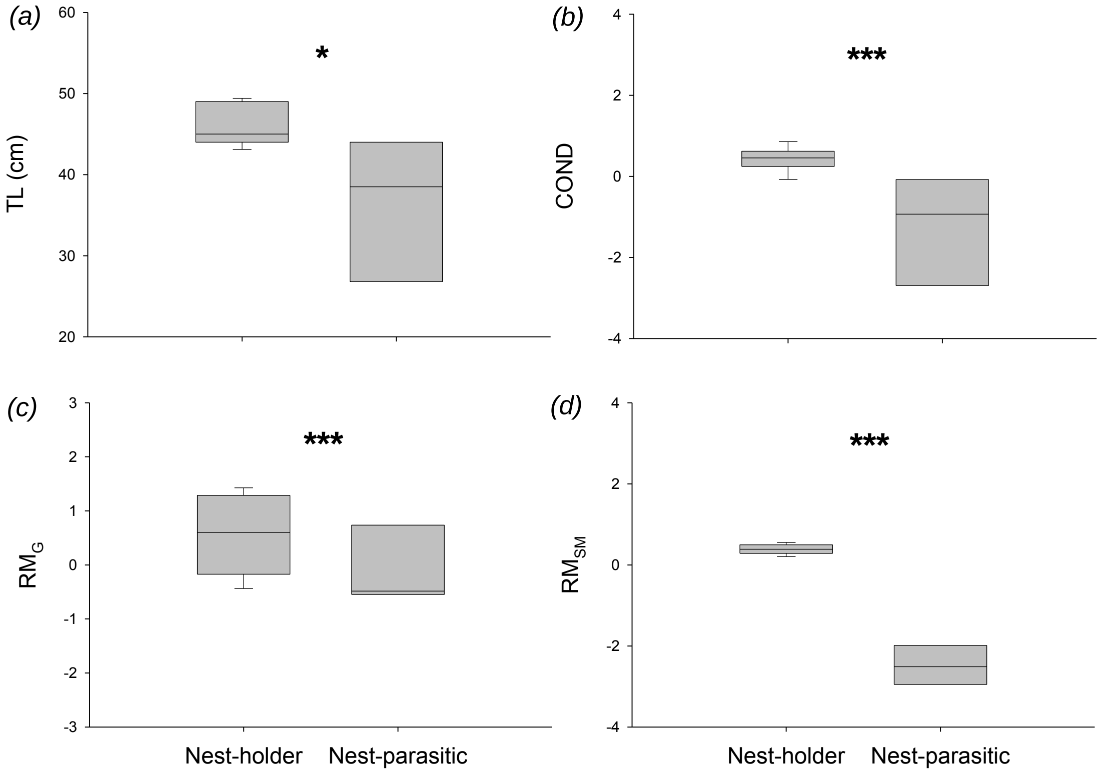

Figure 2 – Comparison of total length, TL (a), body condition, COND (b), residuals of gonads mass, RM_G (c) and residuals of sonic muscle mass, RM_{SM} (d) between nest-holder type I toadfish males and nest-parasitic type I toadfish found outside the nests. Plots show medians, 10th, 25th, 75th and 90th percentiles as boxes and whiskers. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann–Whitney U tests.

Predictors of reproductive success

The mean, active and maximum calling rates were significantly higher in nesting toadfish males with eggs than in males without any clutches (M-W U test, U (CR_{mean}) = 70; U (CR_{active}) = 103; U (CR_{max}) = 66.5; N_{eggs} = 14, N_{noeggs} = 40, $P < 0.001$, Figure 3a). The calling effort (CE) also differed significantly between these two fish groups (U test: U= 102, N_{eggs} = 14, N_{noeggs} = 40, $P < 0.001$, Figure 3b). No differences were found in terms of TL, COND, RM_G and RM_{MS} (U tests: U (TL) = 230.5; U (COND) = 241; U (RM_G) = 277; U (M_{MS}) = 255; N_{eggs} = 14, N_{noeggs} = 40, $P > 0.05$).

Correlation analysis showed that most morphometric features and vocal parameters were not significantly correlated - see Table 1. Calling rates ($CR_{mea,}$ CR_{active} and CR_{max}) and the calling effort (CE) were highly positively correlated with the number of eggs - Table 1.

Androgen levels, T and 11-kT, were neither correlated with any of the several vocal activity parameters, nor with the number of eggs - Table 1. Androgen and cortisol levels were compared between both tested animals and free-swimming fish collected in the same study area and showed that tested animals presented significantly higher cortisol but not significantly different T and 11-kT levels - Table 2. In addition, males with clutches obtained similar number of eggs than free-swimming males.

Moreover, boatwhistle acoustic features were mostly neither correlated with the vocal performance, nor with the number of eggs (Spearman correlations: R=-0.49-0.24, N=24, $P > 0.01$). Only pulse period was significantly negatively correlated with CR_{max} (R=-0.55, N=24, $P = 0.006$). Nevertheless, toadfish males with eggs produced generally boatwhistles with lower dominant frequencies (M-W U test, U=31, N_{eggs} = 13, N_{noeggs} = 11, $P = 0.018$) but similar duration (U test, U= 63, $P > 0.05$), amplitude modulation (U test, U= 47, $P > 0.05$) and pulse period (U tests: U = 65, $P > 0.05$).

The best regression model to predict the number of eggs showed that the maximum calling rate (CR_{max}) and the calling effort (CE) were the best predictors that accounted for most of the variation of the dependent variable - Table 3, Figure 4a,b. CR_{max} was the variable that most explained the variability of the number of eggs, i.e. 52 % (out of 58 % explained by the full model). Secondly, CE explained 6 % out of 58 %.

Although morphometric features did not account for variation of reproductive success, they explained some of the variability found in the vocal parameters. Total length, COND and RM_{SM} explained 29 %, 6 % and 7 % (out of 42 % explained by the full model) of the CR_{max} variability, respectively. In addition, total length was the only variable that explained CE variability, 56 % - Table 3.

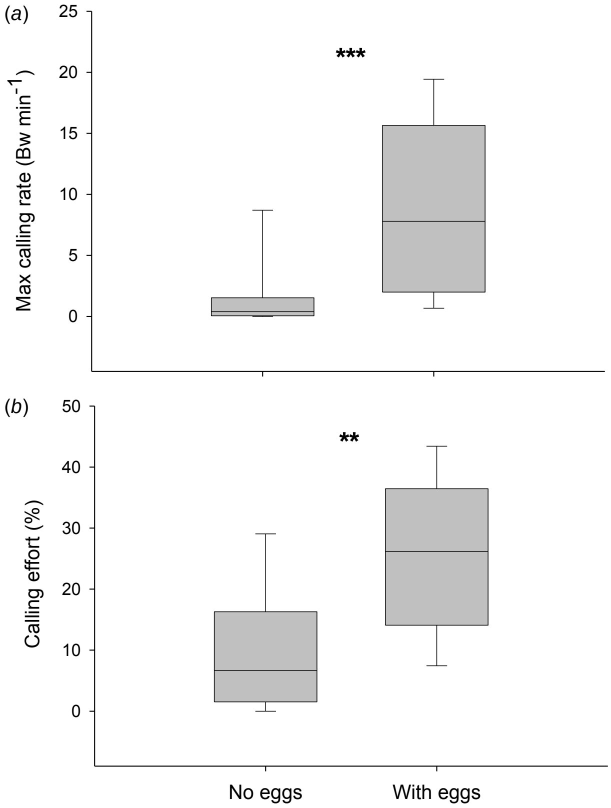

Figure 3 – Comparison of maximum calling rate (a) and calling effort (b) between type I toadfish males with and without clutches in the nests. Plots show medians, 10th, 25th, 75th and 90th percentiles as boxes and whiskers. ** $P < 0.01$, *** $P < 0.001$, Mann–Whitney U tests.

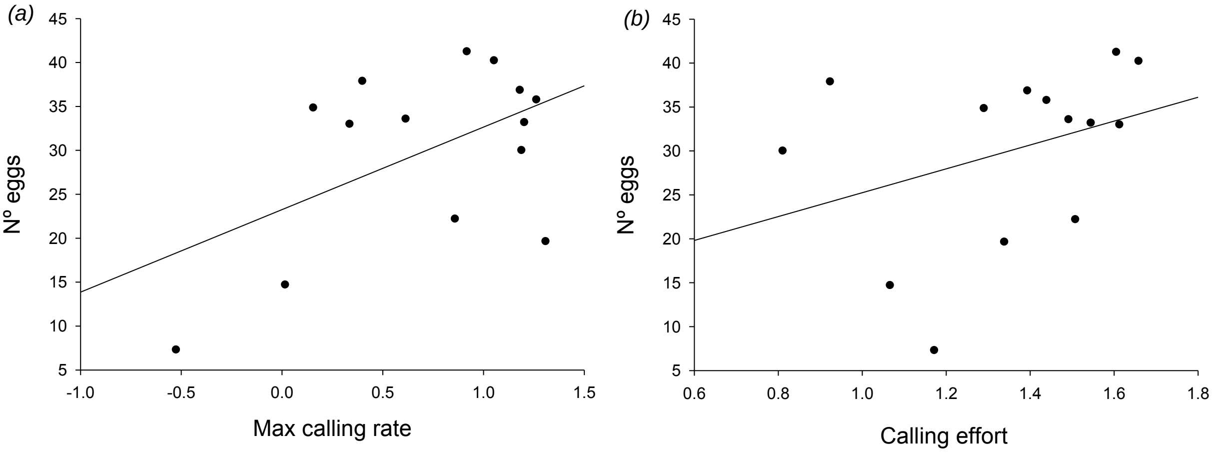

Figure 4 – Relationship between the best predictors, maximum calling rate (a) and calling effort (b), and the reproductive success (number of eggs) in the Lusitanian toadfish. The number of eggs was square-root-transformed and the maximum calling rate was log-transformed. Regression equations: (a) SQRT (nº eggs+0.5) = 9.4 (log CR_{max}) + 23.3; (b) SQRT (nº eggs+0.5) = 9.4 (CE) + 23.3.

Table 1 Correlations between morphometric features, vocal performance and number of eggs in the Lusitanian toadfish males. Values shown are Spearman rank correlation coefficients. Significant differences are indicated by asteristiks: * $P < 0.01$, ** $P < 0.001$. N is indicated below respective correlation coefficients. TL, total length; COND, body condition; RM$_{SM}$, residuals of the sonic muscle mass; RMG, residuals of the gonads mass; CR$_{mean}$, mean calling rate; CR$_{max}$, max calling; CR$_{active}$, active calling rate; CE, calling effort; T, testosterone; 11-kT, 11-ketotestosterone.

	TL	**Cond**	**RM$_{G}$**	**RM$_{SM}$**	**CR$_{mean}$**	**CR$_{max}$**	**CR$_{active}$**	**CE**	**T**	**1-kT**
TL	-	-0.02 (54)	0.27 (54)	-0.05 (54)	-0.22 (55)	-0.25 (55)	-0.34 (55)	-0.06 (55)	-0.42 (24)	-0.17 (24)
Cond		-	0.00 (54)	0.19 (54)	0.02 (54)	-0.01 (54)	-0.02 (54)	0.22 (54)	0.00 (23)	0.01 (23)
RM$_{G}$			-	**0.36*** (54)	0.05 (54)	0.03 (54)	-0.05 (54)	0.11 (54)	0.01 (23)	0.03 (23)
RM$_{SM}$				-	0.23 (54)	0.22 (54)	0.20 (54)	0.21 (54)	0.05 (23)	0.38 (23)
CR$_{mean}$					-	**0.97**** (55)	**0.92**** (55)	**0.86**** (55)	0.11 (23)	0.04 (23)
CR$_{max}$						-	**0.94**** (55)	**0.77**** (55)	0.17 (23)	0.12 (22)
CR$_{active}$							-	**0.68**** (55)	0.08 (23)	0.11 (23)
CE								-	0.26 (23)	0.16 (23)
T									-	0.37 (23)
Nº eggs	-0.12 (54)	-0.08 (54)	0.01 (54)	0.07 (54)	**0.58***** (55)	**0.59***** (55)	**0.49***** (55)	**0.50***** (55)	0.45 (23)	-0.05 (23)

Table 2 Comparison of steroid levels (T, testosterone and 11kT, 11-ketotestosterone) between tested confined fish and free-swimming fish with and without eggs present in the nest. Mann-Whitney U tests performed between groups are indicated. Values are means ± standard deviation and N is indicated in parentheses.

		T (ng)		**11-kT (ng)**		**Cortisol (ng)**		**Number of eggs**	
With eggs	**Test fish**	0.53 ± 0.36 (7)	U=19 $P > 0.05$	0.43 ± 0.40 (7)	U=13 $P > 0.05$	16.66 ± 13.32 (5)	U=3 $P = 0.013$	994 ± 518 (14)	U=78 $P > 0.05$
	Free-swimming fish	1.00 ± 1.05 (8)		2.75 ± 3.53 (8)		3.99 ± 2.47 (8)		810 ± 692 (14)	
No eggs	**Test fish**	0.47 ± 0.24 (17)		0.63 ± 0.59 (17)		21.82 ± 10.37 (18)			
	Free-swimming fish	0.29 (2)		1.54 (2)		1.18 (2)			

Table 3 Predictors of reproductive success (number of eggs), maximum calling rate ($CR_{\mathbf{max}}$) and calling effort (CE) of the Lusitanian toadfish. COND, body condition; TL, total length; RM_{SM}, residuals of the sonic muscle mass.

	Predictor	B	SEM	t	P	r	*F*	Model signif.	R^2	DW	VIF
Nº eggs	CR_{max}	1.20	0.33	3.67	0.001	0.762	$F_{1,53}$= 35.32	<0.001	0.581	2.20	2.03
	CE	0.28	0.10	2.70	0.009						2.03
$\mathbf{CR_{max}}$(1)	log TL	2.10	0.42	0.536	<0.001	0.651	$F_{3,53}$= 12.28	<0.001	0.424	1.92	1.92
	COND	-1.91	0.73	-0.284	0.012						1.03
	RM_{SM}	1.78	0.70	0.275	0.015						1.03
CE(1)	log TL	9.18	1.13	8.104	<0.001	0.747	$F_{1,53}$= 65.68	<0.001	0.558	1.33	1.00

(1) Only morphometric parameters were considered for regression analysis.

DISCUSSION

The relation between characteristics of acoustic signalling that lead to enhanced reproductive success can provide important means to understand how vocal communication systems have evolved. This approach has been well focused in several taxa such as insects, anurans and birds. Although teleost fishes may represent the largest group of sound-producing vertebrates and use acoustic signals during various social interactions including courtship, the relation between vocal behavior and reproductive success has never been clearly shown. This study is the first to experimentally demonstrate that sound production influences reproductive success in a teleost fish.

Inter and intra-sexual attraction

Almost all Lusitanian toadfish tested in this study showed vocal activity and several presented clutches in their nest, indicating that they successfully attracted gravid females. Besides mates, vocal males also attracted other conspecific “satellite” males, which remained outside the nests close to the entrance. In Batrachoididae, nest-parasiting males have been described as type II males that attempt opportunistic fertilizations, which are characterized by exhibiting smaller body size, accessory glands and sonic muscle mass but larger gonads (Brantley and Bass 1994; Modesto and Canário 2003a). Our data, however, showed that the nest-satellite males were mostly type I, but with significantly lower total length, condition, sonic muscle and accessory gland mass and almost significantly lower gonad mass than the nest-holder vocal males. Such finding suggests that smaller toadfish type I males with inferior quality (body size and condition) may adopt a sneaking behaviour during certain periods probably due to space competition. The data presented was collected during a summer season with particular high occupancy of the artificial nests placed along the intertidal study area (personal observations). Alternative mating tactics are not necessarily fixed throughout life and may change depending on the social environment. For instance, in the gobiid fish (*Bathygobius fuscus*), larger males are always nest holders but males of smaller size employ both tactics, nest holder or sneaker (Taru et al. 2002). With the decrement of larger males, sneaking males change their mating tactic to nest holding. Also in cichlid fish, *Oreochromis mossambicus*, males often adopt alternative female-like mating tactics (Oliveira and Almada 1999). Our results provide evidence of a similar mating behavioural plasticity in type I Lusitanian toadfish males, which probably depends on the social contexts and/or environmental constraints. Similar sneaking behaviour has been observed for type I males of the batrachoidid *P. notatus* (Lee and Bass 2004, 2006), although type II males of both batrachoidids species show fixed mating tactics (Brantley and Bass 1994). Species that breed in aggregations typically exhibit higher levels of competition among males that can ultimately result in higher number of males unable to defend a territory, and also in higher sexually motivated territorial males that lose the ability to discriminate between sexes (Oliveira and Almada 1999). Future studies should investigate whether this nest-satellite type I males are truly sneakers and attempt opportunistic fertilizations, whether this tactic is maintained throughout the whole breeding season and, which social/environmental features are responsible for shaping such mating behaviour.

Vocal behaviour and reproductive success

Our data indicated that the calling rate (mean, active and maximum calling rates), as well as the calling effort, were significantly higher in nesting males with eggs than in males without clutches. The physical features of nesting males, such as total length, body condition and gonads and sonic muscle mass, were not correlated with the number of eggs, which indicates that the reproductive success in *H. didactylus* primarily relies on the vocal performance. Higher calling rates and calling in a regular fashion during long periods of time (increased calling effort) results in a more conspicuous vocal output or male advertisement, which probably facilitates detection, localization and selection by females. Higher advertising calling rates might be important to indicate spawning readiness/motivation of males and in synchronizing gamete release (Amorim et al. 2003). Previous studies with other batrachoidids revealed that gravid females show phonotaxis when advertising calls are played back at relatively high rates (eg. *O. tau*, Winn 1972; *P. notatus*, McKibben and Bass 1998).

Ultimately, calling rate and calling effort may provide information about the quality of singing males and it is possible that these vocal features are used as honest signals for mate choice by females. Our data indicated that total length, condition and sonic muscle mass partially explained the variability found in the calling performance. Moreover, besides the likely higher physiological and metabolic costs (Mitchell et al. 2008; but see Amorim et al. 2002), the production of boatwhistles in high rates and in a regular fashion may also impose ecological costs, such as the attraction of predators and the time spent calling and not in other activities (Ryan 1988; Gannon et al. 2005). Females strongly benefit from choosing good males, especially when they are single spawners as batrachoidids (Brantley and Bass 1994; Modesto and Canário 2003a). Fish-unguarded eggs are quickly eaten by predators and females must rely on male brood protection to ensure the survival of the offspring (Sargent and Gross 1993). Also, the presence of a nesting male is critical for eggs' survival as they need to be defended from egg predators and to be aerated to prevent from eventual fungal infections (Ramos et al. unpublished). Therefore, in species where males provide parental care, indicators of male parental quality are expected to be important in intersexual communication and be under strong mate selection by females (Andersson 1994).

Amorim et al. (2010) also reported that increased boatwhistle calling rate and calling effort strongly reflected good male condition given by the lipid content of the somatic muscles in the Lusitanian toadfish, which corroborates the findings obtained in this study. We further demonstrate that these vocal parameters affect the reproductive success in this species and seem to inform receivers, i.e. females and other competing males, about the size and also quality of males. Lusitanian toadfish males that are in good condition advertise their spawning motivation through higher calling rates and increased calling effort, which consecutively affect the reproductive success.

Moreover, our data showed that Lusitanian toadfish males with eggs produced boatwhistles with significantly lower dominant frequencies, which might be explained by the higher calling rates of this fish group (Amorim et al. 2010) and/or possibly because this signal feature may indicate size or quality of vocal male (e.g. Myrberg et al. 1993; Ladich et al. 1992).

Sisneros et al. (2009) reported that, in the batrachoidid *P. notatus*, larger nesting males sampled at the end of the breeding season presented higher body condition (*K*) and larger number of viable late-stage embryos in the nest, suggesting that body condition is an honest indicator of parental ability in batrachoidids. In our study, we did not find a correlation between COND (comparable to *K*, but based on residuals- see methods) and number of eggs. The data obtained in terms of number of eggs was only collected after two weeks of confinement of reproductive males in the experimental nests. At this point, we were only evaluating the ability to attract mates and to provide early parental care, and not the capacity of providing good parental care until later stages of embryo development.

Likewise in other taxa, higher calling rates may signal male quality such as a better immune system (e.g. insects, Jacot et al. 2004) or parental quality (e.g. birds, Dolby et al. 2005) and also higher fertilisation success (e.g. anurans, Pfennig 2000; birds, White et al. 2009).

Steroid plasma levels, vocal behaviour and reproductive success

Our data showed that circulating androgen levels were not significantly related with reproductive success or vocal behaviour in the studied animals. However, androgens levels in tested animals were generally lower (but not significantly different) than those obtained from free-swimming toadfish collected in the same study area and during low tides (see Table 2). In fact, androgen profile of these free-swimming animals was similar to that found for the same species in wild (free-swimming) animals captured by beam trawler during reproductive season (Modesto and Canario 2003). In contrast, cortisol circulating levels of the recorded males were significantly higher than those obtained from free-swimming toadfish at the same study site. This suggests that the confinement in the experimental nests was probably responsible for the increment in the cortisol levels, although animals exhibited vocal activity, successfully attracted mates and presented similar body condition to the free-swimming fish. In addition, subject males with clutches obtained similar number of eggs than free-swimming males from the same site (Table 2), suggesting that spawning success of the studied males was not altered by confinement. Therefore, we consider the hypothesis that in confined animals increased cortisol levels could potentially result in decreased androgen levels concealing any possible relation with the male's reproductive success. In common carp, chronically elevated cortisol levels affected all parts of the brain-pituitary-gonad (BPG)-axis resulting in a strong decrease of the testicular production of androgens, including 11KT (Goos and Consten 2002). Moreover, *in vitro,* physiological levels of cortisol can inhibit the pathways that lead to the production of 11KT (Consten et al. 2002). In this context, measured androgen levels probably did not reflect the hormonal profiles of the specimens throughout the study period and cannot provide accurate information about relations between steroid plasma levels, vocal behaviour, and reproductive success. Future studies should investigate whether androgens affect calling and spawning success in non-confined animals.

This study is the first to experimentally demonstrate that sound production influences reproductive success in a teleost fish. We suggest that signaling at higher rates and in a regular fashion can operate as an honest signal of male size/quality and indicator of elevated motivation/readiness for reproduction in Batrachoididae, and perhaps in other fish. Future studies should address whether vocal behaviour also signals parental quality, such as found in other taxa.

REFERENCES

Amorim MCP, McCracken Ml, Fine ML. 2002. Metabolic costs of sound production in the oyster toadfish, *Opsanus tau*. Can J Zool. 80:830-838.

Amorim MCP, Fonseca PJ, Almada VC. 2003. Sound production during courtship and spawning of *Oreochromis mossambicus*: male-female and male-male interactions. J Fish Biol. 62:658-672.

Amorim MCP, Vasconcelos RO, Marques JF, Almada F. 2006. Seasonal variation of sound production in the Lusitanian toadfish, *Halobatrachus didactylu*s. J Fish Biol. 69:1892-1899.

Amorim MCP, Vasconcelos RO. 2008. Variability in the mating calls of the Lusitanian toadfish *Halobatrachus didactylus*: cues for potential individual recognition. J Fish Biol. 73:1267-1283.

Amorim MCP, Simões JM, Fonseca PJ. 2008. Acoustic communication in the Lusitanian toadfish, *Halobatrachus didactylus*: evidence for an unusual large vocal repertoire. J Mar Biol Assoc UK. 88:1069-1073.

Amorim MCP, Vasconcelos RO, Parreira B. 2009. Variability in the sonic muscles of the Lusitanian toadfish (*Halobatrachus didactylus*): acoustic signals may reflect individual quality. Can J Zool. 87:718-725.

Amorim MCP, Simões JM, Mendonça N, Bandarra NM, Almada VC, Fonseca PJ. 2010. Lusitanian toadfish song reflects male quality. J Exp Biol. 213:2997-3004.

Andersson M. 1994. Sexual Selection. Princeton: Princeton University Press.

Bass AH, McKibben JR. 2003. Neural mechanisms and behaviors for acoustic communication in teleost fish. Prog Neurobiol. 69:1-26.

Bradbury JW, Vehrencamp SL. 1998. Principles of Animal Communication. Massachussets: Sinauer Associates.

Brantley RK, Bass AH. 1994. Alternative male spawning tactics and acoustic signals in the plainfin midshipman fish, *Porichthys notatus* (Teleostei, Batrachoididae). Ethology. 96:213-232.

Chandler CR. 1995. Practical considerations in the use of simultaneous inference for multiple tests. Anim Behav. 49:524-527.

Consten D, Keuning ED, Terlou M, Lambert JGD, Goos HJT. 2002. Cortisol effects on the testicular androgen synthesizing capacity in common carp, *Cyprinus carpio* L. Fish Physiol Biochem. 25:91-98.

Crawford JD. 1997. Hearing and acoustic communication in mormyrid electric fishes. Mar Freshw Behav Physiol. 29:65-86.

Dolby AS, Clarkson CE, Haas ET, Miller JK, Havens LE, Cox BK. 2005. Do song-phrase production rate and song versatility honestly communicate male parental quality in the Gray Catbird? J Field Ornithol. 76:287-292.

dos Santos ME, Modesto T, Matos RJ, Grober MS, Oliviera RF, Canário A. 2000. Sound production by the Lusitanian toadfish, *Halobatrachus didactylus*. Bioacoustics. 10:309-321.

Gannon DP, Barros NB, Nowacek DP, Read AJ, Waples DM, Wells RS. 2005. Prey detection by bottlenose dolphins, *Tursiops truncatus*: an experimental test of the passive listening hypothesis. Anim Behav. 69:709-720.

Goos HJT, Consten D. 2002. Stress adaptation, cortisol and pubertal development in the male common carp, *Cyprinus carpio*. Mol Cell Endocrinol. 197:105-116.

Jacot A, Scheuber H, Brinkhof MWG. 2004. Costs of an induced immune response on sexual display and longevity in field crickets. Evolution. 58:2280-2286.

Lee JSF, Bass AH. 2006. Dimorphic male midshipman fish: reduced sexual selection or sexual selection for reduced characters? Behav Ecol. 17:670-675.

Kime DE, Manning NJ. 1982. Seasonal patterns of free and conjugated androgens in the brown trout *Salmo trutta*. Gen Comp End. 48:222-231.

Knapp R, Wingfield J, Bass AH. 1999. Steroid hormones and parental care in the plainfin midshipman fish (*Porichthys notatus*). Horm Behav. 35:81-89.

Kroodsma DE, Byers BE. 1991. The function(s) of bird song. Am Zool. 31:318-328.

Ladich F, Bischof C, Schleinzer G, Fuchs A. 1992. Intra- and interspecific differences in agonistic vocalization in croaking gouramis (genus: Trichopsis, Anabantoidei, Teleostei). *Bioacoustics*. 4:131-141.

Ladich F, Myrberg AA. 2006: Agonistic behavior and acoustic communication. In: Ladich, F., Collin, S.P., Moller, P., Kapoor, B.G. editors. Communication in Fishes, vol. 1. Enfield, U.S.A.: Science Publishers. p. 121-148.

McKibben JR, Bass AH. 1998. Behavioral assessment of acoustic parameters relevant to signal recognition and preference in a vocal fish. J Acoust Soc Am. 104:3520-3533.

Mitchell S, Poland J, Fine ML. 2008. Does muscle fatigue limit advertisement calling in the oyster toadfish *Opsanus tau*? Anim Behav. 76:1011-1016.

Modesto T, Canário AVM. 2003a. Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, *Halobatrachus didactylus*. Gen Comp End. 131:220-231.

Modesto T, Canário AVM. 2003b. Hormonal control of swimbladder sonic muscle dimorphism in the Lusitanian toadfish *Halobatrachus didactylus*. J Exp Biol. 206:3467-3477.

Myrberg AA, Mohler M, Catala J. 1986. Sound production by males of a coral reef fish (*Pomacentrus partitus*): its significance to females. Anim Behav. 24:923-933.

Myrberg AA, Ha SJ, Shamblott H. 1993. The sounds of bicolor damselfish (*Pomacentrus partitus*): predictors of body size and a spectral basis for individual recognition and assessment. J Acoust Soc Am. 94:3067-3070.

Myrberg AA, Lugli M. 2006. Reproductive Behavior and Acoustical Interactions. In: Ladich, F., Collin, S.P., Moller, P., Kapoor, B.G., editors. Communication in Fishes. vol. 1. Enfield, U.S.A.: Science Publishers. p. 149-176.

Nordby JC, Campbell SE, Beecher MD. 1999. Ecological correlates of song learning in song sparrows. Behav Ecol. 10:287-297.

Oliveira RF, Almada VC. 1999. Male Display Characters, Gonadal Maturation and Androgens in the Cichlid Fish *Oreochromis mossambicus*. Acta Ethologica. 2:67-70.

Pfennig KS. 2000. Female spadefoot toads compromise on mate quality to ensure conspecific matings. Behav Ecol. 11:220-227.

Rice AN, Bass AH. 2009. Novel vocal repertoire and paired swimbladders of the three-spined toadfish, *Batrachomoeus trispinosus*: insights into the diversity of the Batrachoididae. J Exp Biol. 212:1377-1391.

Rotllant JPM, Guerreiro AL, Redruello B, Canário AVM, Power DM. 2005. Stimulation of cortisol release by the N terminus of teleost parathyroid hormone-related protein in interrenal cells in vitro. Endocrinology. 146:71-76.

Ryan MJ. 1988. Energy, calling and selection. Am Zool. 28:88-898.

Sargent RC, Gross MR. 1993.William’s principle: an explanation of parental care in teleost fishes. In: Pitcher, TJ, editor. Behaviour of teleost fishes. London: Chapman and Hall. p. 333-361.

Scott AP, MacKenzie DS, Stacey NE. 1984. Endocrine changes during natural spawning in the white sucker, *Catostomus commersoni*.II. Steroid hormones. Gen Comp End. 56:349-359.

Searcy WA, Andersson M. 1986. Sexual selection and the evolution of song. Annu Rev Ecol Syst. 17:507-533.

Sisneros JA, Alderks PW, Leon K, Sniffen B. 2009. Morphometric changes associated with the reproductive cycle and behaviour of the interidalnesting, male plainfin midshipman fish, *Porichthys notatus*. J Fish Biol. 74:18-36.

Taru M, Kanda T, Sunobe T. 2002. Alternative mating tactics of the gobiid fish *Bathygobius fuscus*. J Ethol. 20: 9-12.

Vasconcelos RO, Simões JM, Almada VC, Fonseca PJ, Amorim MCP. 2010. Vocal behaviour during territorial intrusions in the Lusitanian toadfish: boatwhistles also function as territorial ‘keep-out’ signals. Ethology. 116:155-165.

Winn HE. 1972. Acoustic discrimination by the toadfish with comments on signal systems. In: Winn HE, Olla BL, editors. Behavior of Marine Animals, vol. 2., New York: Plenum. p. 361-385.

White DJ, King AP, West MJ, Gros-Louis J, Tuttled EM. 2010. Effects of singing on copulation success and egg production in brown-headed cowbirds, *Molothrus ater*. Behav Ecol. 21:211-218.

(iv) **Vocal behavior during territorial intrusions in the Lusitanian toadfish: boatwhistles also function as territorial 'keep-out' signals**

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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, gonadsomatic 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/μPa) 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,

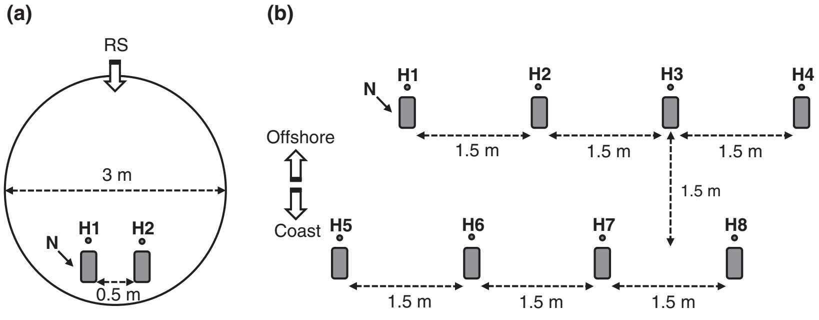

Fig. 1: Diagrams of the setups used for territorial intrusion experiments (a) and to record mate advertising vocal behavior (b). Grey rectangles depict nests (N) with resident fish inside; H, hydrophone position; RS, release site of fish intruders.

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: $U = 63$, $n_{\text{non-parental}} = 14$, $n_{\text{parental}} = 16$, $p = 0.006$) – 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: $U = 109–111$, $n_{\text{non-parental}} = 14$, $n_{\text{parental}} = 16$, $p > 0.05$). Likewise, there were no statistical differences between ABs emitted by parental and non-parental males in any acoustic parameter (U test: $U = 14–17$,

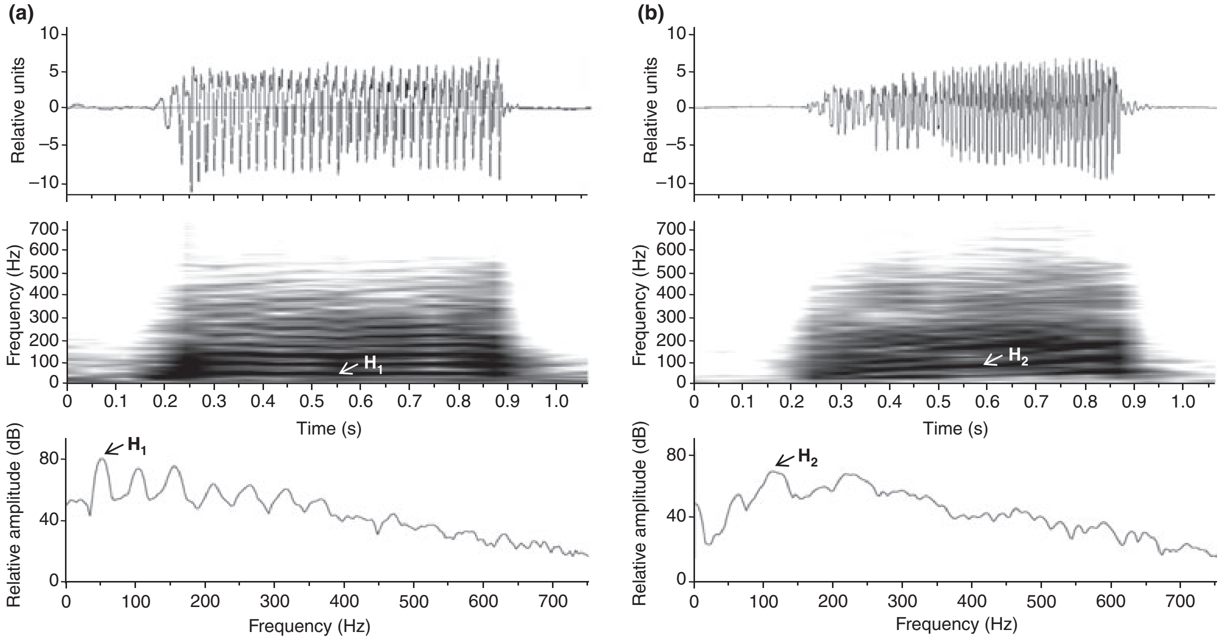

Fig. 2: Oscillogram, sonogram and power spectrum (of the middle tonal phase) of typical agonistic (a) and mating (b) boatwhistles emitted by the Lusitanian toadfish. The spectral energy of the agonistic boatwhistle was almost evenly distributed within the first three harmonics but with a higher peak 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.

$n_{\text{non-parental}} = 5$, $n_{\text{parental}} = 7$, $p > 0.05$). 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 defense 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

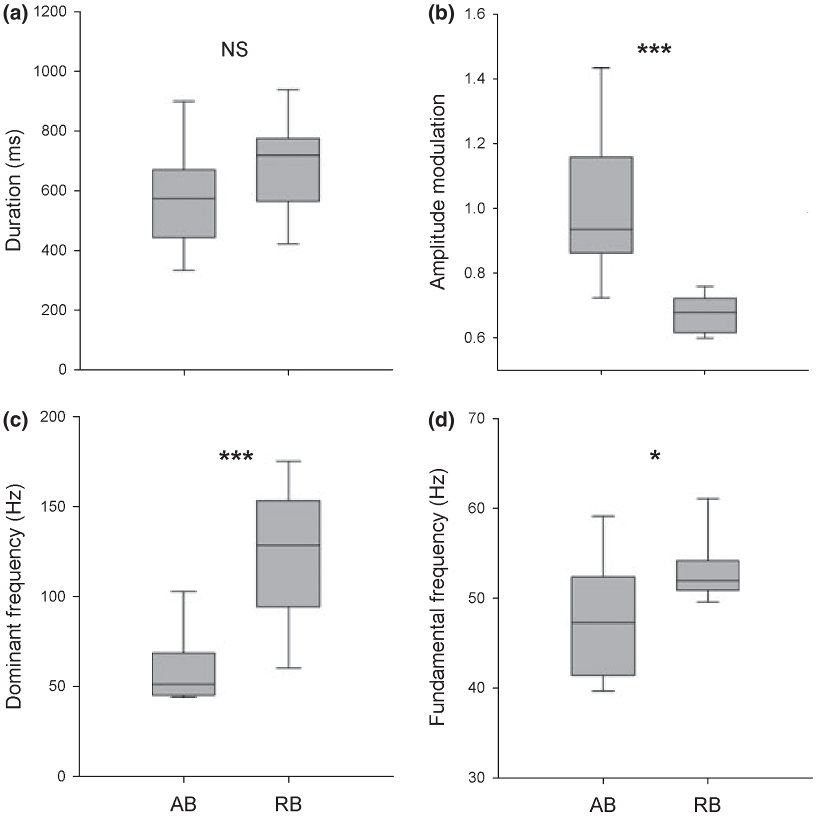

Fig. 3: Median of total sound duration (a), amplitude modulation (b), dominant frequency (c), and fundamental frequency (d) of agonistic (AB) and reproductive (RB) boatwhistles. Plots show 10th, 25th, 75th and 90th percentiles as boxes and whiskers. *p < 0.05, ***p < 0.001, NS: non significant, Mann–Whitney *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.

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Literature Cited

Almada, V. C., Amorim, M. C. P., Almada, F., Matos, R., Pereira, E. & Godinho, R. 1996: Agonistic Behaviour and Sound Production in *Gaidropsarus mediterraneus* (Gadidae). J. Fish Biol. **49**, 363—366.

Amorim, M. C. P. 2006: Diversity of sound production in fish. In: Communication in Fishes (Ladich, F., Collin, S. P., Moller, P. & Kapoor, B. G., eds). Science Publishers, Enfield, pp. 71—104.

Amorim, M. C. P. & Neves, A. S. M. 2007: Acoustic signalling during courtship in the painted goby, *Pomatoschistus pictus*. J. Mar. Biol. Assoc. UK **87**, 1017—1023.

Amorim, M. C. P. & Neves, A. S. M. 2008: Male painted gobies (*Pomatoschistus pictus*) vocalise to defend territories. Behaviour **145**, 1065—1083.

Amorim, M. C. P. & Vasconcelos, R. O. 2008: Variability in the mating calls of the Lusitanian toadfish *Halobatrachus didactylus*: potential cues for individual recognition. J. Fish Biol. **72**, 1355—1368.

Amorim, M. C. P., McCracken, M. L. & Fine, M. L. 2002: Metabolic costs of sound production in the oyster toadfish, *Opsanus tau*. Can. J. Zool. **80**, 830—838.

Amorim, M. C. P., Vasconcelos, R. O., Marques, J. F. & Almada, F. 2006: Seasonal variation of sound production in the Lusitanian toadfish, *Halobatrachus didactylus*. J. Fish Biol. **69**, 1892—1899.

Amorim, M. C. P., Simões, J. M. & Fonseca, P. J. 2008: Acoustic communication in the Lusitanian toadfish, *Halobatrachus didactylus*: evidence for an unusual large vocal repertoire. J. Mar. Biol. Assoc. UK **88**, 1069—1073.

Bailey, W. J. 1991: Acoustic Behaviour of Insects: An Evolutionary Perspective. Chapman & Hall, London.

Bass, A. H. & McKibben, J. R. 2003: Neural mechanisms and behaviors for acoustic communication in teleost fish. Prog. Neurobiol. **69**, 1—26.

Beebee, M. D. 2004: The functions of multiple singing modes: experimental tests in yellow warblers, *Dendroica petechia*. Anim. Behav. **67**, 1089—1097.

Berglund, A., Bisazza, A. & Pilastro, A. 1996: Armaments and ornaments: an evolutionary explanation of traits of dual utility. Biol. J. Linn. Soc. Lond. **58**, 385—399.

Brantley, R. K. & Bass, A. H. 1994: Alternative male spawning tactics and acoustic signals in the plainfin midshipman fish *Porichthys notatus* Girard (Teleostei, Batrachoididae). Ethology **96**, 213—232.

Cohen, M. J. & Winn, H. E. 1967: Electrophysiological observations on hearing and sound production in the fish, *Porichthys notatus*. J. Exp. Zool. **165**, 355—369.

Darwin, C. R. 1871: The Descent of Man, and Selection in Relation to Sex. John Murray, London.

Delaney, K. J., Roberts, J. A. & Uetz, G. W. 2007: Male signaling behavior and sexual selection in a wolf spider (Araneae: Lycosidae): a test for dual functions. Behav. Ecol. Sociobiol. **62**, 67—75.

Fine, M. L. & Thorson, R. F. 2008: Use of passive acoustics for assessing behavioral interactions in individual toadfish. Trans. Amer. Fish. Soc. **137**, 627—637.

Fine, M. L., Malloy, K. L., King, C. B., Mitchell, S. L. & Cameron, T. M. 2001: Movement and sound generation by the toadfish swimbladder. J. Comp. Physiol. A. **187**, 371—379.

Fish, J. 1972: The effect of sound playback in the toadfish. In: Behaviour of Marine Animals (Winn, H. & Olla, B., eds). Plenum Publishing Corporation, New York, pp. 386—434.

Gannon, D. P., Barros, N. B., Nowacek, D. P., Read, A. J., Waples, D. M. & Wells, R. S. 2005: Prey detection by bottlenose dolphins, *Tursiops truncatus*: an experimental test of the passive listening hypothesis. Anim. Behav. **69**, 709—720.

Gerhardt, H. C. 1982: Sound pattern recognition in some North American treefrogs (Anura: Hylidae): implications for mate choice. Am. Zool. **22**, 581—595.

Gerhardt, H. C. 1994: The evolution of vocalization in frogs and toads. Annu. Rev. Ecol. Syst. **25**, 293—324.

Gray, G. A. & Winn, H. E. 1961: Reproductive ecology and sound production of the toadfish *Opsanus tau*. Ecology **28**, 274—282.

Ibara, R., Penny, L., Ebeling, A., Dykhuizen, G. & Cailliet, G. 1983: The mating call of the plainfin midshipman fish, *Porichtys notatus*. In: Predators and Prey in Fishes (Noakes, D., Lindquist, D., Helfman, G. & Ward, J., eds). Dr W. Junk Publishers, The Netherlands, pp. 205—212.

Knapp, R., Wingfield, J. C. & Bass, A. H. 1999: Steroid hormones and parental care in the plainfin midshipman fish (*Porichthys notatus*). Horm. Behav. **35**, 81—89.

Krebs, J., Ashcroft, R. & Weber, M. 1978: Song repertoires and territorial defence in the great tit. Nature **271**, 539—542.

Ladich, F. 1989: Sound production by the river bullhead *Cottus gobio* L. (Cottidae, Teleostei). J. Fish Biol. **35**, 531—538.

Mann, D. A. 2006: Propagation of fish sounds. In: Communication in Fishes (Ladich, F., Collin, S. P., Moller, P. & Kapoor, B. G., eds). Science Publishers, Enfield, pp. 107—120.

Maruska, K., Boyle, K., Dewan, L. & Tricas, T. 2007: Sound production and spectral hearing sensitivity in the Hawaiian sergeant damselfish, *Abudefduf abdominalis*. J. Exp. Biol. **210**, 3990—4004.

McKibben, J. R. & Bass, A. H. 1998: Behavioural assessment of acoustic parameters relevant to signal recognition and preference in a vocal fish. J. Acoust. Soc. Am. **104**, 3520—3533.

Mitchell, S., Poland, J. & Fine, M. L. 2008: Does muscle fatigue limit advertisement calling in the oyster toadfish *Opsanus tau*? Anim. Behav. **76**, 1011—1016.

Modesto, T. & Canário, A. V. M. 2003a: Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, *Halobatrachus didactylus*. Gen. Comp. Endocrinol. **131**, 220—231.

Modesto, T. & Canário, A. V. M. 2003b: Hormonal control of the swimbladder sonic muscles dimorphism in the Lusitanian toadfish *Halobatrachus didactylus*. J. Exp. Biol. **206**, 3467—3477.

Myrberg, A. & Riggio, R. 1985: Acoustically mediated individual recognition by a coral reef fish (*Pomacentrus partitus*). Anim. Behav. **33**, 411—416.

Oliveira, R. F. & Almada, V. C. 1998: Maternal aggression during the mouthbrooding cycle in the cichlid fish, *Oreochromis mossambicus*. Aggress. Behav. **24**, 187—196.

Östlund-Nilsson, S. 2002: Does paternity or paternal investment determine the level of paternal care and does female choice explain egg stealing in the fifteen-spined stickleback? Behav. Ecol. **13**, 188—192.

Patzner, R. A., Seiwald, M., Adlgasser, M. & Kaurin, G. 1986: The reproduction of *Blennius pavo* (Teleostei, Blenniidae) V. Reproductive behavior in natural environment. Zool. Anz. **216**, 338—350.

Pope, D. 2000: Testing function of fiddler crab claw waving by manipulating social context. Behav. Ecol. Sociobiol. **47**, 432—437.

Rodgers, E. W., Earley, R. L. & Grober, M. S. 2006: Elevated 11-ketotestosterone during paternal behaviour in the Bluebanded goby (*Lythrypnus dalli*). Horm. Behav. **49**, 610—614.

Roux, C. 1986: Batrachoididae. In: Fishes of the North-Eastern Atlantic and Mediterranean (Whitehead, P. J. P., Bauchot, M. L., Hureau, J.C., Nielsen, J. & Tortonese, E., eds). UNESCO, Paris, pp. 1360—1361.

Ryan, M. J. 1988: Energy, calling and selection. Am. Zool. **28**, 885—898.

dos Santos, M. E., Modesto, T., Matos, R. J., Grober, M. S., Oliveira, R. F. & Canário, A. 2000: Sound production by the Lusitanian toadfish, *Halobatrachus didactylus*. Bioacoustics **10**, 309—321.

Schmitt, A., Schuster, M. & Barth, F. 1994: Vibratory communication in a wandering spider, *Cupiennius getazi*: female and male preferences for features of the conspecific male's releaser. Anim. Behav. **48**, 1155—1171.

Searcy, W. A. & Andersson, M. 1986: Sexual selection and the evolution of song. Annu. Rev. Ecol. Syst. **17**, 507—533.

Sinn, D., While, G. & Wapstra, E. 2008: Maternal care in a social lizard: links between female aggression and offspring fitness. Anim. Behav. **76**, 1249—1257.

Skoglund, C. R. 1961: Functional analysis of swimbladder muscles engaged in sound production in the toadfish. J. Biophys. Biochem. Cytol. **10**, 187—200.

Sparkes, T. C., Prater, C., Akamatsu, T. & Yan, H. 2002: Acoustic Signals and aggressive conflicts in the skunk loach *Botia morleti*: Integrating sensory and behavioural approaches. Bioacoustics **12**, 257—259.

Stebbins, R. C. & Cohen, N. W. 1997: A Natural History of Amphibians. Princeton Univ., Press, New Jersey.

Townsend, D., Stewart, M. & Pough, F. 1984: Male parental care and its adaptive significance in a neotropical frog. Anim. Behav. **32**, 421—431.

Tricas, T. C., Kajiura, S. M. & Kosaki, R. K. 2006: Acoustic communication in territorial butterflyfish: test of the sound production hypothesis. J. Exp. Biol. **209**, 4994—5004.

Vasconcelos, R. O. & Ladich, F. 2008: Development of vocalization, auditory sensitivity and acoustic communication in the Lusitanian toadfish *Halobatrachus didactylus*. J. Exp. Biol. **211**, 502—509.

Winn, H. E. 1967: Vocal facilitation and the biological significance of toadfish Sounds. In: Marine Bio-Acoustics, vol. 2 (Tavolga, W., ed). Pergamon Press, Oxford, pp. 281—304.

Winn, H. E. 1972: Acoustic discrimination by the toadfish with comments on signal systems. In: Behavior of Marine Animals, vol. 2 (Winn, H. E. & Olla, B. L., eds). Plenum, New York, pp. 361—385.

CHAPTER II

HEARING SENSITIVITY AND ENCODING OF CONSPECIFIC SIGNALS

(i) **Auditory saccular sensitivity of the vocal Lusitanian toadfish: low frequency tuning allows acoustic communication throughout the year**

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ORIGINAL PAPER

Auditory saccular sensitivity of the vocal Lusitanian toadfish: low frequency tuning allows acoustic communication throughout the year

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Abstract A novel form of auditory plasticity for enhanced detection of social signals was described in a teleost fish, *Porichthys notatus* (Batrachoididae, Porichthyinae). The seasonal onset of male calling coincides with inshore migration from deep waters by both sexes and increased female sensitivity to dominant frequencies of male calls. The closely related Lusitanian toadfish, *Halobatrachus didactylus*, (Batrachoididae, Halophryninae) also breeds seasonally and relies on acoustic communication to find mates but, instead, both sexes stay in estuaries and show vocal activity throughout the year. We investigated whether the sensitivity of the inner ear saccule of *H. didactylus* is seasonally plastic and sexually dimorphic. We recorded evoked potentials from populations of saccular hair cells from non-reproductive and reproductive males and females in response to 15–945 Hz tones. Saccular hair cells were most sensitive at 15–205 Hz (thresholds between 111 and 118 dB re. 1 μPa). Both sexes showed identical hearing sensitivity and no differences were found across seasons. The saccule was well suited to detect conspecific vocalizations and low frequencies that overlapped with lateral line sensitivity. We showed that the saccule in *H. didactylus* has major importance in acoustic communication throughout the year and that significant sensory differences may exist between the two batrachoidid subfamilies.

Keywords Hearing · Tuning · Saccule · Acoustic communication · Batrachoididae

Introduction

There is growing evidence that the auditory system of adult vertebrates is dynamic in the way it encodes sound and it is often optimized to detect behaviourally relevant signals that couple sender and receiver in acoustic communication as proposed by the matched filter hypothesis (Capranica and Moffat 1983; Kostarakos et al. 2008). The physiological state of the receiver, namely the hormonal status, can shape the "matched filter" of auditory receivers affecting the response properties of peripheral and/or central auditory systems as reported in anurans, birds and mammals, including humans (Guimarães et al. 2006; Lucas et al. 2007; Miranda and Wilczynski 2009). However, other examples which are not mutually exclusive support the alternative sensory exploitation hypothesis that can explain the mismatch in some Tungara frogs in which the auditory tuning of female receivers does not match the dominant frequencies of mate calls produced by male senders (Ryan et al. 1990; Ryan and Rand 1993). Comparative studies that examine mechanisms of vocal production and auditory reception across closely related species can ultimately provide valuable insight into the diversity and evolution of communication systems.

A novel form of auditory plasticity was demonstrated in a teleost fish, the plainfin midshipman fish *Porichthys*

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notatus (Batrachoididae, subfamily Porichthyinae). During the reproductive season, females migrate from deep offshore and become more sensitive, with increased accuracy of saccular afferent phase-locking, to higher dominant frequencies of male advertising calls that propagate better in the shallow nesting areas (Sisneros and Bass 2003). This seasonal plasticity of the females' auditory sense occurs at the level of the sensory hair-cell receptors in the saccule, the main auditory endorgan, and is sex steroid-dependent (Sisneros et al. 2004a, b, Sisneros 2009). This adaptive mechanism enhances the coupling between the sender and the receiver, promoting the acquisition of acoustic information that is likely needed and probably critical for mate detection, localization and selection (Sisneros et al. 2004a).

The closely related Lusitanian toadfish, *Halobatrachus didactylus*, (Batrachoididae, subfamily Halophryninae) is also a seasonally breeding species (Modesto and Canário 2003) but, contrary to *P. notatus*, may show vocal activity throughout the year depending on the temperature (Amorim et al. 2006) and both sexes stay in shallow estuary areas even during the winter non-reproductive season (Pereira 2006; Amorim et al. 2010). Males of this species exhibit an unusually rich vocal repertoire, producing acoustic signals to attract mates, defend territories and during other agonistic contexts (Amorim and Vasconcelos 2008; Amorim et al. 2008; Vasconcelos et al. 2010). The onset of sound production starts early in life, along with the development of agonistic behaviours and territorial activity (Vasconcelos and Ladich 2008). A recent study shows that both sexes can accurately perceive fine temporal, spectral and amplitude features of conspecific vocalizations (Vasconcelos et al. 2011).

Here, we investigated whether auditory saccular sensitivity of *H. didactylus* varies seasonally and if saccular sensitivity is sexually dimorphic at the level of the hair-cells receptors. We tested the hypothesis that saccular sensitivity does not change across seasons or sexes since both male and female of this species maintain vocal communication all year round despite seasonal variation of circulating sex steroid levels (Modesto and Canário 2003). We provide detailed data on the auditory sensitivity of the saccule, the main auditory endorgan in *H. didactylus* and in most teleosts (reviewed in Wysocki 2006), across a wide range of behaviourally relevant frequencies from 15 Hz to 945 Hz.

Methods

Test subjects

The test subjects were 93 Lusitanian toadfish adults caught in the Tagus estuary (Portugal) during the breeding season at the end of April–June (32 females: 25–35 cm total length (TL), 279–651 g total body mass (BM); 22 type I males: 23–43 cm TL, 197–1,267 mg BM), and winter, December-February (23 females: 23–41 cm TL, 188–655 g BM; 16 type I males: 24–38 cm TL, 274–896 g BM) by local fishermen (trawl) or collected directly from nests in intertidal areas during low tide. Fish were transported during the same day to the stock tanks (80 l) and maintained at 21 ± 1°C up to 15 days prior to the saccular potential recordings, in order to avoid effects of stress and captivity that may affect saccular sensitivity, namely possible reduction in sensitivity (Sisneros and Bass 2003). Females were first identified by their larger abdomens and/or their wider genital papilla with a genital pore. Type I males or parental nesting males were typically bigger than females, presented elongated genital papilla and generally released a secretion of their larger accessory glands when gently pressed near the anus (Modesto and Canário 2003). Type I males differed in size from type II males (sneaker males that attempt opportunistic fertilizations), which are similar in size to females but with more elongated genital papilla. Both sex and male type (type I vs. type II) were always confirmed by the dissection of the specimen after each experiment when the fish were sacrificed by immersion in a 0.025% ethyl *p*-aminobenzoate saltwater bath. The total length (TL), total (TW) and eviscerated (EW) weights, gonad (GW) and accessory testicular glands (AGW) weights were measured for each individual. The reproductive state of the specimens and the male type was verified according to Modesto and Canário (2003), by calculating the gonadosomatic index (GSI) as 100*GW/EW, as well as the accessory glands index (AGI) as 1,000*AGW/EW.

During the breeding season, GSI varied between 14 and 37% in non-spent females (before releasing the eggs) and 0.02–4% for type I males; during the winter, GSI was 0.7–10% in females and 0.06–4% in type I males. Consistent with Modesto and Canário (2003), a marked seasonal difference in GSI was found among females, but not in type I males. Spent females (GSI: 1–6%) collected from the nests during the breeding season were considered reproductive or breeding fish since they were found in the nest with type I males. No differences in saccular sensitivity were observed between spent and non-spent breeding female *P. notatus* collected during the breeding season (Sisneros, personal observation). Only four type II males were collected during the whole year and they were identified by their larger gonads and generally smaller accessory testicular glands (Apr–Jun: 3 fish, GSI: 8–9%, AGI: 1.1–1.2‰; Dec–Feb: 1 fish, GSI: 5%, AGI: 0.1‰), which was in contrast to type I males (Apr–Jun: AGI: 0.7–5‰; Dec–Feb: 0.6–4‰).

Saccular potential recording setup

The method for recording the saccular potential in the Lusitanan toadfish was based on the experimental procedure adopted by Sisneros (2007, 2009). Surgical procedures for exposing the inner ear saccule followed those in previous studies (Sisneros and Bass 2003; Sisneros 2007). Briefly, fish were first anesthetized in a 0.025% ethyl *p*-aminobenzoate saltwater bath and then immobilized by an intramuscular injection of pancuronium bromide (circa 2-4 mg/kg). The saccule was then exposed by dorsal craniotomy (see Fig. 1) and a barrier of denture cream was built up around the cranial cavity to allow the fish to be lowered below the water surface. A saline solution was used to prevent the cranial cavity from drying out and to clean eventual bleeding.

Test subjects were positioned below the water surface in the middle of a round plastic experimental tank (diameter: 36 cm, water depth: circa 18 cm), with the saccule kept at about 7 cm above the vibrating disc of the sound generating device. The tank was placed on a vibration isolated table inside a Faraday cage. All recording and sound generating equipment was located outside the recording room. Fish gills were perfused with saltwater through the mouth using a simple temperature-controlled (21 ± 2°C) gravity-fed water system.

Acoustic stimuli generated via the reference output signal of a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA) was delivered to the underwater sound generating device through a custom built amplifier. The sound generating device was composed of an immersed plexiglass disc (diameter: 8 cm) driven by a mechanical wave driver (SF9324, PASCO, Roseville, CA, USA) kept below the experimental tank. The disc was attached to the wave driver by a stainless steel rod which crossed the tank bottom through a water restraining flexible device, which not only prevented water drainage but also kept the rod vertically positioned. The sound stimuli were delivered via the vibrating disc placed at the midpoint of the tank. Auditory stimuli were calibrated before each experiment. Sound measurements were performed using a calibrated mini-hydrophone (8103 Brüel and Kjaer, Naerum, Denmark) positioned 7 cm above the disc, a position normally occupied by the fish's head during the recordings. The hydrophone was connected to an amplifier (2692 Nexus, Naerum, Denmark) and the acoustic signal recorded was digitized (Edirol UA-25, Roland Corporation, Tokyo, Japan) and monitored by a laptop running Audition 2.0 (Adobe Systems Inc., CA, USA), which was used to verify stimuli spectra and control the relative amplitudes of the auditory stimuli. Sound pressure levels (SPL) were measured using a sound level meter (Mediator, 2238 Brüel and Kjaer, Naerum, Denmark) connected to the mini-hydrophone. Stimuli amplitudes were equalized (130 ± 1 dB re 1 μPa) at all tested frequencies by a custom MatLab script (MathWorks, Inc., Natick, MA, USA) that controlled the signal output of the lock-in amplifier. Auditory stimuli consisted of eight repetitions of single 500 ms tones from 15 to 945 Hz (in 5–80 Hz increments) presented randomly at a rate of one every 1.5 s.

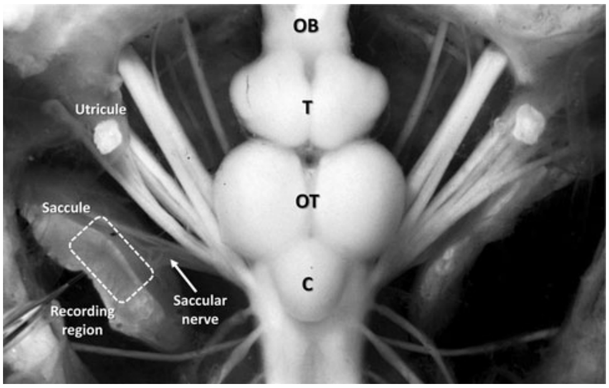

Fig. 1 Dorsal view of the brain and inner ears of the Lusitanian toadfish, *Halobatrachus didactylus*, obtained from a perfusion fixed specimen with paraformaldehyde. The recording region is indicated in the saccule, which was slightly deflected to improve visibility of the saccular nerve. *OB* olfactory bulb, *T* Telencephalon, *OT* optic tectum, *C* cerebellum

Although toadfishes possess no hearing specializations and thus are primarily sensitive to particle motion (Fay and Edds-Walton 1997), we report in this study hearing thresholds based on pressure measurements for both technical reasons and comparison purposes with previous studies using batrachoidid fish (e.g. Sisneros 2007, 2009). The intention of this study was to compare the saccular sensitivity of *H. didactylus* between sexes (male vs. female) and across seasons (non-reproductive vs. reproductive state) under identical experimental conditions. The data presented in sound pressure levels (SPL) to describe hearing sensitivities (i.e., thresholds based on saccular potential measurements) should not be considered in terms of absolute values but instead should be used as a mean to make quantifiable comparisons of relative differences in hearing sensitivity between the different groups. Moreover, the saccular potential recording technique used here and developed by Sisneros (2007) provide comparable data with other recently published studies that also adopted this technique (Sisneros 2007, Sisneros 2009; Rohmann and Bass unpublished). After finishing the data collection, calibration tests were performed using an underwater miniature acoustic pressure–particle acceleration (*p*–*a*) sensor S/N 2007-001 (Applied Physical Sciences Corp., Groton, CT, USA) provided by F. Ladich and showed that pressure and particle acceleration were positively correlated below the water surface in the experimental tank. In the primary axis of particle motion (the vertical *z* axis), a 3 dB change in SPL was generally accompanied by a 3 dB change in particle acceleration level for all stimuli.

Saccular potentials were recorded with glass electrodes filled with 3 M KCl (0.5–6 MΩ). Electrodes were visually guided and placed in the middle region of the saccular macula (see Fig. 1) in either the left or right saccule. The hair-cell orientation patterns are not known for *H. didactylus* and thus variations in the placement of the recording electrode in the saccular region could have resulted in variability in the auditory responses. In the closely related batrachoidid *Opsanus tau*, hair-cell orientation pattern in the saccule changes from rostrally oriented cells in the rostral region to vertical oriented cells in the middle to caudal regions (Edds-Walton and Popper 1995). Also in *P. notatus*, sensitivity differences were found among the rostral, middle and caudal regions of the saccule (Sisneros 2007, 2009). Hence, all the recordings performed in this study were from the middle recording region of the saccule in order to obtain data that could be comparable between different specimens. Saccular potentials were preamplified 10× (Getting 5A, Getting Instruments, San Diego, CA, USA), band-pass filtered (20–5,000 Hz, further 10× gain, SR650, Stanford Research Systems, Sunnyvale, CA, USA), inputted into the digital signal processor of the Lock-in amplifier (SR830) and stored on a PC computer running the custom MatLab control program. The auditory stimuli, evoked saccular potentials, and background noise were continuously monitored with an oscilloscope. The Lock-in amplifier converts the saccular potential response (RMS) into a DC voltage output signal that is proportional to the component of the signal whose frequency is exactly locked to the reference frequency. The second harmonic of the stimulus frequency was set as the reference frequency because the maximum evoked saccular potentials typically occur at twice the stimulation frequency. This phenomenon results from opposite-oriented hair cells within the saccular macula that respond in opposite phases of the stimulus in teleost fishes (Cohen and Winn 1967; Furukawa and Ishii 1967; Fay 1974).

To estimate auditory thresholds, the saccular potentials were recorded in response to single tone stimuli that were reduced in 3 dB steps until the saccular response (mean voltage of eight evoked saccular potential measurements) was no longer above background noise (mean voltage measured without acoustic stimulation) ± 2 SD (standard deviation). Background noise measurements were made prior to the recording of each threshold tuning curve and were averaged across eight measurements. The background noise levels (recorded with no auditory stimulation present) were consistently between 2 and 5 μV.

Iso-level responses were obtained by presenting all the single-tones at a sound pressure of 130 dB re 1 μPa and plotting the amplitude of the saccular responses. This sound amplitude was selected because it corresponds approximately to the recorded amplitude of a boatwhistle in close proximity (<0.5 m) to a vocalizing nesting fish (personal observations) and it was previously used in similar studies with other batrachoidids (McKibben and Bass 1999; Sisneros 2009). In order to calculate the relative gain of the saccular responses, the iso-level frequency response profiles were first converted to dB and then normalized by subtracting the dB value obtained for the best frequency. Best frequency (BF) was defined as the stimulation frequency that induced the greatest evoked saccular potential at the specific stimulus level of 130 dB re 1 μPa. This procedure allows a comparison of profiles with different absolute values but preserves the relation between frequencies (within a profile), thus cancelling the effect of the distance from the electrode to the macula epithelium. Average relative gain allowed comparing auditory amplitude responses between sexes and seasons.

Statistical analysis

The overall effects of sex (females vs. type I males), reproductive state (reproductive vs. non-reproductive) and stimulus frequency on the auditory thresholds and relative gain were analyzed with a repeated-measures ANOVA. This test analyzed responses (auditory thresholds or relative gain) to several frequencies in each subject fish (within-subject factor) of different sex and reproductive state (two between-subject factors). For the auditory threshold data analysis, only the frequency range up to 625 Hz was considered because some thresholds were missing at the higher frequencies. For the relative gain data, the whole frequency range tested (15–945 Hz) was considered for comparison purpose between groups. Parametric tests were used preferentially since data were normally distributed and variances were homogenous. Statistical tests were performed with Statistica 7.1 for Windows (StatSoft, Inc. 2005).

Results

Iso-level frequency responses

The evoked saccular potential response typically occurred at twice the stimulation frequency as expected (Fig. 2). Examples of iso-level response profiles of the evoked saccular potentials recorded in response to frequencies between 15 and 945 Hz at 130 dB re 1 μPa are shown in Fig. 3. The best frequency (BF) varied widely between specimens ranging from 15 to 205 Hz, but the majority or mode of BFs occurred at 15 Hz in both reproductive fish

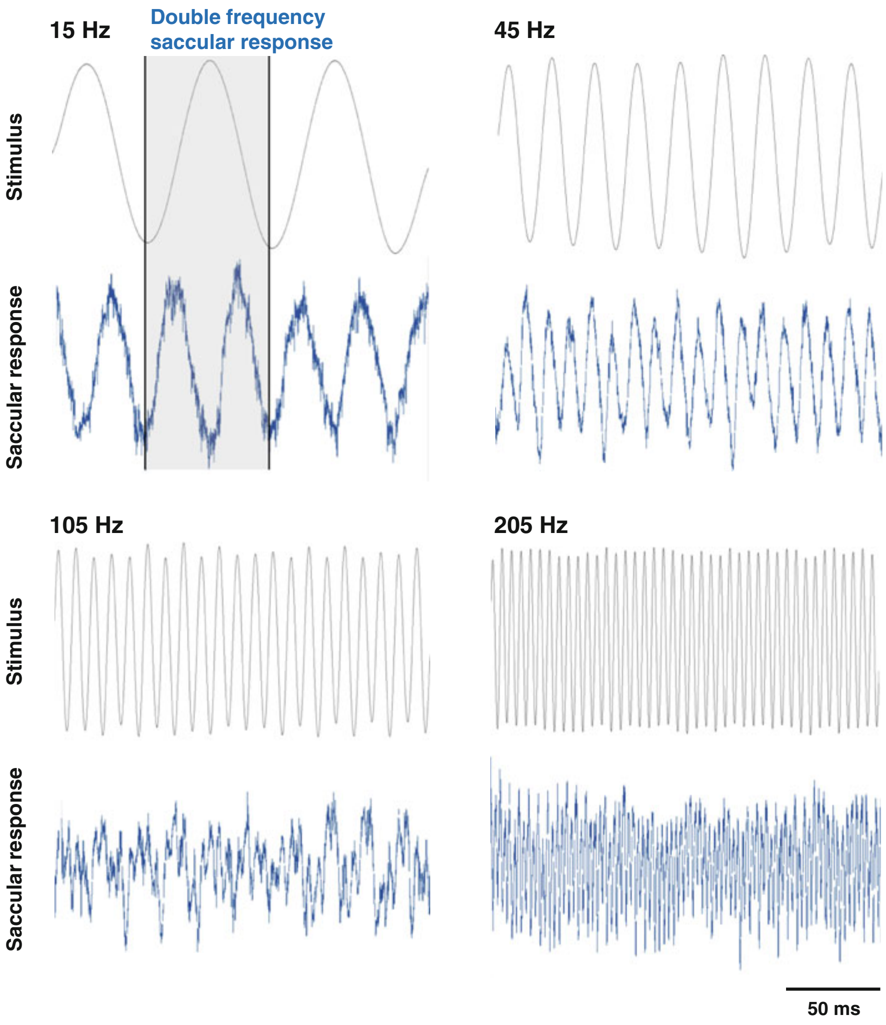

Fig. 2 Representative examples of the acoustic stimulation and evoked auditory responses obtained from the Lusitanian toadfish saccule under 130 dB re 1 μPa single tones. Note the frequency doubling effect present in the saccular responses most likely resulting from opposite oriented hair cells. Sound waveform and saccular response amplitudes were adjusted to enhance visibility

(24% of recordings) and non-reproductive fish (21% of recordings).

The relative gain (iso-level saccular potential data obtained at 130 dB re 1 μPa normalized to BF in each recording) varied on average between −7.2 dB (15 Hz) and −39.7 dB (865 Hz). The relative gain did not reveal overall significant differences between sexes or seasons (repeated measures ANOVA, $F_{1,159} = 0.2–2.1$, $P > 0.05$), with no interaction between the two variables (repeated measures ANOVA, $F_{1,159} = 1.1$, $P > 0.05$) as shown in Fig. 4.

Auditory saccular sensitivity

The auditory thresholds determined from populations of hair-cells located in the middle region of the saccule were on average between 111 and 118 dB re 1 μPa for the lowest frequencies (15–205 Hz) and increased gradually to thresholds of 145 dB re 1 μPa (the maximum amplitude tested) at the highest frequencies (Fig. 5). There were no significant differences in auditory thresholds between the sexes or seasons (repeated measures ANOVA, $F_{1,150} = 0–2.5$, $P > 0.05$, see Fig. 5) as well as no interaction between the two variables (repeated measures ANOVA, $F_{1,150} = 0.1$, $P > 0.05$).

The comparison between both sexes audiograms with the power spectra of conspecific vocalizations, recorded previously at 10–20 cm from calling adult toadfish (Vasconcelos et al. 2010), revealed that the main energy of boatwhistles and grunts coincided with the greatest saccular sensitivity range, i.e. frequencies lower than 205 Hz. Most of the energy associated with typical boatwhistles and grunts are well above the hearing thresholds for saccule hair cells reported here, up to 20 and 10 dB for peak frequencies contained in the boatwhistle and grunt, respectively (Fig. 6). In addition, saccular hair cells showed a remarkable sensitivity to frequencies as low as 15 Hz, where sound energy of conspecific calls is either greatly reduced or absent.

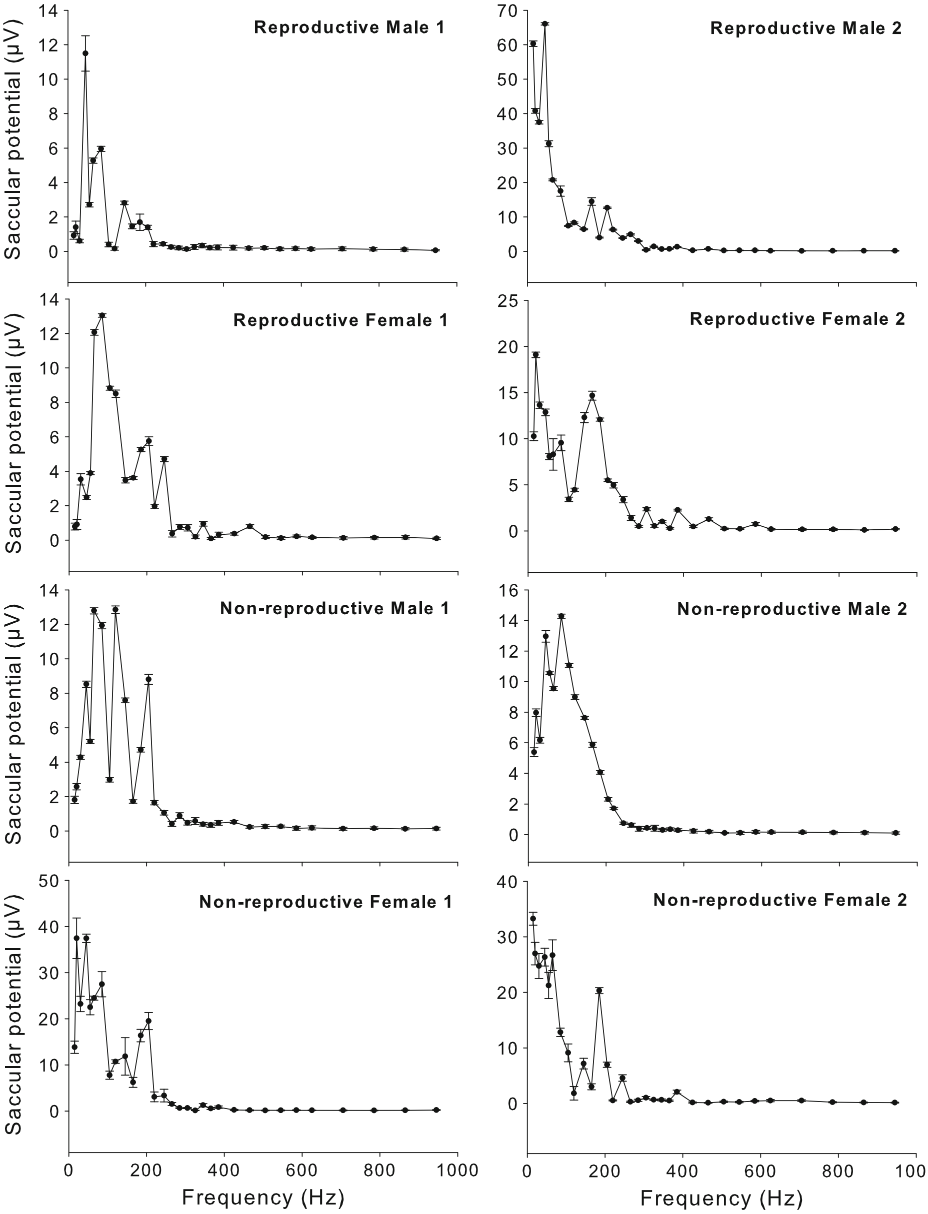

Fig. 3 Representative examples of iso-level curves (mean ± SD) of evoked saccular potentials recorded from the middle region of the saccule in different Lusitanian toadfish. Auditory responses shown were obtained at 130 dB re 1 µPa from both males and females, during reproductive and non-reproductive seasons

Discussion

The physiological state of the receiver is known to shape the auditory response properties in several vertebrates such that behaviourally relevant signals can be better detected and encoded by the auditory system (matched filter hypothesis, Capranica and Moffat 1983; Kostarakos et al. 2008). A novel form of auditory plasticity that enhances the coupling between sender and receiver has been reported in the teleost fish, *P. notatus* (Batrachoididae), and revealed that females become more sensitive to the dominant frequency components of male advertising calls during the breeding season (Sisneros et al. 2004a; Sisneros 2009). However, it is not known whether other seasonal-reproductive teleost species that rely heavily on acoustic signalling during social life show similar auditory plasticity to optimize vocal communication.

The closely related Lusitanian toadfish *H. didactylus*, although from a different subfamily, is also a seasonal breeding species where mate finding is mediated by acoustic communication. Since vocal communication is maintained throughout the year in *H. didactylus* (Amorim et al. 2006, 2010), contrary to *P. notatus*, we hypothesised that saccular sensitivity does not change seasonally, despite the seasonal variation of sex steroids levels related to the reproductive state (Modesto and Canário 2003).

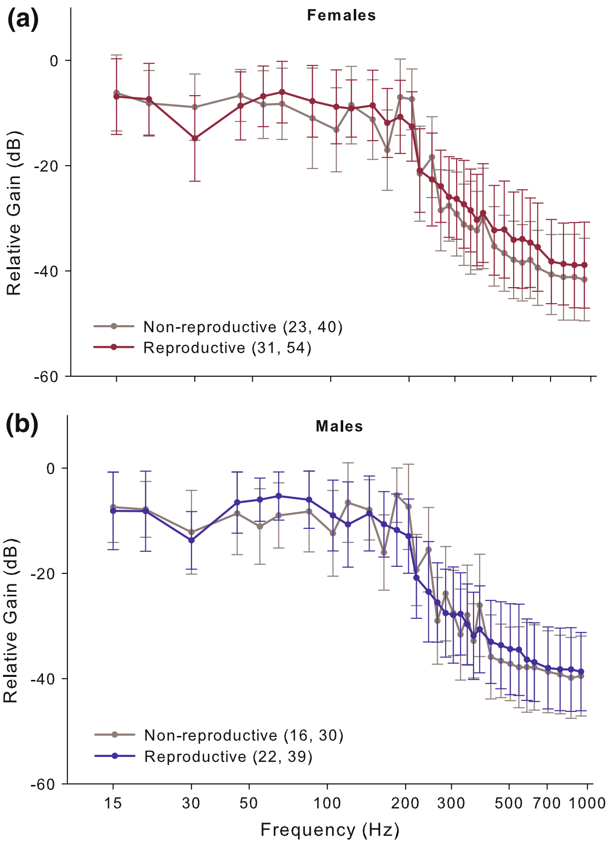

Fig. 4 Mean (±SD) iso-level response curves of saccular evoked potentials obtained at 130 dB re 1 μPa in Lusitanian toadfish females (**a**) and males (**b**) during reproductive and non-reproductive seasons. Iso-level response data were normalized to a relative value of 0 dB assigned to the peak response for each record and then expressed in relative dB, i.e. relative to the Best Frequency sensitivity for that record. Number of animals and records per group are indicated in *parentheses*

Toadfish auditory sensitivity: seasonal plasticity or sensory stability?

Our data indicated that the saccular sensitivity of the Lusitanian toadfish does not change seasonally between end of April-June (breeding season) and December–February (non-breeding season). Similarly to other species that display conspicuous reproductive behaviour, the Lusitanian toadfish undergoes marked seasonal changes in circulating levels of sex steroids. In females, circulating plasma levels of estradiol (E_2) increases considerably prior to the breeding season (around May) during vitellogenesis, whereas in nesting males testosterone (T) and 11-KT (11-ketotestosterone) levels peak in June during spermatogenesis (Modesto and Canário 2003). Our results suggest that the seasonal variations of circulating sex steroids levels in this species (Modesto and Canário 2003) are not correlated with any changes in auditory sensitivity throughout the annual reproductive cycle. Nevertheless, future studies that examine both auditory saccular sensitivity and hormonal levels would be useful to resolve whether there are any slight effects of sex steroids on auditory hair cell tuning or phase-locking accuracy in this teleost species.

The unchanging perception of acoustic communication signals, independent of the reproductive state, in the Lusitanian toadfish is consistent with behavioural and ecological observations. Lusitanian toadfish exhibits an unusually large vocal repertoire composed of at least five distinct vocalisations produced in different social contexts, such as mate attraction, establishment of territories and other agonistic interactions (Amorim and Vasconcelos 2008; Vasconcelos et al. 2010) and the full acoustic repertoire can be detected all year round (Amorim et al. 2006, 2010; Vasconcelos and Ladich 2008). This species breeds in estuarine shallow waters and remains in the estuaries (Pereira 2006), including in subtidal areas (Amorim et al. 2010), during the winter non-breeding season. Vocal behaviour outside the breeding season is most likely

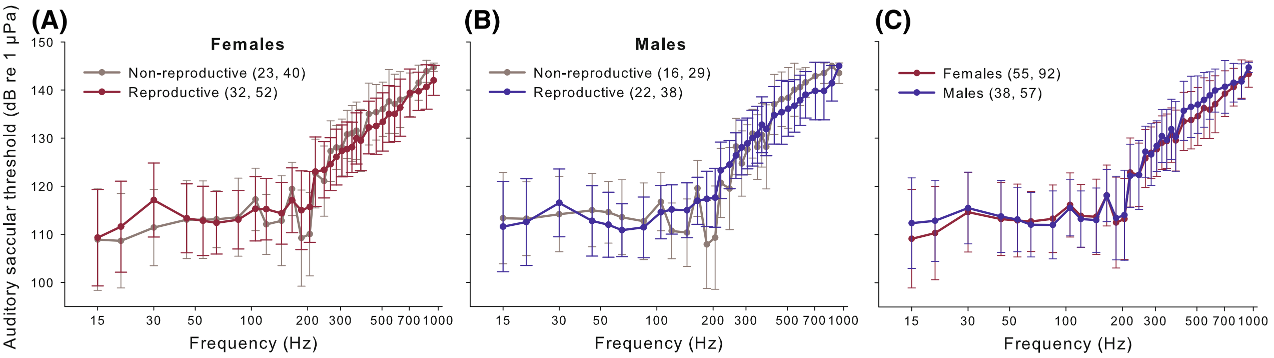

Fig. 5 Comparison between mean (±SD) auditory threshold curves from reproductive and non-reproductive Lusitanian toadfish females (**a**) and males (**b**). All data obtained from both females and males were plotted together to compare threshold curves between sexes (**c**). Number of animals and records per group are indicated in *parentheses*

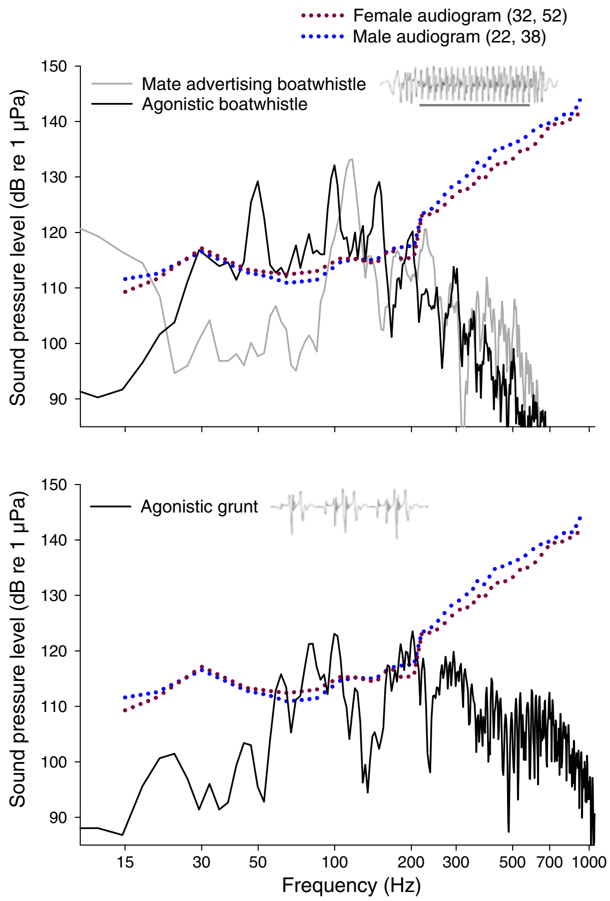

Fig. 6 Comparison between the saccular sensitivity of reproductive toadfish and the power spectra of conspecific mate advertising boatwhistle and agonistic grunt recorded at 10–20 cm from a calling adult toadfish. Sampling frequency 6 kHz, filter bandwidth 10 Hz, 75% overlap, Hamming window. Number of animals and records per group are indicated in *parentheses*

associated with disputes for the access of suitable shelters and feeding sites. In addition, sound production seems remarkably important throughout life, starting in early developmental stages when it is used during food and space competition (personal observations). Moreover, *H. didactylus* can only be found along the Eastern Atlantic, from the Tagus estuary (Portugal) down to the Guinea Gulf, and in the Mediterranean (Roux 1986). Such geographical distribution suggests that this species probably evolved to communicate also in tropical areas where temperature remains more stable and thus a seasonal reproduction and a plastic auditory sensitivity are not required.

Conversely, *P. notatus* females inhabit deeper low-temperature waters during most of the year along the Pacific coast of North America and experience a dramatic change in their environment when they migrate to the intertidal nesting areas to spawn (Sisneros et al. 2004a). Females undergo seasonal sex steroid-dependent changes in the hearing thresholds at the level of hair-cell receptors within the saccule macula (Sisneros 2009). Estrogen receptor alpha was found in the inner ear's saccular epithelium in females and the annual spike in estrogen levels prior to the breeding season appears to be responsible for the induced changes that enhance the encoding of the dominant harmonics of male advertising calls (Sisneros and Bass 2003; Sisneros 2004a, b). Moreover, nothing has been described in terms of vocal activity in adult *P. notatus* during the non-breeding season or in juveniles that are clearly non-territorial. Vocal activity in this species seems to start later in life probably associated with sexual maturity and reproduction behaviours (personal observations).

Although *H. didactylus* and *P. notatus* belong to the same family Batrachoididae, they are classified in different subfamilies (Halophryninae and Porichthyinae, revised by Greenfield et al. 2008) and nothing is known about when these subfamilies diverged and how closely related they are. In fact, previous work showed that there is a notable difference in the forebrain nuclei responsible for generating vocalizations in species from two different subfamilies within Batrachoididae (Batrachoidinae and Porichthinae, Fine and Perini 1994). Such differences in the vocal motor now combined with our auditory sensitivity data suggest that there may be major differences in the nervous system of the subfamilies within the Batrachoididae.

The auditory plasticity and the influence of the hormonal or reproductive state (and sex) on the auditory receptors and processing in acoustic communication systems remains poorly understood. The influence of seasonal changes in the response properties of the peripheral and/or central auditory system have been described in other vertebrate taxa. For example, Lucas et al. (2002, 2007) measured auditory evoked potentials in several bird species and described seasonal changes in the response amplitude and latency correlated with their vocal complexity. Goense and Feng (2005) reported seasonal changes in frequency tuning and temporal processing in the midbrain torus semicircularis of the Northern leopard frog where a smaller number of neurons sensitive to low-frequencies (100–500 Hz) in winter show increased phase locking in late spring. Although no differences were found in the auditory thresholds, these seasonal changes in phase locking accuracy may facilitate call recognition and/or localization of conspecific calling males. Recently, Gordon and Gerhardt (2009) showed hormonal-induced phonotaxis in green treefrog females treated with estradiol and progesterone. Similar hormone-induced changes in hearing sensitivity have been reported in humans. Psychoacoustic experiments in women showed differences in the auditory perception depending on the phase of the menstrual cycle (e.g. Swanson and Dengerink 1988). Conversely, other examples in vertebrates, namely in frogs, point to a divergence between the auditory system sensitivity and the frequency content of the vocal output that results in the mismatch between the auditory tuning of the receiver and the dominant frequencies of the mate call produced by the sender. These types of mismatches have been used to support the sensory exploitation model for sexual selection and evolution of communication signals (Ryan et al. 1990). However, remains unclear whether there is a hormonal influence in the frequency tuning of the two sexes (eg. Narins and Capranica 1976), as well as the presence of the estrogen receptor in the frog's inner ear.

Auditory sensitivity among different sexes

Our results also point to identical saccular sensitivity between nesting type I males and females. The lack of sexual dimorphism in the auditory sense of *H. didactylus* suggests that the perception of acoustic communication signals is potentially important for both sexes. Parental males nest in aggregations and vocalize in choruses to attract females and behavioural observations suggest that nesting males interact acoustically altering their own boatwhistle calling rate in response to other calling males (Amorim et al. 2011). Moreover, nesting males produce boatwhistles as well as other sound types during territorial intrusions indicating that acoustic signals also have an important role in male–male assessment and territorial defense (Vasconcelos et al. 2010). These facts suggest that the auditory system of nesting males must be adapted to detect and resolve acoustic parameters of boatwhistles (and other sound types) similar to females, which probably select mates based on acoustic cues (Amorim and Vasconcelos 2008; Amorim et al. 2010). Moreover, Vasconcelos et al. (2011) measured auditory evoked potentials (AEP), i.e. overall synchronous neural electric activity from the endorgans, VIII nerve and CNS auditory nuclei induced by acoustic stimulation, and demonstrated that both females and males can accurately resolve fine temporal features of both pulsed grunts and tonal boatwhistles and spectral content and amplitude modulation of boatwhistles.

Sisneros (2009), based on recordings of saccular potentials, showed that reproductive *P. notatus* females had saccular thresholds 8–13 dB lower than non-reproductive females across a wide frequency range, which included the dominant higher harmonics of the male advertising call. This most likely enhances mate detection, recognition, and localization during the breeding season. Recent work by Rohmann and Bass (unpublished) suggest that *P. notatus* nesting (type I) males also undergo reproductive-dependent changes in auditory saccular sensitivity. Here, we provide the first data on the saccular tuning in the Lusitanian toadfish and show that similar saccular sensitivity is found in both sexes.

Functional significance of the saccule's frequency tuning

The iso-intensity potential recordings indicated that saccular hair-cells of the Lusitanian toadfish were most sensitive to frequencies below 205 Hz, at a behaviourally relevant sound level of 130 dB re 1 μPa. We demonstrated not only that saccular sensitivity matches the frequencies with greatest energy in agonistic and advertising vocalizations, but also that hearing thresholds were well below (circa 10–20 dB) the peak amplitudes of the dominant frequencies that compose the conspecific vocalizations. A similar match between audiograms and conspecific sound spectra of the Lusitanian toadfish, but based on AEPs, was also described in Vasconcelos et al. (2007) and Vasconcelos and Ladich (2008).

Correlation between saccular sensitivity and the main energy of conspecific sounds was also found in *P. notatus* (Sisneros 2007). Similarly, Fay and Edds-Walton (1997) reported an identical match between saccular afferent sensitivity and the dominant (fundamental) frequency of vocal signals in the batrachoidid *O. tau*. Other studies in batrachoidids, based on extracellular recordings of saccular afferents or auditory brainstem units described similar observations (e.g. *O. tau*, Yan et al. 2000; Fay and Edds-Walton 2000; *P. notatus,* McKibben and Bass 1999; Weeg et al. 2002; Sisneros and Bass 2005). Such correspondence between the spectral content of vocalizations and hearing sensitivity has been reported not only in other fish taxa (e.g. Stabentheiner 1988; Ladich and Yan 1998), but also in other animal groups (e.g. insects, anurans, birds, Dooling et al. 2000; Gerhardt and Huber 2002).

Additionally, we provide evidence that the saccule can detect very low frequencies outside the frequency range of conspecific vocalizations, potentially overlapping with the lateral line sensitivity (e.g. Coombs and Janssen 1990; Weeg and Bass 2002). Prior to these experiments, saccular sensitivity of batrachoidid fish has only been investigated at frequencies as low as 30–75 Hz (e.g. Cohen and Winn 1967; Fay and Edds-Walton 1997; Sisneros 2007). Here, we present data on the saccular sensitivity as low as 15 Hz, which corresponded to the best stimulation frequency that induced the greatest evoked saccular potential (BF) in most specimens. Cohen and Winn (1967), using the saccular potential recording technique, also reported best auditory sensitivity at frequencies as low as 30 Hz in *P. notatus*. Although lateral line sensitivity has never been determined in *H. didactylus*, we predict an overlap in sensitivity between auditory and this other mechanosensory sense. Our finding suggests a potential adaptation of this species to detect low frequencies potentially important to enhance detection of conspecifics and/or predator/prey items when close to the biologically relevant sound source.

Phylogenetic analysis indicated that *H. didactylus* probably represents a basal lineage in the Batrachoididae (Rice and Bass 2009), providing an excellent model for understanding integrated mechanisms underlying the evolution of hearing and acoustic communication in fishes. Future studies should evaluate the contribution of the different otolithic endorgans, also utricle and lagena, for the auditory sensory coding; as well as verify the specific role of the inner ear and lateral line for sound detection.

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References

Amorim MCP, Vasconcelos RO (2008) Variability in the mating calls of the Lusitanian toadfish *Halobatrachus didactylus*: cues for potential individual recognition. J Fish Biol 73:1267–1283

Amorim MCP, Vasconcelos RO, Marques JF, Almada F (2006) Seasonal variation of sound production in the Lusitanian toadfish *Halobatrachus didactylus*. J Fish Biol 69:1892–1899

Amorim MCP, Simões JM, Fonseca PJ (2008) Acoustic communication in the Lusitanian toadfish, *Halobatrachus didactylus*: evidence for an unusual large vocal repertoire. J Mar Biol Assoc UK 88:1069–1073

Amorim MCP, Simões JM, Fonseca PJ, Almada VC (2010) Patterns of shelter usage and social aggregation by the vocal Lusitanian toadfish. Mar Biol 157:495–503

Amorim MCP, Simões JM, Almada VC, Fonseca PJ (2011) Stereotypy and variation of the mating call in the Lusitanian toadfish, *Halobatrachus didactylus*. Behav Ecol Sociobiol 65:707–716

Capranica RR, Moffat JM (1983) Neurobehavioral correlates of sound communication in anurans. In: Ewert JP, Capranica RR, Ingle DJ (eds) Advances in vertebrate neuroethology. Plenum Press, New York, pp 701–730

Cohen MJ, Winn HE (1967) Electrophysiological observations on hearing and sound production in the fish, *Porichthys notatus*. J Exp Zool 165:355–369

Coombs S, Janssen J (1990) Behavioral and neurophysiological assessment of lateral line sensitivity in the mottled sculpin *Cottus bairdi*. J Comp Physiol 167:557–568

Dooling RJ, Lohr B, Dent ML (2000) Hearing in birds and reptiles. In: Dooling RJ, Fay RR, Popper AH (eds) Comparative hearing: birds and reptiles. Springer, New York, pp 308–359

Edds-Walton PL, Popper AN (1995) Hair cell orientation patterns on the saccules of juvenile and adult toadfish *Opsanus tau*. Acta Zool 7:257–265

Fay RR (1974) Sound Reception and processing in the carp: saccular potentials. Comp Biochem Physiol A 49:29–42

Fay RR, Edds-Walton PL (1997) Diversity in frequency properties of saccular afferents of the toadfish, *Opsanus tau*. Hear Res 113:235–246

Fay RR, Edds-Walton PL (2000) Frequency response of auditory brainstem units in toadfish (*Opsanus tau*). Biol Bull 199:173–174

Fine ML, Perini MA (1994) Sound production evoked by electrical stimulation of the forebrain in the oyster toadfish. J Comp Physiol A 174:173–185

Furukawa T, Ishii Y (1967) Neurophysiological studies on hearing in goldfish. J Neurophysiol 30:1377–1403

Gerhardt HC, Huber F (2002) Acoustic communication in insects and anurans. University of Chicago Press, Chicago

Goense JBM, Feng AS (2005) Seasonal changes in frequency tuning and temporal processing in single neurons in the frog auditory midbrain. J Neurobiol 65:22–36

Gordon NM, Gerhardt HC (2009) Hormonal modulation of phonotaxis and advertisement-call preferences in the gray treefrog (*Hyla versicolor*). Horm Behav 55:121–127

Greenfield DW, Winterbottom R, Collette BB (2008) Review of the toadfish genera (Teleostei: Batrachoididae). Proc Calif Acad Sci Fourth Series 59:665–710

Guimarães P, Frisina ST, Mapes F, Tadros SF, Frisina DR, Frisina RD (2006) Progestin negatively affects hearing in aged women. Proc Natl Acad Sci USA 103:14246–14249

Kostarakos K, Hartbauer M, Römer H (2008) Matched filters, mate choice and the evolution of sexually selected traits 8. PLoS ONE 3(8):e3005

Ladich F, Yan HY (1998) Correlation between auditory sensitivity and vocalization in anabantoid fishes. J Comp Physiol A 182:737–746

Lucas JR, Freeberg TM, Krishnan A, Long GR (2002) A comparative study of avian auditory brainstem responses: correlations with phylogeny and vocal complexity, and seasonal effects. J Comp Physiol A 188:981–992

Lucas JR, Freeberg TM, Long GR, Krishnan A (2007) Seasonal variation in avian auditory evoked responses to tones: a comparative analysis of carolina chickadees, tufted titmice, and white-breasted nuthatches. J Comp Physiol A 193:201–215

McKibben JR, Bass AH (1999) Peripheral encoding of behaviorally relevant acoustic signals in a vocal fish: single tones. J Comp Physiol A 184:563–576

Miranda JA, Wilczynski W (2009) Female reproductive state influences the auditory midbrain response. J Comp Physiol A 195:341–349

Modesto T, Canário AVM (2003) Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, *Halobatrachus didactylus*. Gen Comp Endocrinol 131:220–231

Narins PM, Capranica RR (1976) Sexual differences in the auditory system of the tree frog *Eleutherodactylus coqui*. Science 192:378

Pereira T (2006) Biology and reproductive ecology of the Lusitanian toadfish *Halobatrachus didactylus* (Bloch & Schneider, 1801) in the Tagus estuary. M.Sc. Dissertation, University of Lisbon

Rice AN, Bass AH (2009) Novel vocal repertoire and paired swimbladders of the three-spined toadfish, *Batrachomoeus trispinosus*: insights into the diversity of the Batrachoididae. J Exp Biol 212:1377–1391

Roux C (1986) Batrachoididae. In: Whitehead PJP, Bauchot ML, Hureau JC, Nielsen J, Tortonese E (eds) Fishes of the North-Eastern Atlantic and Mediterranean, vol 3. UNESCO, Paris, pp 1360–1361

Ryan MJ, Rand AS (1993) Phylogenetic patterns of behavioral mate recognition systems in the *Physalaemus pustulosus* species group (Anura: Leptodactylidae): the role of ancestral and derived characters and sensory exploitation. In: Lees DR, Edwards D (eds) Evolutionary patterns and processes. Academic Press, London, pp 251–267

Ryan MJ, Fox JH, Wilczynski W, Rand AS (1990) Sexual selection for sensory exploitation in the frog *Physalaemus pustulosus*. Nature 343:66

Sisneros JA (2007) Saccular potentials of the vocal plainfin midshipman fish, *Porichthys notatus*. J Comp Physiol A 193:413–424

Sisneros JA (2009) Seasonal plasticity of auditory saccular sensitivity in the vocal plainfin midshipman fish, *Porichthys notatus*. J Neurophysiol 102:1121–1131

Sisneros JA, Bass AH (2003) Seasonal plasticity of peripheral auditory frequency sensitivity. J Neurosci 23:1049–1058

Sisneros JA, Bass AH (2005) Ontogenetic changes in the response properties of individual, primary auditory afferents in the vocal plainfin midshipman fish *Porichtys notatus* Girard. J Exp Biol 208:3121–3131

Sisneros JA, Forlano PM, Deitcher DL, Bass AH (2004a) Steroid-dependent auditory plasticity leads to adaptive coupling of sender and receiver. Science 305:404–407

Sisneros JA, Forlano PM, Knapp R, Bass AH (2004b) Seasonal variation of steroid hormone levels in an intertidal-nesting fish, the vocal plainfin midshipman. Gen Comp Endocrinol 136:101–116

Stabentheiner A (1988) Correlations between hearing and sound production in piranhas. J Comp Physiol A 162:67–76

Swanson SJ, Dengerink HA (1988) Changes in pure-tone thresholds and temporary thresholds shifts as a function of menstrual cycle and oral contraceptives. J Speech Hear Res 31:569–574

Vasconcelos RO, Ladich F (2008) Development of vocalization, auditory sensitivity and acoustic communication in the Lusitanian toadfish *Halobatrachus didactylus*. J Exp Biol 211:502–509

Vasconcelos RO, Amorim MCP, Ladich F (2007) Effects of ship noise on the detectability of communication signals in the Lusitanian toadfish. J Exp Biol 210:2104–2112

Vasconcelos RO, Simões JM, Almada VC, Fonseca PJ, Amorim MCP (2010) Vocal behaviour during territorial intrusions in the Lusitanian toadfish: boatwhistles also function as territorial 'keep-out' signals. Ethology 116:155–165

Vasconcelos RO, Fonseca PJ, Amorim MCP, Ladich F (2011) Representation of complex vocalizations in the Lusitanian and amplitude discrimination toadfish auditory system: evidence of fine temporal, frequency and amplitude discrimination. Proc R Soc B 278:826–834

Weeg MS, Bass AH (2002) Frequency response properties of lateral line superficial neuromasts in a vocal fish, with evidence for acoustic sensitivity. J Neurophysiol 88:1252–1262

Weeg M, Fay R, Bass AH (2002) Directionality and frequency tuning of primary saccular afferents of a vocal fish, the plainfin midshipman (*Porichthys notatus*). J Comp Physiol A 188:631–641

Wysocki LE (2006) Detection of communication sounds. In: Ladich F, Collin SP, Moller P, Kapoor BG (eds) Communication in fishes, vol 1. Science Publishers, Enfield, pp 177–205

Yan HY, Fine ML, Horn NS, Colon WE (2000) Variability in the role of the gasbladder in fish audition. J Comp Physiol A 187:371–379

CHAPTER II

HEARING SENSITIVITY AND ENCODING OF CONSPECIFIC SIGNALS

(ii) **Representation of complex vocalizations in the Lusitanian toadfish auditory system: evidence of fine temporal, frequency and amplitude discrimination**

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Representation of complex vocalizations in the Lusitanian toadfish auditory system: evidence of fine temporal, frequency and amplitude discrimination

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Many fishes rely on their auditory skills to interpret crucial information about predators and prey, and to communicate intraspecifically. Few studies, however, have examined how complex natural sounds are perceived in fishes. We investigated the representation of conspecific mating and agonistic calls in the auditory system of the Lusitanian toadfish *Halobatrachus didactylus*, and analysed auditory responses to heterospecific signals from ecologically relevant species: a sympatric vocal fish (meagre *Argyrosomus regius*) and a potential predator (dolphin *Tursiops truncatus*). Using auditory evoked potential (AEP) recordings, we showed that both sexes can resolve fine features of conspecific calls. The toadfish auditory system was most sensitive to frequencies well represented in the conspecific vocalizations (namely the mating boatwhistle), and revealed a fine representation of duration and pulsed structure of agonistic and mating calls. Stimuli and corresponding AEP amplitudes were highly correlated, indicating an accurate encoding of amplitude modulation. Moreover, Lusitanian toadfish were able to detect *T. truncatus* foraging sounds and *A. regius* calls, although at higher amplitudes. We provide strong evidence that the auditory system of a vocal fish, lacking accessory hearing structures, is capable of resolving fine features of complex vocalizations that are probably important for intraspecific communication and other relevant stimuli from the auditory scene.

Keywords: hearing; temporal encoding; amplitude modulation detection; auditory evoked potential; conspecific sounds; Batrachoididae

1. INTRODUCTION

Fishes depend on their auditory system to interpret information from the acoustic environment, including predator and prey detection (e.g. [1]), and to communicate acoustically. Many teleosts have evolved a variety of sound-producing mechanisms and vocalizations that are crucial not only for mate attraction but also during social interactions [2,3]. Temporal characteristics of sounds are thought to be the most important for acoustic communication in fishes because most calls consist of series of short broad-band pulses (e.g. gudgeons, gouramis, catfishes [4,5]). Sound variability, however, also relies on other differences, such as dominant frequency, and less commonly on frequency and amplitude modulation [6]. This variability plays a role in the social life of fishes by providing information to assess the size of the calling individual (e.g. dominant frequency [7,8]), to identify motivation for mating (e.g. calling rate [9,10]) and to recognize conspecifics from other vocally closely related species (e.g. [11–13]). Behavioural observations have shown that fishes can respond selectively to acoustic stimuli varying in temporal patterns and frequency content. Playback experiments with the toadfish *Opsanus tau* and the midshipman fish *Porichthys notatus* (Batrachoididae) demonstrated that males may alter their own calling rate in response to another male calling [14] and that females move towards the sound source depending on the signal temporal content, frequency and amplitude (including modulation) [15]. Temporal patterns, frequency and amplitude modulation of sounds are clearly important for acoustic communication in other taxa such as insects, anurans, birds and mammals (e.g. [16–20]).

Most studies on fish audition have used artificial stimuli to test hearing abilities (e.g. [12,21–26]). Accordingly, the representation of complex conspecific sounds in a fish's auditory system remains almost uninvestigated. To date, only two studies have examined how conspecific sounds (mostly short and pulsed calls) are represented in the auditory pathway. Wysocki & Ladich [27] analysed auditory evoked potentials (AEPs) in response to conspecific sounds in fishes possessing accessory morphological structures for enhancing hearing sensitivity (catfishes *Platydoras costatus* and *Pimelodus pictus*, loach *Botia modesta* and gourami *Trichopsis vittata*) and in a species lacking specializations, the sunfish *Lepomis gibbosus*. Species possessing hearing specializations generally showed an

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accurate representation of sound temporal patterns, amplitude fluctuations and, solely in *P. pictus*, a clear representation of the harmonics of its drumming sounds. In contrast, *L. gibbosus* did not exhibit an accurate temporal or frequency resolution. More recently, Maruska & Tricas [28] analysed the response properties of central auditory neurons to conspecific signals in a species without specializations, the damselfish *Abudefduf abdominalis*. The authors found that thresholds were lower in the midbrain than the hindbrain, and that the species was most sensitive to the frequency and temporal components of its natural pulsed calls.

Representatives of the family Batrachoididae (Teleostei, Actinopterygii), which include toadfishes and the plainfin midshipman fish, have emerged as one of the main study models for both behavioural and neurobiological studies in fish acoustic communication [29]. The rich vocal repertoire in this group is rare among fishes and includes long tonal advertising sounds. This suggests that the sensory system of batrachoidids is probably adapted to encode different sound characteristics of communication signals. The present study was designed to investigate the representation of complex conspecific sounds in the auditory system of the Lusitanian toadfish *Halobatrachus didactylus* (Bloch & Schneider 1801). This species produces at least five different vocalizations [13,30] and some sound characteristics, such as pulse interval, dominant frequency and amplitude of the agonistic calls, are correlated with fish size [31]. Besides, the complex amplitude-modulated advertising sounds (boatwhistles) reveal individual differences that may provide cues for mate choice and assessment of opponents [32].

Our primary goal was to investigate whether temporal patterns, amplitude modulation and frequency content of agonistic grunts and mating boatwhistles are encoded by the toadfish auditory brainstem. We also analysed the auditory responses to sounds from two ecologically relevant species—a sympatric vocal fish (meagre *Argyrosomus regius*) and a potential predator (bottlenose dolphin *Tursiops truncatus* [1])—in order to evaluate the extent to which this species is adapted to interpret other relevant information from its acoustic environment.

2. MATERIAL AND METHODS

(a) *Animals*

The test subjects were 16 Lusitanian toadfish: eight females (23–28 cm total length, TL) and eight type I males (25–32 cm TL), caught in the Tagus estuary (Portugal) during the breeding season (late June) by local fishermen. Fish were kept in two 250 l tanks for two weeks prior to the auditory recordings. Females were identified by their larger abdomens and/or their wider genital papilla. Type I males, which possess smaller gonads but larger accessory glands and more developed sonic muscles [33], were recognized by the secretion of their accessory glands.

(b) *Auditory evoked potential recording setup*

The AEP technique is a non-invasive method that records the overall synchronous neural electric activity induced by acoustic stimulation [34] and proved to be valuable for studying the perception of conspecific vocalizations (e.g. porpoises [35]; teleost fishes [27]). Test subjects were mildly immobilized with Flaxedil (gallamine triethiodide; Sigma-Aldrich, Austria; dosage 4.8–15.0 $\mu g\ g^{-1}$) and placed just below the water surface in an oval-shaped plastic tub (diameter 45 × 30 cm; water depth 12 cm) with the exception of the electrode contacting points. Fish respiration was secured through a simple temperature-controlled ($21 \pm 1°C$) gravity-fed water system using a pipette inserted into the subject's mouth. The recording electrode was located above the brainstem and the reference electrode approximately 2 cm rostrally (silver wire, 0.25 mm diameter). Shielded electrode leads were attached to the differential input of an AC pre-amplifier (Grass P-55, Grass Instruments, USA; gain 100×, high-pass at 30 Hz, low-pass at 1 kHz). A ground electrode was placed in the water near the fish body. A hydrophone (Brüel and Kjaer 8101, Denmark; voltage sensitivity −184 dB re 1 V μPa^{-1}) was placed on the right side of the subject near the inner ear (approx. 2 cm away) in order to control for stimulus characteristics. The experimental tub was positioned on an air table (TMC Micro-g 63–540, Technical Manufacturing Corporation, USA) inside a walk-in soundproof room. Both sound stimuli and AEP waveforms were recorded using a Tucker-Davis Technologies (USA) modular rack-mount system (TDT System 3) controlled by a computer containing a TDT digital processing board and running TDT BioSig RP software.

(c) *Sound stimuli presentation*

Two advertising boatwhistles with different dominant frequencies of 93 Hz (bw1) and 44 Hz (bw2), produced by nesting toadfish males (35–48 cm; 963–1819 g) in the Tagus estuary (Portugal), were chosen among previously field-recorded sounds [36]. An agonistic grunt train composed of three grunts recorded from an adult female (25.0 cm TL, 492 g) and a single grunt produced by a juvenile (10.5 cm TL, 15.5 g) was also selected from previous laboratory recordings [31]. To test for temporal encoding, we also considered two other modified boatwhistles created from the original bw1 that was shortened by 149 ms (bw1short) or extended by 298 ms (bw1long) in the middle of the tonal phase. An additional sound presentation consisted of two boatwhistles emitted in sequence (i.e. bw1 followed by bw2 after a 50 ms interval), mimicking two vocalizing male neighbours.

Heterospecific calls consisted of a segment of a sequence of pops produced by a bottlenose dolphin *T. truncatus* during conspecific social interactions and foraging in the Sado estuary, provided by M. E. dos Santos. The bottlenose dolphin has been described as a potential predator of batrachoidids [1], including *H. didactylus* in Sado River, Portugal [37]. We also considered a mate advertising call emitted by a male meagre *A. regius* (Sciaenidae) previously recorded in the Guadiana River, Portugal (N. Prista & M. C. P. Amorim). Breeding meagre males are relatively large (up to 2 m long), emit high-amplitude long tonal calls (probably used for mate attraction [38]), and inhabit the coastal areas in the eastern Atlantic and Mediterranean where Lusitanian toadfish breeding aggregations are also found (e.g. Tagus River; R. O. Vasconcelos 2006–2008, personal observations).

Sound wave stimuli files (25 kHz sampling frequency) were imported into TDT SigGen 3.2 software and fed through a real-time processor (RP 2.1) into a programmable attenuator (PM 5). Two speakers including a sub-woofer (Fostex PM-0.5 Sub and PM-0.5 MKII, Fostex Corporation, Japan) were positioned 50 cm above the experimental tub and used to play back sounds. Stimuli repetition rate varied from 0.8 to 2.7 per second. Each stimulus was presented at least 500 times at opposite polarities and the two AEP traces obtained were then averaged. This procedure using natural sounds at opposite polarities efficiently eliminated eventual stimulus artefacts in the AEPs recorded in our setup because auditory responses are not affected by polarity changes [27]. Sound pressure levels (SPLs) used were monitored with a hydrophone (Brüel and Kjaer 8101) connected to the sound level meter (Brüel and Kjaer 2238 Mediator). Sounds were first presented at 123–129 dB re 1 μPa (depending on the stimulus), and then attenuated in 4 dB steps until recognizable and repeatable auditory response could no longer be detected. The lowest SPL at which a repeatable AEP trace correspondent to specific sound pulses could be obtained, as determined by overlaying replicate traces, was considered the threshold. This method of visual inspection/correlation of hearing thresholds has been traditionally used in AEP audiometry [27,34].

Toadfishes possess no known hearing specialization and are most probably sensitive to particle motion [39,40]. We therefore provide hearing thresholds in sound pressure and particle acceleration levels. For this purpose, a calibrated underwater miniature acoustic pressure–particle acceleration (p–a) sensor S/N 2007-001 (Applied Physical Sciences Corp., Groton, CT, USA) was placed at the fish's position in the test tub. Particle acceleration levels (L_a) were determined for all sound stimuli at various levels, including the hearing threshold levels of the species, with the acceleration sensor oriented in all three orthogonal directions. Similar to Wysocki *et al.* [41], the total acceleration level was calculated based on the acceleration level of each axis in micrometers per second square as $20\log(\sqrt{(x^2+y^2+z^2)})$. Pressure and particle acceleration were positively correlated to each other below the water surface in the experimental tub, and any 4 dB change in SPL was generally accompanied by a 4 dB change in particle acceleration level for all stimuli.

(d) *Auditory response waveform analysis and statistics*

Detailed waveform and spectral analysis were performed using Audition 2.0 (Adobe Systems Inc., CA, USA) and Raven 1.2 (Cornell Laboratory of Ornithology, NY, USA) at the maximum amplitude tested (123–129 dB re 1 μPa 84–92 dB re 1 $\mu m\,s^{-2}$, approx. 14–31 dB above hearing thresholds, depending on the stimuli). Stimuli and AEP durations were determined to evaluate temporal resolution. The onset of the auditory response was considered the beginning of the first positive peak, which was typically delayed by approximately 7–11 ms relative to the onset of the sound stimulus. The end of the AEP trace was considered the last peak clearly distinguished from the ongoing noise.

Spectral peaks of sound and corresponding AEP (sampling frequency 20 kHz, 8192 points FFT size, Hamming window) were compared to verify whether the main frequency content of sounds was represented within the auditory response [27,42].

To evaluate the representation of the boatwhistles' amplitude modulation (bw1, bwshort, bwlong, bw1 + bw2) in the auditory responses, the envelopes of both acoustic stimuli and AEPs were compared. Stimuli and response envelopes were extracted by calculating a moving average of maximum amplitude values of the waveforms using a moving window of 7 ms. The choice of window length is critical and in this case 7 ms was used as a compromise between the period at 93 Hz (stimulus dominant frequency) and the period expected if a double frequency occurs in the AEP response. The stimulus and corresponding AEP envelopes with the same duration or number of points (21 484–47 606 points) were compared using Pearson's correlations. This method was validated by correlating the envelopes of sound stimuli with envelopes of white noise sequences with the same duration (three different white noise sequences for each stimulus), but also by correlating the envelopes of boatwhistles of other toadfish (e.g. bw2, with different dominant frequency but similar amplitude modulation) with AEP responses to bw1. We also correlated the envelope of another mate advertisement boatwhistle (bw3) produced by a nesting toadfish male previously recorded in the Tagus estuary [36], with different dominant frequency (227 Hz) and amplitude modulation, with AEPs to bw1. This validation should produce low correlation coefficients in both cases, in contrast to the high coefficients expected for the stimulus versus corresponding AEP response.

Thresholds to all sound stimuli were compared with a one-way ANOVA performed with all data (from males and females) followed by a Bonferroni post hoc test to verify specific differences between sound stimuli. Mann–Whitney *U* tests were used to compare hearing thresholds to conspecific stimuli (bw1, bw2 and grunt train) between males and females.

Parametric tests were performed when data were normally distributed and variances were homogeneous. The statistical tests were run using Statistica 7.1 for Windows (StatSoft, Inc., USA).

3. RESULTS

(a) *Representation of temporal patterns*

The temporal structure of conspecific sounds was accurately represented in the auditory responses in both males and females ($n = 16$ fish). Each sound pulse generally elicited a separate AEP waveform. Auditory responses to the boatwhistle bw1 showed a representation of both parts of the call, namely the pulsed part and the longer tonal part (see figure 1*a*, a_1 and a_2 for waveform details). Changes in the boatwhistle duration were accurately represented in the auditory system (figure 1*b*,*c* and table 1).

Agonistic grunt trains elicited AEP waveforms that corresponded exactly to each single grunt with similar durations and pulse structure (figure 1*d* and table 1). AEPs obtained in response to the juvenile grunt also revealed identical, precise temporal resolution (figure 1*e* and table 1).

Heterospecific sounds elicited AEP waves that generally followed the temporal patterns of the stimuli (figure 1*f*,*g*), although in most cases the waves could not be attributed to separate sound pulses, especially when responding to dolphin foraging pops. A clear auditory response was only verified at relatively high sound amplitudes, usually above 119 dB for the meagre advertising call and 124 dB for dolphin pops.

(b) *Representation of amplitude modulation*

Amplitude modulation of conspecific boatwhistles was well represented in the auditory responses (figure 1*a*–*c*). The amplitude of these calls, represented by their envelope, was highly correlated with the amplitude of the AEP waveforms for all 16 specimens analysed: bw1 ($r = 0.619$–0.842, $p < 0.001$), bw1short ($r = 0.556$–0.780, $p < 0.001$) and bw1long ($r = 0.654$–0.785, $p < 0.001$).

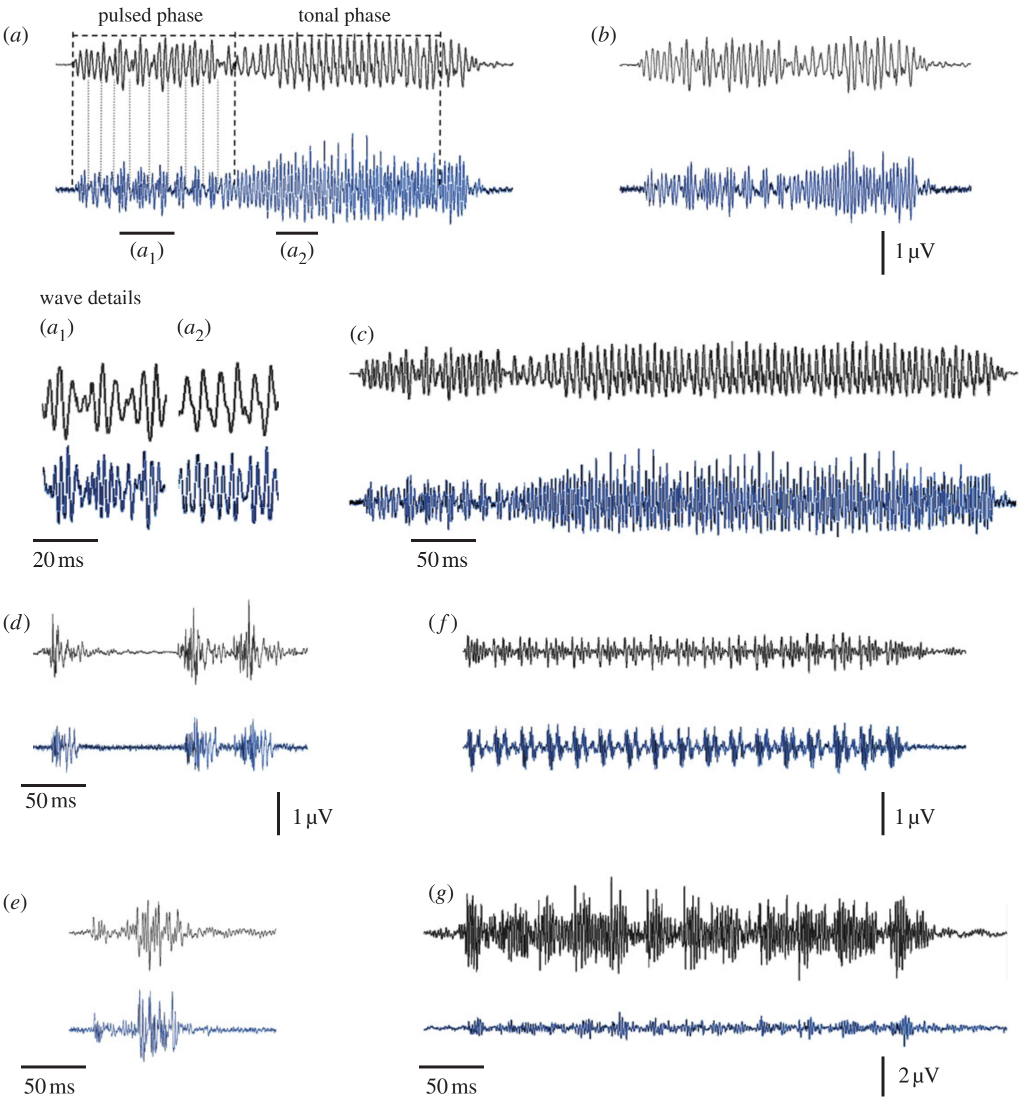

Figure 1. Oscillograms of each sound stimulus (upper black trace) and corresponding auditory evoked response (lower blue trace) recorded from *H. didactylus*. Sound stimuli shown consist of: (*a–c*) conspecific mate advertising boatwhistles ((*a*) original bw1; waveform details of the (a_1) pulsed and (a_2) tonal phase; and modified boatwhistles, (*b*) bw1short and (*c*) bw1long); (*d,e*) conspecific agonistic calls ((*d*) adult grunt train and (*e*) juvenile grunt); and (*f,g*) heterospecific sounds ((*f*) mate advertising call of meagre *A. regius* and (*g*) foraging pops of bottlenose dolphin *T. truncatus*). Averaged stimuli and AEPs depicted resulted from 1000 recordings in one specimen obtained at 123–129 dB re 1 mPa, approximately 14–31 dB above hearing thresholds depending on the stimuli. The amplitude of sound waveforms was adjusted to better fit AEP traces.

Table 1. Duration (ms) of sound stimuli and corresponding AEP responses (mean ± s.e. and range). bw1, original boatwhistle; bw1short, bw1 shortened in the tonal phase; bw1long, bw1 extended in the tonal phase; gr1–gr3, grunts emitted in a train by an adult; juv gr, juvenile grunt.

	mating boatwhistles			agonistic grunts			
	bw1	bw1short	bw1long	gr1	gr2	gr3	juv gr
stimulus	617	430	988	80	77	84	88
AEP	614 ± 2 (601–632)	439 ± 4 (421–477)	998 ± 3 (976–1015)	67 ± 1 (57–82)	74 ± 1 (65–80)	80 ± 2 (58–101)	85 ± 1 (80–89)

Moreover, amplitude values of the stimulus composed of two different boatwhistles (i.e. bw1 followed 50 ms after by bw2) were highly correlated with the amplitude values of AEPs ($r = 0.517–0.691$, $p < 0.001$). This indicated that the toadfish auditory system is capable of resolving amplitude fluctuations of different boatwhistles emitted sequentially. As expected, simulations with white noise (instead of AEP responses) and amplitude values of the different stimuli revealed no significant correlations: bw1 ($r = -0.017–0.020$, n.s.), bw1short

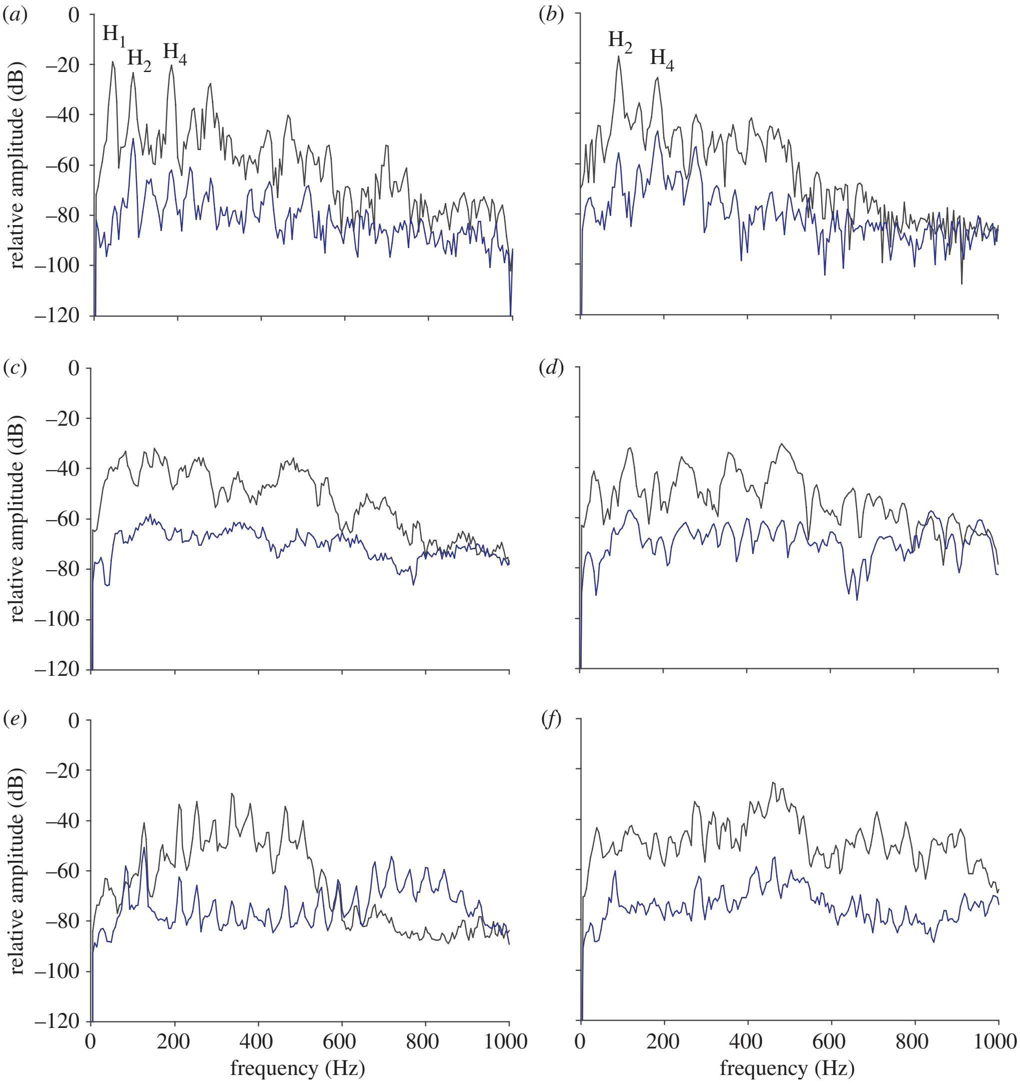

Figure 2. Power spectra of sound stimuli and AEP responses to conspecific mate advertising boatwhistles ((*a*) bw2 and (*b*) bw1), (conspecific agonistic sounds (*c*) adult grunt train and (*d*) juvenile grunt; and heterospecific sounds (*e*) mate advertising call of meagre *A. regius* and (*f*) foraging pops of bottlenose dolphin *T. truncatus*. Averaged stimuli and AEPs depicted resulted from 1000 recordings in one specimen obtained at 123–129 dB re 1 μPa, circa 14–31 dB above hearing thresholds depending on the stimuli. Sampling frequency 20 kHz, 4096 points FFT, 50% overlap, Hamming window. Black lines, sound stimulus; blue lines, AEP.

Table 2. Dominant frequency (Hz) of sound stimuli and corresponding AEP responses (mean ± s.e. and range). bw1, bw2, boatwhistles; gr train, adult grunt train; juv gr, juvenile grunt; *Ar*, *A. regius*; *Tt*, *T. truncatus*. Sampling frequency 20 kHz, 8192 FFT size.

	conspecific sounds				heterospecific sounds	
	bw1	bw2	gr train	juv gr	*Ar* call	*Tt* pops
stimulus	93	44	151	481	339	461
AEP	180 ± 1 (173–183)	100 ± 6 (83–139)	141 ± 9 (93–225)	310 ± 71 (81–845)	300 ± 26 (127–381)	590 ± 95 (239–918)

($r = -0.115$–0.032, n.s.), bw1long ($r = -0.043$–0.031, n.s.) and bw1 + bw2 ($r = -0.068$–0.045, n.s.). Moreover, correlations between bw2 and AEP to bw1 were not significant ($r = -0.354$–0.502, n.s.), nor were they between bw3 and AEP to bw1 ($r = -0.029$–0.082, n.s.).

(c) *Representation of frequency content*

AEP waveforms evoked by bw1 and bw2 showed spectral peaks corresponding exactly to the several harmonics presented in the sound spectra (figure 2*a*,*b*). As expected, the dominant frequency of each AEP spectrum was typically

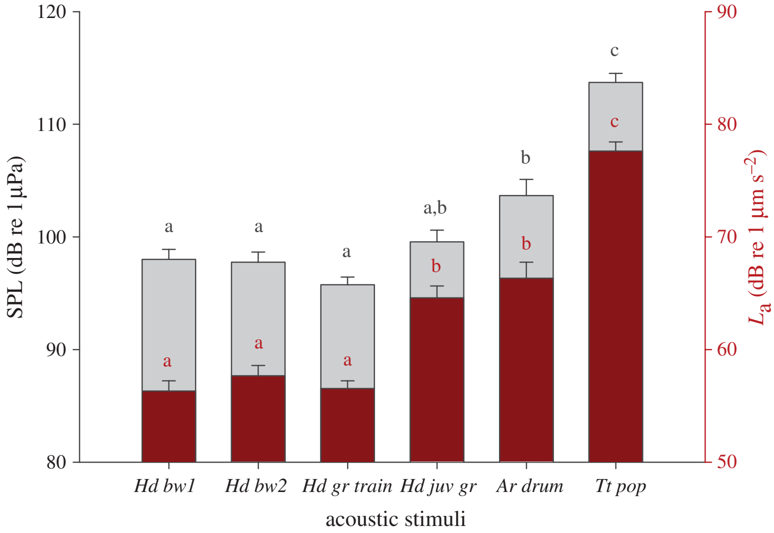

Figure 3. Mean (± s.e.) hearing thresholds, given as sound pressure levels (SPL, grey bars) and as particle acceleration levels (L_a, dark red bars), to conspecific and heterospecific sounds. Conspecific stimuli (*Hd*, *H. didactylus*): *Hd bw1*, mate advertising boatwhistle with 93 Hz dominant frequency; *Hd bw2*, boatwhistle with 44 Hz dominant frequency; *Hd gr train*, agonistic adult grunt train; *Hd juv gr*, agonistic juvenile grunt. Heterospecific stimuli (*Ar*, *A. regius*; *Tt*, *T. truncatus*): *Ar drum*, mate advertising sound; *Tt pop*, foraging pop sound. Groups that are significantly different ($p < 0.01$) are indicated by different letters (results from Bonferroni post hoc tests).

twice the dominant frequency of the respective sound stimulus (table 2 and figure 2*a*,*b*).

The other conspecific agonistic sounds—adult grunt train and juvenile grunt—did not present a harmonic structure. Although the spectrum of AEPs showed some corresponding energy peaks to the sound spectrum, an association between both spectra was generally less clear (figure 2*c*,*d*). Dominant frequencies were more variable within AEPs obtained with these stimuli (table 2). Nevertheless, lower frequency adult grunts mainly generated AEPs with lower frequency energy than did higher frequency juvenile grunts. This suggests that the frequency content of agonistic sounds was also represented in the auditory system.

Heterospecific sounds exhibited relatively high dominant frequencies. The drumming sound of *A. regius* was harmonic, and a good match was observed between the AEP spectrum and each spectral peak of the sound stimulus. However, the dominant frequencies of both spectra differed considerably (figure 2*e* and table 2). AEPs elicited by *T. truncatus* foraging pops showed a general correspondence in some spectral peaks, but the main energy varied considerably (figure 2*f* and table 2).

(d) *Auditory sensitivity*

Mean (±s.e., standard error) hearing thresholds for conspecific boatwhistles were 98.0 ± 0.9 dB re 1 µPa (56.3 ± 0.9 dB re 1 µm s^{-2}) for bw1, 97.8 ± 0.9 dB re 1 µPa (57.7 ± 0.9 dB re 1 µm s^{-2}) for bw2, 95.8 ± 0.7 dB re 1 µPa (56.5 ± 0.7 dB re 1 µm s^{-2}) for adult grunt trains and 99.6 ± 1.0 dB re 1 µPa (64.6 ± 1.0 dB re 1 µm s^{-2}) for juvenile grunts. Heterospecific calls evoked responses at higher levels: 103.7 ± 1.4 dB re 1 µPa (66.3 ± 1.4 dB re 1 µm s^{-2}) for *A. regius* calls and 113 ± 0.8 dB re 1 µPa (77.6 ± 0.8 dB re 1 µm s^{-2}) for *T. truncatus* pops. Thresholds varied significantly between sound stimuli (SPL: one-way ANOVA, $F_{5,70} = 30.50$, $p < 0.001$; L_a: one-way ANOVA, $F_{5,70} = 51.6$, $p < 0.001$) and revealed significant differences (Bonferroni post hoc tests, $p < 0.01$) between conspecific and heterospecific calls. The exceptions were the toadfish juvenile grunt and the *A. regius* call (figure 3). Hearing thresholds (for bw1, bw2 and grunt train) did not differ between males and females (SPL, L_a: Mann–Whitney *U* test, $U = 22 - 29$, $n_1 = n_2 = 8$, n.s.).

4. DISCUSSION

Most studies that have investigated the hearing abilities of fishes have used only artificial stimuli, such as pure tones [21–24,26], tone bursts [34,43,44] and clicks [12,25]. These studies have mostly aimed to describe species-specific audiograms, but also examine other aspects of auditory processing such as coding of temporal and intensity patterns, as well as spectral content. Some artificial stimuli approached the characteristics of conspecific calls [12,22], but did not fully reflect the overall complexity of vocalizations that animals produce and detect in their environment. To date, only two studies have analysed how conspecific sounds, mostly short-pulsed calls, are encoded by the auditory system in fishes [27,28]. The present study provides first data on the representation of complex conspecific vocalizations, including amplitude-modulated tonal calls, in the auditory system of a strongly vocal fish that lacks accessory hearing structures.

We showed that, in *H. didactylus*, both sexes can accurately resolve temporal patterns of conspecific signals. Auditory responses to the advertising boatwhistle showed a fine representation of each pulse and the distinct phases of the call (pulsed and tonal). Changes in boatwhistle duration were also accurately perceived. Agonistic grunts, including the juvenile call, were well encoded in their temporal characteristics (number of pulses, interval between grunts). The temporal pattern is thought to be the most important sound characteristic for acoustic communication in fishes, especially in noisy and/or shallow waters, where low frequencies do not propagate well and the spectral content of signals is easily altered [45]. Temporal information, such as the pulse period, seems to be important for intraspecific communication and species recognition (e.g. damselfishes [46]; gouramis [11]; electric fish [12]; cichlids [13]). According to Wysocki & Ladich [27], in the sunfish *L. gibbosus*, a species lacking accessory hearing structures, AEPs elicited by conspecific sound pulses were very long and did not follow specific pulses. In contrast, the results obtained with *H. didactylus* point to a fine temporal resolution comparable to those of species possessing hearing specializations (e.g. *P. pictus* and *T. vittata* [27]). This species exhibits an unusually complex acoustic repertoire that mostly varies in its temporal features (i.e. pulse interval, duration and repetition rate [13,30]). One of the parameters most probably used to distinguish between advertising nesting males is the boatwhistle duration and pulse period [32]. Moreover, other sound features such as repetition rate and duration of the agonistic grunts are correlated with fish size [31]. Therefore, detecting the temporal patterns of sounds is likely to be valuable for social interactions and mate attraction in *H. didactylus*. Previous behavioural studies reported that toadfishes (*O. tau* and *O. beta*) are able to produce an agonistic grunt on top of another toadfish's call after an average latency of 69 ms. This phenomenon (termed acoustic tagging) indicates a rapid response of the auditory component of a behavioural (sensory-motor) loop [47,48]. Our study confirmed that temporal patterns of both tonal advertising boatwhistles and pulsed agonistic grunts are precisely perceived and may help fish to extract important information during acoustic communication.

Amplitude modulation of advertising boatwhistles was also well represented in the auditory responses. Amplitudes of boatwhistles were highly correlated with the amplitudes of the auditory responses, independent of signal duration. A significant amplitude correlation was also found when two different boatwhistles were played back in sequence. This suggests that this parameter is well encoded even in the presence of more than one calling male, which typically occurs in toadfish breeding aggregations [32]. Marked amplitude modulation is found in boatwhistles produced by competing males in an advertising context. This contrasts with boatwhistles emitted during territorial defence, suggesting that this sound characteristic might be important for mate attraction but also informative of the social context in *H. didactylus* [36]. The perception of amplitude modulation has been poorly investigated in fish, probably because most species do not produce long amplitude-modulated sounds. Bodnar & Bass [22,24] investigated the neural responses in the batrachoidid *P. notatus* to simultaneous pure tones that form acoustic beats, similar to what occurs in a natural chorus. The authors found that midbrain units encode spectral and temporal features of concurrent signals (i.e. intensity and depth of modulation of beats).

We also showed that the frequency content of sounds, especially the multi-harmonic boatwhistles, can be perceived by *H. didactylus*. AEPs evoked by the boatwhistles showed spectral peaks corresponding exactly to the harmonics presented in the sound spectrum. The dominant frequency of the AEP spectrum was typically twice the dominant frequency of the corresponding sound stimulus. Such a frequency-doubling effect of AEPs, which is a further reassurance of a biological response, can be explained by the fact that saccular hair cells are oriented in opposite directions [49,50]. This phenomenon has also been observed in other fish species using the same AEP recording technique [51–53]. The frequency content of agonistic sounds was not as clearly represented in the auditory system, although a general match between the main energy of the stimulus and the AEP spectrum was detected, along with a distinct auditory response to juvenile and adult grunts. The dominant frequency of agonistic grunts is related to the body size in *H. didactylus* [31], similar to other teleosts [7,8]. Detection of the spectral content of vocalizations might be important in assessing the fighting ability of opponents and the quality of potential mates [8,54,55].

Hearing thresholds to conspecific signals did not differ between sexes in Lusitanian toadfish. Type I males nest in aggregations and vocalize in choruses to attract females. Behavioural evidence with this species showed that nesting males interact acoustically and alter their own boatwhistle calling rate in response to other calling males (J. M. Jordão, P. J. Fonseca & M. C. P. Amorim 2008, personal observations). These acoustic interactions suggest that the auditory system of nesting males must be adapted to detect and resolve acoustic parameters of boatwhistles similar to females, which probably select mates based on acoustic cues [15]. Hearing thresholds to higher-frequency heterospecific calls were higher than thresholds to conspecific signals, indicating that the Lusitanian toadfish is better adapted to detect intraspecific low-frequency vocalizations. Nevertheless, this species not only detected but also to some extent resolved temporal features of heterospecific sounds, namely of the advertising calls of the sympatric sciaenid *A. regius* and foraging sounds of *T. truncatus*. *Argyrosomus regius* inhabits coastal areas where Lusitanian toadfish breeding aggregations are usually found and produces advertising calls often at the same time (R. O. Vasconcelos 2006–2008, personal observations). Our results indicated that toadfish can discriminate between both conspecific and heterospecific multi-harmonic calls, in terms of temporal and amplitude patterns, and spectral content. The bottlenose dolphin *T. truncatus* has been described as a potential predator of batrachoidids [56], including *H. didactylus* [37]. Remage-Healey *et al.* [1] reported that playbacks of *T. truncatus* foraging pops considerably reduced the calling rate of the Gulf toadfish *O. beta* and induced an increment in cortisol levels. Our data indicate that the Lusitanian toadfish intercepts dolphin foraging sounds and support the previous behavioural observations.

In summary, we provide strong evidence that the auditory system of a highly vocal fish, lacking accessory hearing structures, can detect the fine temporal, amplitude and spectral features of complex vocalizations that are potentially important for acoustic communication. Future studies will determine the encoding properties of specific regions of the Lusitanian toadfish auditory system as AEP only reflects overall responses of the auditory pathway (saccule hair cells, eight nerve and brainstem auditory nuclei) up to the midbrain [57].

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REFERENCES

1 Remage-Healey, L., Douglas, R., Nowacek, P. & Bass, A. H. 2006 Dolphin foraging sounds suppress calling and elevate stress hormone levels in a prey species, the Gulf toadfish. *J. Exp. Biol.* **209**, 4444–4451. (doi:10.1242/jeb.02525)

2 Ladich, F. & Myrberg, A. A. 2006 Agonistic behavior and acoustic communication. In *Communication in fishes*, vol. 1 (eds F. Ladich, S. P. Collin, P. Moller & B. G. Kapoor), pp. 121–148. Enfield, NH: Science Publishers.

3 Myrberg, A. A. & Lugli, M. 2006 Reproductive behavior and acoustical interactions. In *Communication in fishes*, vol. 1 (eds F. Ladich, S. P. Collin, P. Moller & B. G. Kapoor), pp. 149–176. Enfield, NH: Science Publishers.

4 Ladich, F. 2004 Sound production and acoustic communication. In *The senses of fishes: adaptations for the reception of natural stimuli* (eds G. van der Emde, J. Mogdans & B. G. Kapoor), pp. 210–230. New Delhi, India: Narosa Publishing House.

5 Bass, A. H. & Ladich, F. 2008 Vocal-acoustic communication: from neurons to behavior. In *Fish bioacoustics* (eds J. F. Webb, R. R. Fay & A. N. Popper). Springer Handbook on Auditory Research, no. 32, pp. 253–278. New York, NY: Springer-Verlag.

6 Amorim, M. C. P. 2006 Diversity of sound production in fish. In *Communication in fishes*, vol. 1 (eds F. Ladich, S. P. Collin, P. Moller & B. G. Kapoor), pp. 71–104. Enfield, NH: Science Publishers.

7 Myrberg, A. A., Ha, S. J. & Shamblott, H. S. 1993 The sounds of bicolor damselfish (*Pomacentrus partitus*): predictors of body size and a spectral basis for individual recognition and assessment. *J. Acoust. Soc. Am.* **94**, 3067–3070. (doi:10.1121/1.407267)

8 Ladich, F. 1998 Sound characteristics and outcome of contests in male croaking gouramis (Teleostei). *Ethology* **104**, 517–529. (doi:10.1111/j.1439-0310.1998.tb00087.x)

9 Fish, J. F. 1972 The effect of sound playback on the toadfish. In *Behavior of marine animals*, vol. 2 (eds H. E. Winn & B. Olla), pp. 386–434. New York, NY: Plenum Press.

10 Amorim, M. C. P., Fonseca, P. J. & Almada, V. C. 2003 Sound production during courtship and spawning of *Oreochromis mossambicus*: male–female and male–male interactions. *J. Fish Biol.* **62**, 658–672. (doi:10.1046/j.1095-8649.2003.00054.x)

11 Ladich, F., Bischof, C., Schleinzer, G. & Fuchs, A. 1992 Intra- and interspecific differences in agonistic vocalisation in croaking gouramis (genus: *Trichopsis*, *Anabantoidei*, *Teleostei*). *Bioacoustics* **4**, 131–141.

12 Marvit, P. & Crawford, J. D. 2000 Auditory discrimination in a sound producing electric fish (*Pollimyrus*): tone frequency and click-rate difference detection. *J. Acoust. Soc. Am.* **108**, 1819–1825. (doi:10.1121/1.1287845)

13 Amorim, M. C. P., Simões, J. M., Fonseca, P. J. & Turner, G. F. 2008 Species differences in acoustic signals among five Lake Malawi cichlid species (*Pseudotropheus* spp.). *J. Fish Biol.* **72**, 1355–1368. (doi:10.1111/j.1095-8649.2008.01802.x)

14 Winn, H. E. 1964 The biological significance of fish sounds. In *Marine bio-acoustics*, vol. 2 (ed. W. N. Tavolga), pp. 213–231. New York, NY: Pergamon Press.

15 McKibben, J. R. & Bass, A. H. 1998 Behavioral assessment of acoustic parameters relevant to signal recognition and preference in a vocal fish. *J. Acoust. Soc. Am.* **104**, 3520–3533. (doi:10.1121/1.423938)

16 Eggermont, J. J. 1998 Representation of spectral and temporal sound features in three cortical fields of the cat. Similarities outweigh differences. *J. Neurophysiol.* **80**, 2743–2764.

17 Boatright-Horowitz, S. S., Garabedian, C. E., Odabashian, K. H. & Simmons, A. M. 1999 Coding of amplitude modulation in the auditory midbrain of the bullfrog (*Rana catesbeiana*) across metamorphosis. *J. Comp. Physiol. A* **184**, 219–231. (doi:10.1007/s003590050320)

18 Fonseca, P. J. & Revez, M. A. 2002 Song discrimination by male cicadas *Cicada barbara lusitanica* (Homoptera, Cicadidae). *J. Exp. Biol.* **205**, 1285–1292.

19 Rose, G. J. & Brenowitz, E. A. 2002 Pacific treefrogs use temporal integration to differentiate advertisement from encounter calls. *Anim. Behav.* **63**, 1183–1190. (doi:10.1006/anbe.2002.3025)

20 Henry, K. S. & Lucas, J. R. 2008 Coevolution of auditory sensitivity and temporal resolution with acoustic signal space in three songbirds. *Anim. Behav.* **76**, 1659–1671. (doi:10.1016/j.anbehav.2008.08.003)

21 Fay, R. R. 1995 Perception of spectrally and temporally complex sounds by the goldfish (*Carassius auratus*). *Hear. Res.* **89**, 146–154. (doi:10.1016/0378-5955(95)00132-8)

22 Bodnar, D. A. & Bass, A. H. 1997 Temporal coding of concurrent acoustic signals in auditory midbrain. *J. Neurosci.* **17**, 7553–7564.

23 Bodnar, D. A. & Bass, A. H. 1999 Midbrain combinatorial code for temporal and spectral information in concurrent acoustic signals. *J. Neurophysiol.* **81**, 552–563.

24 Bodnar, D. A. & Bass, A. H. 2001 Coding of concurrent vocal signals by the auditory midbrain: effects of stimulus level and depth of modulation. *J. Acoust. Soc. Am.* **109**, 809–825. (doi:10.1121/1.1340646)

25 Wysocki, L. E. & Ladich, F. 2002 Can fishes resolve temporal characteristics of sounds? New insights using auditory brainstem responses. *Hear. Res* **169**, 36–46. (doi:10.1016/S0378-5955(02)00336-2)

26 Plassmann, W. 1985 Coding of amplitude-modulated tones in the central auditory system of catfish. *Hear. Res.* **17**, 209–217. (doi:10.1016/0378-5955(85)90065-6)

27 Wysocki, L. E. & Ladich, F. 2003 The representation of conspecific sounds in the auditory brainstem of teleost fishes. *J. Exp. Biol.* **206**, 2229–2240. (doi:10.1242/jeb.00417)

28 Maruska, K. P. & Tricas, T. C. 2009 Encoding properties of auditory neurons in the brain of a soniferous damselfish: reponse to simple tones and complex conspecific signals. *J. Comp. Physiol. A* **195**, 1071–1088. (doi:10.1007/s00359-009-0480-1)

29 Bass, A. H. & McKibben, J. R. 2003 Neural mechanisms and behaviors for acoustic communication in teleost fish. *Prog. Neurobiol.* **69**, 1–26. (doi:10.1016/S0301-0082(03)00004-2)

30 dos Santos, M. E., Modesto, T., Matos, R. J., Grober, M. S., Oliveira, R. F. & Canário, A. 2000 Sound production by the Lusitanian toadfish, *Halobatrachus didactylus*. *Bioacoustics* **10**, 309–321.

31 Vasconcelos, R. O. & Ladich, F. 2008 Development of vocalisation, auditory sensitivity and acoustic communication in the Lusitanian toadfish *Halobatrachus didactylus*. *J. Exp. Biol.* **211**, 502–509. (doi:10.1242/jeb.008474)

32 Amorim, M. C. P. & Vasconcelos, R. O. 2008 Variability in the mating calls of the Lusitanian toadfish *Halobatrachus didactylus*: potential cues for individual recognition. *J. Fish Biol.* **73**, 1267–1283. (doi:10.1111/j.1095-8649.2008.01974.x)

33 Modesto, T. & Canário, A. V. M. 2003 Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, *Halobatrachus didactylus*. *Gen. Comp. Endocrinol.* **131**, 220–231. (doi:10.1016/S0016-6480(03)00027-3)

34 Kenyon, T. N., Ladich, F. & Yan, H. Y. 1998 A comparative study of hearing ability in fishes: the auditory brainstem response approach. *J. Comp. Physiol. A* **182**, 307–318. (doi:10.1007/s003590050181)

35 Bullock, T. H. & Ridgway, S. H. 1972 Evoked potentials in the central auditory system of alert porpoises to their own and artificial sounds. *J. Neurobiol.* **3**, 79–99. (doi:10.1002/neu.480030107)

36 Vasconcelos, R. O., Simões, J. M., Almada, V. C., Fonseca, P. J. & Amorim, M. C. P. 2010 Vocal behavior during territorial intrusions in the Lusitanian toadfish: boatwhistles also function as territorial 'keep-out' signals. *Ethology* **116**, 155–165. (doi:10.1111/j.1439-0310.2009.01722.x)

37 dos Santos, M. E., Coniglione, C. & Louro, S. 2007 Feeding behaviour of the bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821) in the Sado estuary, Portugal, and a review of its prey species. *Zoociências* **9**, 31–40.

38 Lagardère, J. P. & Mariani, A. 2006 Spawning sounds in meagre *Argyrosomus regius* recorded in the Gironde estuary, France. *J. Fish Biol.* **69**, 1697–1708. (doi:10.1111/j.1095-8649.2006.01237.x)

39 Fay, R. R. & Edds-Walton, P. L. 1997 Diversity in frequency properties of saccular afferents of the toadfish, *Opsanus tau*. *Hear. Res.* **113**, 235–246. (doi:10.1016/S0378-5955(97)00148-2)

40 Weeg, M., Fay, R. & Bass, A. H. 2002 Directionality and frequency tuning of primary saccular afferents of a vocal fish, the plainfin midshipman (*Porichthys notatus*). *J. Comp. Physiol. A* **188**, 631–641.

41 Wysocki, L. E., Codarin, A., Ladich, F. & Picciulin, M. 2009 Sound pressure and particle acceleration audiograms in three marine fish species from the Adriatic Sea. *J. Acoust. Soc. Am.* **126**, 2100–2107. (doi:10.1121/1.3203562)

42 Krishnan, A. 2002 Human frequency-following responses: representation of steady-state synthetic vowels. *Hear. Res.* **166**, 192–201. (doi:10.1016/S0378-5955(02)00327-1)

43 Ladich, F. & Yan, H. Y. 1998 Correlation between auditory sensitivity and vocalisation in anabantoid fishes. *J. Comp. Physiol. A* **182**, 737–746. (doi:10.1007/s003590050218)

44 Vasconcelos, R. O., Amorim, M. C. P. & Ladich, F. 2007 Effects of ship noise on the detectability of communication signals in the Lusitanian toadfish. *J. Exp. Biol.* **210**, 2104–2112. (doi:10.1242/jeb.004317)

45 Mann, D. A. 2006 Propagation of fish sounds. In *Communication in fishes* (eds F. Ladich, S. P. Collin, P. Moller & B. G. Kapoor), pp. 107–120. Enfield, NH: Science Publishers.

46 Myrberg, A. A., Spanier Jr, E. & Ha, S. J. 1978 Temporal patterning in acoustical communication. In *Contrasts in behavior: adaptations in the aquatic and terrestrial environment.* (eds E. S. Reese & F. J. Lighter), pp. 138–179. New York, NY: John Wiley & Sons, Inc.

47 Thorson, R. F. & Fine, M. L. 2002 Acoustic competition in the gulf toadfish *Opsanus beta*: acoustic tagging. *J. Acoust. Soc. Am.* **111**, 2302–2307. (doi:10.1121/1.1466865)

48 Fine, M. L. & Thorson, R. F. 2008 Use of passive acoustics for assessing behavioral interactions in individual toadfish. *Trans. Am. Fish. Soc.* **137**, 627–637. (doi:10.1577/T04-134.1)

49 Sand, O. 1976 Microphonic potentials as tool for auditory research in fish. In *Sound reception in fish* (eds A. Schuijf & A. D. Hawkins), pp. 27–48. Amsterdam, The Netherlands: Elsevier Scientific Publishing Company.

50 Sisneros, J. A. 2007 Saccular potentials of the vocal plainfin midshipman fish, *Porichthys notatus*. *J. Comp. Physiol. A* **193**, 413–424. (doi:10.1007/s00359-006-0195-5)

51 Mann, D. A., Higgs, D. M., Tavolga, W. N., Souza, M. J. & Popper, A. N. 2001 Ultrasound detection by clupeiform fishes. *J. Acoust. Soc. Am.* **109**, 3048–3054. (doi:10.1121/1.1368406)

52 Higgs, D. M., Rollo, A. K., Souza, M. J. & Popper, A. N. 2003 Development of form and function in peripheral auditory structures of the zebrafish (*Danio rerio*). *J. Acoust. Soc. Am.* **113**, 1145–1154. (doi:10.1121/1.1536185)

53 Egner, S. A. & Mann, D. A. 2005 Auditory sensitivity of sergeant major damselfish *Abudefduf saxatilis* from post-settlement juvenile to adult. *Mar. Ecol. Prog. Ser.* **285**, 213–222. (doi:10.3354/meps285213)

54 Davies, N. B. & Halliday, T. R. 1978 Deep croaks and fighting assessment in toads *Bufo bufo*. *Nature* **274**, 683–685. (doi:10.1038/274683a0)

55 Myrberg, A. A., Mohler, M. & Catala, J. D. 1986 Sound production by males of a coral reef fish (*Pomacentrus partitus*): its significance to females. *Anim. Behav.* **34**, 913–923.

56 Barros, N. B. & Myrberg, A. A. 1987 Prey detection by means of passive listening in bottlenose dolphins (*Tursiops truncatus*). *J. Acoust. Soc. Am.* **82**, S65.

57 Corwin, J. T. 1981 Audition in elasmobranchs. In *Hearing and sound communication in fishes* (eds W. N. Tavolga, A. N. Popper & R. R. Fay), pp. 81–102. New York, NY: Springer.

CHAPTER III

ONTOGENY OF ACOUSTIC COMMUNICATION

(i) **Development of vocalization, auditory sensitivity and acoustic communication in the Lusitanian toadfish *Halobatrachus didactylus***

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Development of vocalization, auditory sensitivity and acoustic communication in the Lusitanian toadfish *Halobatrachus didactylus*

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SUMMARY

The ontogenetic development of acoustic communication has so far only been investigated in one fish species. In order to determine whether detectability of conspecific sounds changes during growth in a species with limited hearing abilities (generalist), we investigated the development of auditory sensitivity and agonistic vocalizations in the Lusitanian toadfish *Halobatrachus didactylus*. Agonistic grunts were recorded, their sound pressure levels determined, and auditory sensitivities measured in five different size groups ranging from 3 to 32 cm standard length. Hearing thresholds were obtained using the auditory evoked potentials (AEP) recording technique. Dominant frequency, sound duration and number of pulses decreased, whereas pulse period and sound level increased with increasing fish size. The best hearing was below 300 Hz in all groups. Lower hearing sensitivity was found in the smallest juveniles at 100 Hz as well as at higher frequencies (800 and 1000 Hz). Comparisons between audiograms and sound spectra within the same-sized fish revealed that smaller juveniles would be barely able to detect agonistic grunts, while these vocalizations were clearly perceived by larger fish. In the latter, the main energy of sounds was found at the most sensitive frequencies. This study demonstrates that acoustic communication in the Lusitanian toadfish might be absent in early developmental stages and seems to start when juveniles are able to generate grunts of higher sound level and lower dominant frequency.

Key words: ontogeny, sound spectra, hearing, auditory evoked potential, acoustic communication, *Halobatrachus didactylus*.

INTRODUCTION

While numerous studies have examined developmental changes in vocalizations or hearing in mammals and birds (e.g. Dimitrieva and Gottlieb, 1992; Dimitrieva and Gottlieb, 1994; Podos et al., 1995; Ruben, 1995; Reimer, 1996; Moss et al., 1997; Branchi et al., 2001), few have focused on similar changes in other vertebrates such as fishes. Ontogenetic development of vocalization has been investigated in detail in the croaking gourami *Trichopsis vitatta*. Sound duration, number of pulses, pulse period and sound level increased, while dominant frequency decreased with age (Henglmüller and Ladich, 1999; Wysocki and Ladich, 2001). Such a negative correlation between dominant frequency and size was also found in other fish species (e.g. Ladich et al., 1992; Myrberg et al., 1993; Crawford, 1997; Amorim and Hawkins, 2005).

Whereas sound characteristics change with age and size in all fishes investigated, no clear picture exists on whether auditory sensitivity changes during development. Using whole nerve action potential recordings, Corwin (Corwin, 1983) first described an increment in vibrational sensitivity with growth for the elasmobranch *Raja clavata*. Improved hearing with increasing size was reported in the damselfish *Stegastes partitus*, the labyrinth fish *T. vittata* and the batrachoidid *Porichthys notatus* (Kenyon, 1996; Wysocki and Ladich, 2001; Sisneros and Bass, 2005), whereas no improvement was observed in the otophysines *Carassius auratus* and *Danio rerio* (Popper, 1971; Higgs et al., 2002; Higgs et al., 2003) or in the damselfish *Abudefduf saxatilis* (Egner and Mann, 2005).

Furthermore, the relationship between development of hearing and sound production is almost unknown in fishes. The only study correlating both processes was in *T. vitatta* (Wysocki and Ladich, 2001), where auditory sensitivity develops prior to the ability to vocalize and sound production occurs prior to the ability to communicate acoustically.

The aims of the present study were to (1) describe the developmental changes of temporal, spectral and intensity characteristics of agonistic grunt sounds emitted by the Lusitanian toadfish, *Halobatrachus didactylus* (Bloch and Schneider 1801), in a distress situation; (2) analyze the development of auditory sensitivity with growth; and (3) determine whether the ability to communicate acoustically changes across the life history in this species.

The Lusitanian toadfish (Batrachoididae) possesses a relatively complex acoustic repertoire of different low-frequency vocalizations, i.e. at least three sounds likely used in agonistic contexts (grunt call, croak and double-croak), and one for mate attraction (boatwhistle) (Dos Santos et al., 2000). Males are territorial and defend nests under rocks in shallow waters during the breeding season, from May to July (Dos Santos et al., 2000; Palazón-Fernández et al., 2001; Modesto and Canário, 2003a). Grunt calls (or trains of grunts) are detectable almost the year round but are more frequent early in the reproductive season, and are therefore thought to be important for occupation of territories and nest defence (Amorim et al., 2006).

MATERIALS AND METHODS

Animals

The test subjects were 79 Lusitanian toadfish, *H. didactylus*, caught by local fishermen in the estuaries of the Mira and Tagus (only the largest fish size group) Rivers (Portugal). Fish were kept in 250 l tanks separately according to their size for at least 2 weeks before starting the auditory experiments. The bottoms of aquaria were covered with sand and equipped with several half flowerpots and plastic shelters (for larger specimens). The aquaria were filtered by external filters and protein skimmers and a 12 h:12 h L:D cycle was maintained. Animals were fed every second or third day with cod and occasionally shellfish.

Sound recordings were obtained in 73 fish (standard length, *SL*=3.8–31.8 cm; body mass=2.14–800 g), whereas sound pressure levels (SPL) were measured from 38 calling specimens (*SL*=3.8–23.8 cm; body mass=2.14–323 g).

For auditory sensitivity measurements and comparison with sound spectra, tested animals were classified by size into five different groups (G); G1: *SL*=2.8–3.8 cm, body mass=0.60–2.14 g (*N*=6); G2: *SL*=5.4–6.6 cm, body mass=4.2–7.0 g (*N*=6); G3: *SL*=8.0–10.2 cm, body mass=11–27 g (*N*=7); G4: *SL*=12.4–15.3 cm, body mass=43–84 g (*N*=6); and G5: *SL*=20.2–31.8 cm, body mass=221–800 g (*N*=9). Individuals of these groups were probably just a few months, 1 year, 1–2 years, 2–3 years and 5–8 years old, respectively (based on J. L. Costa, unpublished). Hearing thresholds from the largest size group (G5) are reported elsewhere (Vasconcelos et al., 2007).

All experiments were performed with the permission of the Austrian Commission on Experiments in Animals (GZ 68.10/50-Pr/4/2002 and GZ 66.006/2-BrGT/2006).

Sound recordings and sound pressure level measurements

Test subjects were handheld by the investigator and positioned inside an oval plastic tub (diameters: 45×30 cm, water depth: 12 cm) covered with sand on the bottom and lined on the inside with acoustically absorbent material (air-filled packing wrap) to reduce resonances and reflections. Fish were positioned underwater in the center of the experimental tub at a distance of 10 cm from the hydrophone fixed at the right side of the animal. We chose this recording procedure because agonistic fish–fish interactions typically take place at roughly this distance, in particular during nest defense in aquaria (R.O.V. and F.L., personal observations).

Most of sound recordings were performed in the laboratory (*N*=44 fish, *SL*=3.8–27.0 cm, body mass=2.14–579 g). However, in order to avoid any lab artifacts in terms of frequency content of sounds from larger specimens, vocalizations from 29 fish (*SL*=8.0–31.8 cm, body mass=11–800 g) were also recorded at the field near an intertidal toadfish nesting area inside the experimental tub over the sand substrate. These field recordings were used for dominant frequency determinations and spectral analysis (groups 3–5).

Fish sounds were recorded for over 1–4 min (at least 10 sounds) per specimen using a hydrophone (Brüel and Kjaer 8101, Naerum, Denmark; frequency range: 1 Hz–80 kHz, ±2 dB; voltage sensitivity: –184 dB re. 1 V/μPa) connected to a Brüel and Kjaer 2804 power supply and a DAT recorder (Sony TCD-D100, Sony Corporation, Tokyo, Japan) or a flashcard recorder (Marantz PMD 660, Eindhoven, The Netherlands). Field recordings were performed with a hydrophone (High Tech 94 SSQ, Gulfport, MS, USA; frequency range: 30 Hz–6 kHz, ±1 dB; voltage sensitivity: –165 dB re. 1 V/μPa) connected to an amplifier (Edirol UA-25, Roland Corporation, Tokyo, Japan) and a portable computer. Instantaneous SPL values, i.e. L_{LFP} (linear frequency weighting, RMS fast time weighting), were measured for 10 sounds per fish using a sound level meter (Brüel and Kjaer 2804 Mediator) connected to the power supply.

Sound analysis

Sound recordings (sampling frequency 6 kHz) were analyzed using Raven 1.2 for Windows (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, NY, USA). The following sound characteristics (see Fig. 1) were determined from 10 grunts per fish: total duration of single grunts (ms), from the start of the first pulse to the end of the last pulse; number of pulses within a single grunt; pulse period (ms), as the average time period between two up to six consecutive peaks (depending on number of pulses within a grunt); dominant frequency (Hz), as the highest amplitude within the sound power spectrum (Blackman-Harris window, filter bandwidth 10 Hz).

Cepstrum-smoothed sound power spectra (Noll, 1967) were calculated for each size group. A sound file composed of vocalizations emitted by different specimens (10 sounds per individual) was created separately for each size groups (number of fish per group: G1, *N*=1; G2, *N*=5; G3, *N*=9; G4, *N*=6; G5, *N*=8) and used to create group-specific sound spectra. These were determined using the acoustic analysis software S_TOOLS-STx 3.7 (Acoustics Research Institute, Austrian Academy of Sciences, Vienna, Austria). Absolute sound spectra of the recordings were calculated as described previously (Amoser et al., 2004; Wysocki and Ladich, 2005a).

Auditory sensitivity measurements

The auditory evoked potential recording protocol was based on that originally reported and evaluated (Kenyon et al., 1998) and subsequently modified (Wysocki and Ladich, 2005a; Wysocki and Ladich, 2005b). Hence, just a shortened description of the experimental procedure will be given.

In order to immobilize fish, Flaxedil (gallamine triethiodide; Sigma-Aldrich, Vienna, Austria) diluted in a Ringer solution (see Walsh, 1987) was administered intramuscularly, i.e. 5–6 μg g^{-1} body mass for groups 1–4 and 10–15 μg g^{-1} body mass for group 5. This still enabled the fish to produce slight opercular movements. The subjects were positioned below the water surface in the center of an oval plastic tub (diameters: 45×30 cm, water depth: 12 cm, 1.5 cm layer of sand) lined on the inside with air-filled packing wrap. The contacting points of the electrodes were maximally 1–2 mm above the water surface. A small piece of Kimwipes™ tissue paper was placed on the fish head to keep it moist and ensure proper contact of electrodes. Respiration pipettes with different dimensions were inserted into the subjects' mouth

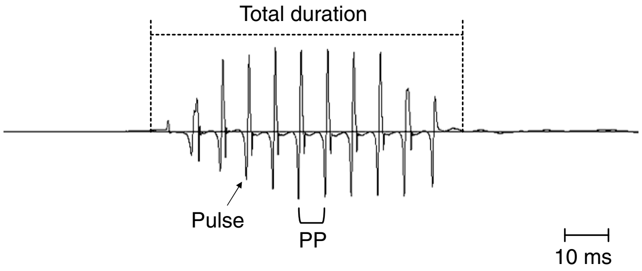

Fig. 1. Oscillogram of a single grunt of a juvenile *H. didactylus* showing temporal sound characteristics analyzed (PP, pulse period).

according to their size. Respiration was achieved through a simple temperature-controlled (22±1°C), gravity-fed water system. The recording electrode was placed at the brainstem region and the reference electrode cranially close to the nares (silver wire, 0.25 mm diameter), pressed firmly against the subject's skin. Shielded electrode leads were attached to the differential input of an a.c. preamplifier (Grass P-55, Grass Instruments, West Warwick, RI, USA; gain 100×, high-pass at 30 Hz, low-pass at 1 kHz). A grounding electrode was placed underwater near the fish body. A hydrophone (Brüel and Kjaer 8101) was placed on the right side of the fish (circa 1 cm away) near the inner ear in order to determine absolute stimulus SPL values underwater in close proximity to the subjects. The experimental tub was positioned on an air table (TMC Micro-g 63–540, Technical Manufacturing Corporation, Peabody, MA, USA), which rested on a vibration-isolated concrete plate. The entire experimental setup was enclosed in a walk-in soundproof room (interior dimensions, 3.2 m× 3.2 m×2.4 m), which was constructed as a Faraday cage.

Acoustic stimuli consisted of tone bursts presented at a repetition rate of 21 s^{-1}. The hearing thresholds were determined at the following frequencies: 50, 100, 200, 300, 500, 800 and 1000 Hz, always presented at random. Duration of sound stimuli increased from 2 cycles at 50 Hz (40 ms) up to 5 cycles at 1000 Hz (5 ms). All bursts were gated using a Blackman window. For each test condition, one thousand stimuli were presented at opposite polarities (180° phase shifted) and were averaged together by the BioSig RP Software, yielding a 2000-stimulus trace to eliminate any stimulus artifact. At frequencies close to the threshold, this procedure was performed at least twice and the AEP traces were overlaid to examine if they were repeatable. SPL values of tone burst stimuli were reduced in 4 dB steps. The lowest SPL where a recognizable and repeatable AEP trace could be obtained was considered the hearing threshold.

Sound stimuli presentation and AEP waveform recording were accomplished using a Tucker-Davis Technologies (Gainesville, FL, USA) modular rack-mount system (TDT System 3) controlled by Pentium 4 PC containing a TDT digital processing board and running TDT BioSig RP Software. A dual-cone speaker (Wharfedale Pro Twin 8, frequency response: 65 Hz–20 kHz ±3 dB), mounted 1 m above subjects in the air, was used to present tone stimuli during testing.

Hearing thresholds were obtained using the auditory evoked potentials (AEP) recording technique. Although hearing generalists, such as batrachoidids, primarily detect particle motion of sounds (Fay and Edds-Walton, 1997; Weeg et al., 2002), for technical reasons we determined hearing thresholds of the Lusitanian toadfish in pressure units. This experimental procedure is acceptable because our study emphasized a comparison of hearing abilities of different-sized fish with their corresponding absolute sound power spectra of agonistic vocalizations, which are also given in pressure units. Moreover, this approach with hearing generalists has frequently been adopted in similar studies, e.g. the Lusitanian toadfish *Halobatrachus didactylus* (Vasconcelos et al., 2007), the oyster toadfish *Opsanus tau* (Yan et al., 2000), the bluegill sunfish *Lepomis macrochirus* (Scholik and Yan, 2002), the gobies *Padogobius martensii* and *Gobius nigricans* (Lugli et al., 2003), the European perch *Perca fluviatilis* (Amoser et al., 2004; Amoser and Ladich, 2005) and the damselfish *Abudefduf saxatilis* (Egner and Mann, 2005). Even so, the hearing thresholds should not be considered as absolute values. Calibration tests were performed later on using an uniaxial pressure acceleration sensor (p-a probe, Applied Physical Sciences Corporation, Groton, CT, USA) and showed that pressure and particle velocity were positively correlated to each other below the water surface in our experimental tub. Any 4 dB change in SPL was accompanied by a 4 dB change in particle acceleration at any frequency (re. 1 $\mu m\ s^{-2}$).

Statistical analysis

Means of sound characteristics were calculated for each fish (based on 10 sounds per individual) and used for further analyses. Relationships between fish size (*SL* or log*SL*) and sound characteristics (or log of the measured variables) were determined by Pearson's correlation coefficients and linear regressions.

Audiograms from different fish groups were compared by a repeated-measures ANOVA, which analyzed responses (hearing thresholds) to several frequencies in each subject fish (within-subject factor) of different size groups (between-subject factor).

In addition, a one-way ANOVA was performed separately at each test frequency, followed by a Bonferroni *post-hoc* test, in order to verify group-specific differences.

Parametric tests were used preferentially since data were normally distributed and variances homogeneous. All SPL values obtained (in dB) were converted to sound pressure (μPa), used for calculations, and then converted back to dB. Therefore, two different values for s.e.m. are given (see Table 1). The statistical tests were performed with Statistica 7.1 for Windows (StatSoft, Inc., 2005).

RESULTS

Sound production

Lusitanian toadfish were territorial at early stages of development. Small specimens from G3 exhibited several agonistic displays during shelter occupation and feeding, such as opening the mouth and spreading of pectoral fins and opercula during confrontation in aquaria. Sounds were produced in all groups tested and started almost immediately when handling the specimens. However, within the G1 size range, only one specimen measuring 3.8 cm *SL* (body mass=2.14 g) showed vocal activity, whereas the others did not utter sounds during the experimental procedure (*SL*=2.8–3.8 cm, body mass=0.60–1.80 g, *N*=6).

Agonistic vocalizations in groups G1 and G2 consisted primarily of single grunts, whereas in groups G4 and G5 they were often produced in series with shorter intervals between consecutive grunts (Fig. 2).

The total duration of single grunts ($r=-0.469$, $N=44$, $P=0.001$) and the number of pulses within grunts ($r=-0.761$, $N=44$, $P<0.001$, Fig. 3) decreased with growth, in contrast to pulse period ($r=0.693$, $N=44$, $P<0.001$, Fig. 4). Sound pressure levels were positively correlated with fish size ($r=0.944$, $N=38$, $P<0.001$, Fig. 5).

Sound spectra showed that in G1 sound energy was concentrated at the third and fourth harmonics (420–570 Hz), while in G5, the main energy was mostly found at the first harmonic at about 110 Hz. Intermediate groups showed a gradual change as fish grew (Fig. 6).

Auditory sensitivity

Auditory evoked potentials were recorded in all test groups between 50 and 1000 Hz, with the exception of G1 and G2, where a recognizable and repeatable AEP trace could not be obtained at 1000 Hz (Table 1, Fig. 7). All size groups revealed best hearing at 50 Hz and a sensitivity decrease towards 1000 Hz. The mean hearing thresholds increased from about 77 dB re. 1 μPa at 50 Hz (G3–G5) up to 132 dB at 1000 Hz (G3).

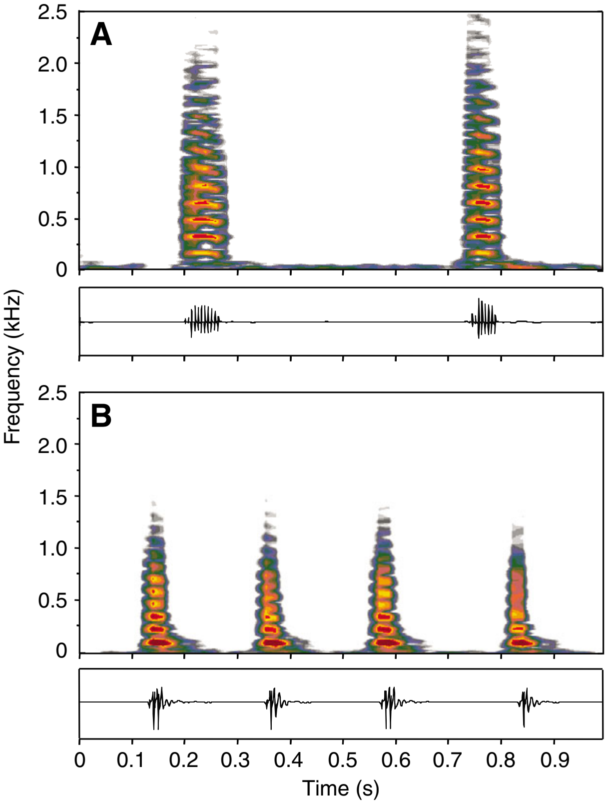

Fig. 2. Sonogram and oscillogram of a grunt call produced by a representative *H. didactylus* of (A) group G2 (6.1 cm *SL*) and (B) group G5 (28.5 cm *SL*), showing two single grunts (A) and a part of a grunt train (B). Note the shorter grunt duration and lower dominant frequency in B. Sampling frequency 6 kHz, filter bandwidth 15 Hz, 70% overlap, Blackman–Harris window.

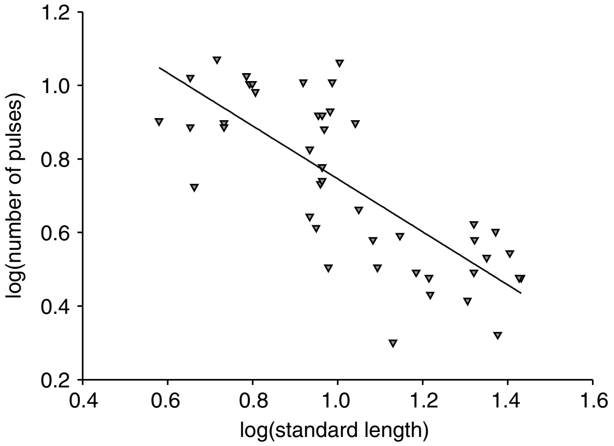

Fig. 3. Log–log plot of mean number of pulses against standard length (*SL*, in cm). Regression equation: log number of pulses=–0.72×log(*SL*)+1.47. N=44, P<0.001, r^2=0.579.

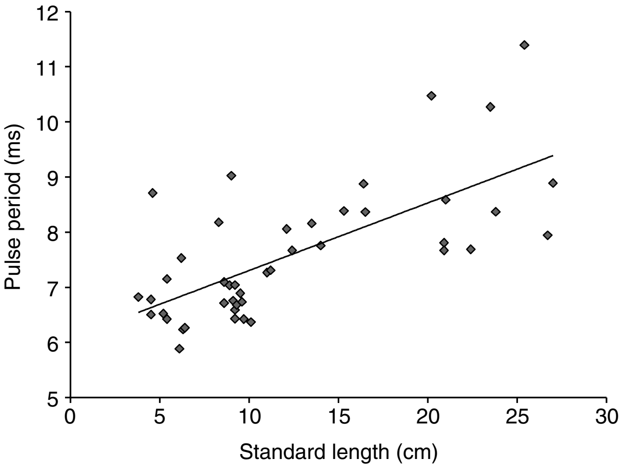

Fig. 4. Correlation between mean pulse period and standard length (*SL*). Regression equation: pulse period=0.12×*SL*+6.08. N=44, P<0.001, r^2=0.480.

Comparisons between audiograms obtained from all size groups (at the frequency range 50–800 Hz) showed significant overall differences (repeated-measures ANOVA, $F_{4,27}$=9.01, P<0.001) and significant interactions between size and frequency ($F_{20,135}$=8.99, P<0.001). Namely, the audiogram of the smallest size group (G1) differed significantly from those of G4 (repeated measures ANOVA, $F_{1,10}$=9.77, P=0.011) and G5 (repeated measures ANOVA, $F_{1,12}$=21.58, P<0.001).

Comparing groups at each frequency separately revealed significant differences at 100 Hz (one-way ANOVA, $F_{4,28}$=11.85, P<0.001) and at the highest test frequencies, 800 Hz (one-way ANOVA, $F_{4,29}$=9.80, P<0.001) and 1000 Hz (one-way ANOVA, $F_{2,19}$=27.58, P<0.001) (Fig. 7). Bonferroni *post-hoc* tests revealed significant group-specific differences, namely: at 100 Hz, between G1 and all the others; at 800 Hz, between groups G1 and G3 and groups G4 and G5; and at 1000 Hz, between G3 and groups G4 and G5. At 50 Hz, inter-group differences were close to significance (one-way ANOVA, $F_{4,28}$=2.98, P=0.036; Bonferroni *post-hoc* test: between G1 and G5: P=0.061; between G1 and G3: P=0.073).

Comparison between sound spectra and audiograms

Comparison between audiograms and sound power spectra within the same size group (Fig. 8), calculated for a distance of 10 cm, showed that the agonistic vocalizations were clearly detectable in groups G4 and G5. Sound spectra were considerably above hearing thresholds in the frequency range below 200 Hz (up to circa 20–30 dB re. 1 μPa at 100 Hz), where the main energy of agonistic vocalizations was concentrated. In G3, sound energy was up to about 5 dB re. 1 μPa above hearing thresholds, at approx. 160 Hz. However, within G2 and G1 juveniles, the sound spectrum was more than 5 and 15 dB re. 1 μPa below the auditory curve, respectively.

DISCUSSION

Development of sound production

Agonistic vocalizations are produced in numerous contexts, such as distress or disturbance situations (e.g. while being attacked or grabbed by potential predators), competitive feeding and competition for space (Ladich and Myrberg, 2006). Competition for food and space is important for both adults and all juveniles, and sound production during agonistic contexts has been reported in juvenile stages of several non-related families such as tigerperches, cobitids, gouramis and gurnards (Schneider, 1964; Valinski and Rigley, 1981; Henglmüller and Ladich, 1999; Wysocki and Ladich, 2001; Amorim and Hawkins, 2005).

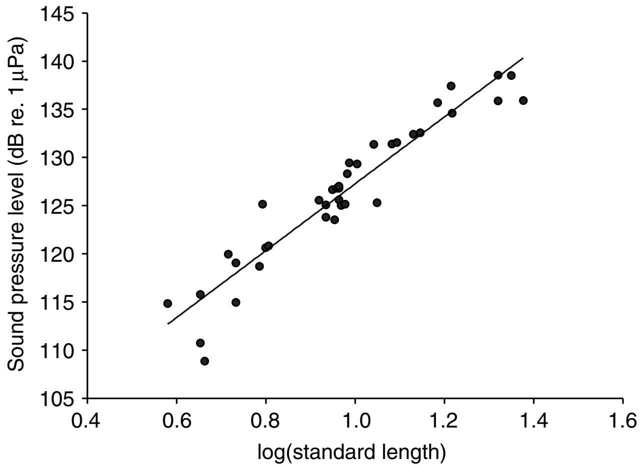

Fig. 5. Correlation between mean sound pressure level and log standard length (*SL*, in cm). Regression equation: sound pressure level=34.70×log(*SL*)+92.56. N=38, P<0.001, r^2=0.903.

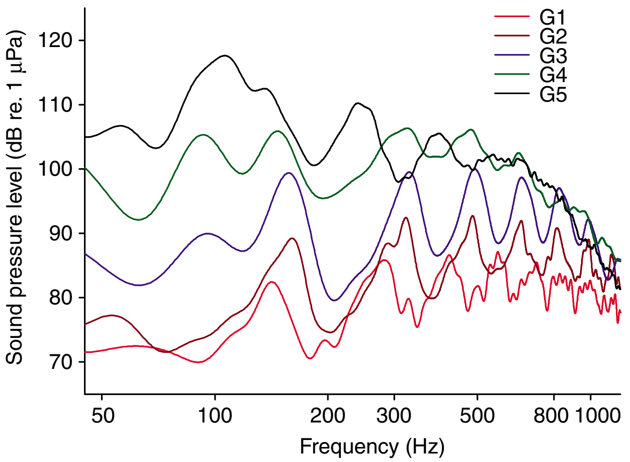

Fig. 6. Cepstrum-smoothed sound power spectra (mean) of the Lusitanian toadfish grunt call from groups G1 (3.8 cm *SL*, N=1); G2 (5.4–6.6 cm *SL*, N=5); G3 (8.0–10.2 cm *SL*, N=9); G4 (12.4–15.3 cm *SL*, N=6); and G5 (20.2–31.8 cm *SL*, N=8). Sampling frequency 6 kHz, filter bandwidth 1 Hz, 75% overlap, Blackman–Harris window.

Lusitanian toadfish juveniles were extremely territorial and exhibited agonistic displays (at least starting at *SL*=8 cm, probably 1–2 years old), including opening the mouth and extension of pectoral fins during confrontation with similar-sized conspecifics. When handling the fish, agonistic vocalizations were uttered in all different size/age classes studied (from *SL* 4–32 cm, a few months up to *circa* 5–8 years old). However, in the smallest size group (*SL*=2.8–3.8 cm), most of the tested animals did not exhibit vocal activity and only the heaviest specimen uttered sounds during the experimental proceeding. These data suggest that either in this early stage the sound-producing apparatus was not sufficiently developed to produce sounds or it could be too risky demonstrate toughness when the fish are too small and vulnerable to potential predators.

In general, sounds consisted mostly of single grunts in juveniles (groups G1–3), whereas in sexually mature specimens, i.e. G5 and probably G4 (total length more than 15 cm), were often produced series or trains of grunts. The minimum maturity sizes are 16 cm and 19 cm total length for males and females, respectively (Palazón-Fernández et al., 2001).

Agonistic calls of adults recorded in the laboratory by handling the specimens were similar to those obtained from field recordings at the nesting places of *H. didactylus*, which are important during agonistic contexts and for territorial occupation (see Dos Santos et al., 2000; Amorim et al., 2006). This similarity in terms of temporal and spectral characteristics between handheld fish calls underwater and field-recorded grunt trains has also been described in other batrachoidids, e.g. *Opsanus tau* (Cohen and Winn, 1967). In addition, through brain stimulation in *Opsanus beta* (Demski and Gerald, 1972; Demski and Gerald, 1974) and in *O. tau* (Fine, 1979; Fine and Perini, 1994), grunts were produced in the laboratory and shown to be similar to field-recorded calls of the species. Interestingly, the other agonistic vocalizations of the Lusitanian toadfish, such as croak and double-croak, were not emitted during sound recordings, because they are probably related to spacing functions and not distress.

The vocalizations produced during different developmental stages showed clear changes in temporal characteristics, spectral content and intensities. These changes are perhaps associated with

Table 1. Auditory thresholds in the five different test groups

	Test frequency (Hz)													
Group*	50		100		200		300		500		800		1000	
G1	85	+2.22 –2.99	98	+1.19 –1.37	97	+1.37 –1.63	102	+1.13 –1.31	114	+0.72 –0.79	132	+1.74 –2.18	NR	
G2	80	+1.56 –1.90	91	+1.34 –1.59	97	+1.83 –2.33	97	+1.10 –1.26	115	+2.20 –2.95	126	+0.86 –0.96	NR	
G3	77	+1.24 –1.44	89	+1.24 –1.45	97	+2.52 –3.56	101	+1.49 –1.79	117	+1.99 –2.59	130	+1.48 –1.78	132	+1.03 –1.17
G4	77	+2.07 –2.72	87	+0.78 –0.85	98	+1.29 –1.52	100	+0.19 –0.19	115	+0.08 –0.09	120	+0.12 –0.12	120	+0.41 –0.43
G5	77	+1.96 –2.53	91	+1.03 –1.17	98	+0.11 –0.11	102	+0.25 –0.25	111	+0.24 –0.24	117	+0.24 –0.25	121	+0.61 –0.65

Values are mean ± s.e.m.

*Group G1, *SL*=2.8–3.8 cm (N=6); G2, *SL*=5.4–6.6 cm (N=6); G3, *SL*=8.0–10.2 cm (N=7); G4, *SL*=12.4–15.3 cm (N=6); G5, *SL*=20.2–31.8 cm (N=9). NR, no response.

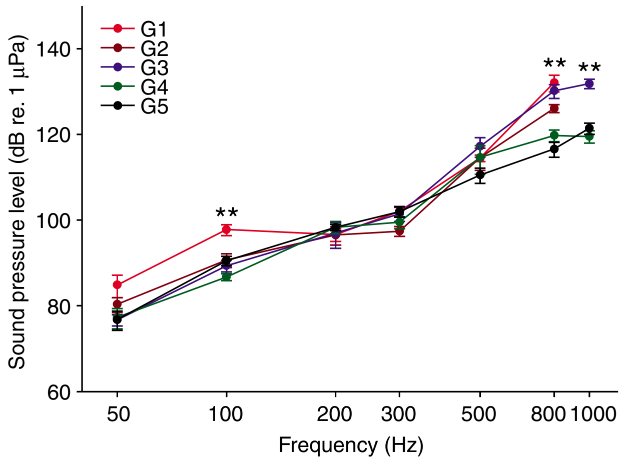

Fig. 7. Auditory thresholds of juveniles from the five different size groups. G1, 2.8–3.8 cm *SL* (*N*=6); G2, 5.4–6.6 cm *SL* (*N*=6); G3, 8.0–10.2 cm *SL* (*N*=7); G4, 12.4–15.3 cm *SL* (*N*=6); and G5, 20.2–31.8 cm *SL* (*N*=9). Values are mean ± s.e.m. Asterisks indicate highly statistically significant differences between groups (one-way ANOVA); $**P<0.001$.

the swimbladder and intrinsic sonic muscles, which both increase in size throughout life in *H. didactylus* (Modesto and Canário, 2003b).

The duration and therefore number of pulses within a grunt diminished with toadfish growth, contrary to other fish species such as the croaking gourami *T. vittata* and the grey gurnard *Eutrigla gurnardus*, where these parameters increased with size (Henglmüller and Ladich, 1999; Amorim and Hawkins, 2005). This difference is probably because larger toadfish emitted long trains of grunts with shorter intervals between consecutive grunts. These trains may indicate elevated aggression but also higher development of the sonic neuromuscular system, i.e. sonic motor nucleus (SMN) and intrinsic swimbladder sonic muscles (Fine et al., 1984; Fine, 1989).

On the other hand, pulse period within a grunt increased with size in our study species, similar to the gourami (Henglmüller and Ladich, 1999); this points to a lower sonic muscle contraction rate in larger toadfish (Fine et al., 2001) (for a review, see Ladich and Fine, 2006).

The dominant frequency of sounds decreased with increasing fish size. Comparing sound spectra of agonistic vocalizations obtained at different stages of development indicated a clear gradual shift in main energies of sounds from higher harmonics (between 420 and 570 Hz, groups G1–3, <10 cm *SL*) down to the first harmonic (at approx. 110 Hz) with increasing size (G5, >20 cm *SL*). Correlations between dominant frequencies of sounds and size are also known in other fish species, e.g. bicolor damselfish (Myrberg et al., 1993), croaking gouramis (Ladich et al., 1992), mormyrids (Crawford, 1997) and grey gurnard (Amorim and Hawkins, 2005). However, a decrease in dominant frequency during ontogeny since early developmental stages has only been reported in the croaking gourami (Henglmüller and Ladich, 1999; Wysocki and Ladich, 2001).

SPL values increased significantly during growth. This allowed larger fish to produce louder signals to deter opponents. A similar positive relationship between size and sound amplitude was reported for the croaking gourami *T. vittata* (Wysocki and Ladich, 2001), as well as for the weakfish *Cynoscion regalis* (Connaughton et al., 2002).

Our data suggest that sound characteristics may inform conspecifics about the size of sound producers. In addition to visual cues, this information can be valuable for assessing the fighting ability of opponents and thus to decide contests before they escalate to more costly phases, i.e. damaging combat (Ladich, 1998).

Development of hearing

Auditory evoked potentials could be obtained in all size groups, including the smallest juveniles with, for instance, 2.8 cm *SL* (the maximum size of *H. didactylus* exceeds 50 cm). In general, this species revealed best auditory sensitivity at low frequencies in all stages of development, namely below 300 Hz (with hearing

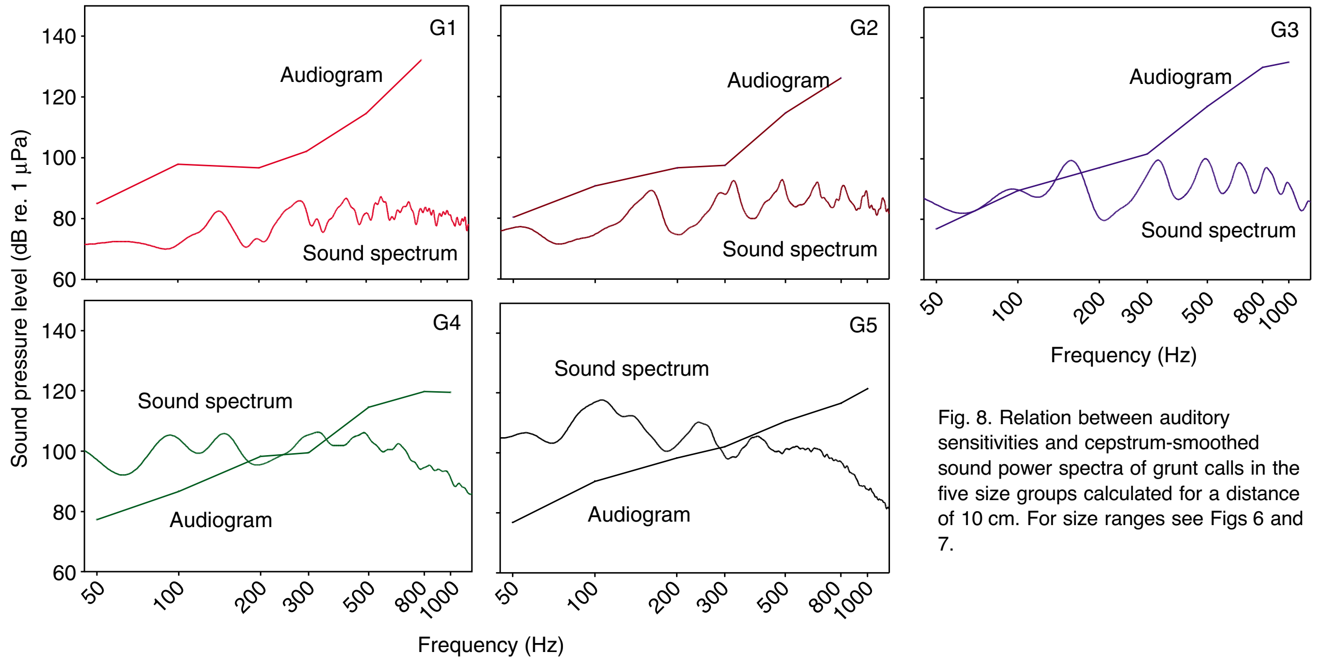

Fig. 8. Relation between auditory sensitivities and cepstrum-smoothed sound power spectra of grunt calls in the five size groups calculated for a distance of 10 cm. For size ranges see Figs 6 and 7.

thresholds under 100 dB re. 1 μPa), with a decrease in sensitivity by up to 55 dB re. 1 μPa observed towards 1000 Hz. Although earlier stages were not investigated (the fish did not hatch in the laboratory), our data indicated that hearing sensitivity changes only slightly during growth. Only the smallest toadfish group revealed higher hearing thresholds within the best hearing range (100 Hz). Moreover, at higher frequencies (i.e. 800 and 1000 Hz) younger fish demonstrated either absence of auditory response or lower sensitivity.

Batrachoidids are classified as hearing non-specialists or generalists (Fish and Offutt, 1972; McKibben and Bass, 1999; Weeg et al., 2002; Sisneros and Bass, 2005); they lack accessory hearing structures to enhance auditory abilities and therefore likely respond to the particle motion component of low frequency sounds at relatively high sound intensities (Hawkins and Myrberg, 1983; Ladich and Popper, 2004). The Lusitanian toadfish, similar to other generalists, possesses limited auditory abilities and, as a consequence, probably does not show considerable sensitivity changes during life history. According to the calibration tests carried out using a particle acceleration sensor it can be assumed that the slight changes in pressure thresholds observed during ontogeny are paralleled by particle acceleration changes of the same degree. In an ontogenetic study, Sisneros and Bass (Sisneros and Bass, 2005) investigated the response properties of individual primary auditory afferents in the plainfin midshipman fish *P. notatus* (Batrachoididae) and showed that the best hearing range was between 60 and 200 Hz in small juveniles and large juveniles as well as adults. Similar to our results in the Lusitanian toadfish, the most sensitive frequencies did not change during ontogeny. The same authors reported an increment in auditory sensitivity in *P. notatus* at the most sensitive frequency (from 118 to 104 dB re. 1 μPa) from small to large juveniles. No difference was found between large juveniles and adults. Congruently, our study revealed significant hearing differences between size groups, i.e. *circa* 7 dB re. 1 μPa at 100 Hz (and 8 dB at 50 Hz close to significance) between the smallest and largest fish. This smaller hearing difference during growth of the European toadfish relative to the Californian batrachoidid might reflect genus-specific differences or the different age groups chosen.

Studies on other species, including hearing specialists, are contradictory, with no straightforward conclusions. Auditory sensitivity increases dramatically during development, by about 50 dB re. 1 μPa in the bicolor damselfish *S. partitus* (Kenyon, 1996), whereas the opposite was found in another damselfish, the sergeant major *Abudefduf saxatilis* (Egner and Mann, 2005). Egner and Mann revealed that sensitivity decreases at low frequencies in larger fish. Different developmental tendencies were also reported among non-related hearing specialists, namely improvements as well as no changes in hearing sensitivity. Hearing sensitivity improves by about 14 dB re. 1 μPa in croaking gourami and the most sensitive frequency drops from 2.5 kHz to 1.5 kHz (Wysocki and Ladich, 2001). In contrast, no changes were observed in differently sized cyprinids. Neither the goldfish *Carassius auratus* nor the zebra fish *Danio rerio* exhibited improved hearing during growth (Popper, 1971; Higgs et al., 2002; Higgs et al., 2003).

Relationship between development of hearing and sound production: onset of acoustic communication

Comparing audiograms and sound spectra in larger size groups (G4 and G5) revealed that the main energy of sounds was located within their most sensitive frequencies, i.e. below 300 Hz. In small juveniles (groups G1–2), however, dominant frequencies were found between 420–570 Hz and did not match as well with their best hearing range.

According to our results, adults were able to detect vocal agonistic signals of same-sized conspecifics, as sound energies were up to 30 dB re. 1 μPa (at about 110 Hz) above hearing thresholds. In the smallest juveniles analyzed (<4 cm *SL* and just a few months old) the sound spectrum was somewhat below the auditory curve, suggesting that the ability to perceive sounds and therefore to communicate acoustically with same-sized conspecifics is lacking or only possible at very short distances. This is due to the low SPL values of vocalizations and to the high dominant frequency. Although we determined sound pressure levels in our ontogenetic study we assume that our conclusion also hold for particle acceleration levels because these two acoustical parameters were proportional in our tanks according to calibration tests. Additionally, pressure and particle velocity spectra of ambient noise and vocalizations of the goby *Padogobius bonelli* are relatively similar in terms of main energy distribution (Lugli and Fine, 2007).

The onset of the development of acoustic communication is still poorly investigated in fishes. Hearing develops prior to the onset of sound production in the croaking gourami and the ability of juveniles to communicate acoustically starts gradually when thresholds decrease and sound intensities increase (Wysocki and Ladich, 2001). The species investigated so far (croaking gouramis and Lusitanian toadfish) reveal similar developmental trends. The results suggest that, in both cases, sound detection develops prior to the ability to generate sounds and that acoustic communication might be absent in earliest developmental stage because of low hearing sensitivities or low sound levels. Nevertheless, juveniles of both hearing specialist and generalist start early to communicate acoustically during agonistic interactions.

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REFERENCES

Amorim, M. C. P. and Hawkins, A. D. (2005). Ontogeny of acoustic and feeding behaviour in the Grey Gurnard, *Eutrigla gurnardus. Ethology* **111**, 255-269.

Amorim, M. C. P., Vasconcelos, R. O., Marques, J. F. and Almada, F. (2006). Seasonal variation of sound production in the Lusitanian toadfish, *Halobatrachus didactylus. J. Fish Biol.* **69**, 1892-1899.

Amoser, S. and Ladich, F. (2005). Are hearing sensitivities of freshwater fish adapted to the ambient noise in their habitats? *J. Exp. Biol.* **208**, 3533-3542.

Amoser, S., Wysocki, L. E. and Ladich, F. (2004). Noise emission during the first powerboat race in an Alpine lake and potential impact on fish communities. *J. Acoust. Soc. Am.* **116**, 3789-3797.

Branchi, I., Santucci, D. and Alleva, E. (2001). Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. *Behav. Brain Res.* **125**, 49-56.

Cohen, M. J. and Winn, H. E. (1967). Electrophysiological observations on hearing and sound production in the fish, *Porichthys notatus. J. Exp. Zool.* **165**, 355-369.

Connaughton, M. A., Fine, M. L. and Taylor, M. H. (2002). Weakfish sonic muscle: influence of size, temperature and season. *J. Exp. Biol.* **205**, 2183-2188.

Corwin, J. T. (1983). Postembryonic growth of the macula neglecta auditory detector in the ray, *Raja clavata*: continual increases in hair cell number, neural convergence and physiological sensitivity. *J. Comp. Neurol.* **217**, 345-356.

Crawford, J. D. (1997). Hearing and acoustic communication in mormyrid electric fishes. *Mar. Freshw. Behav. Physiol.* **29**, 65-86.

Demski, L. S. and Gerald, J. W. (1972). Sound production evoked by electric stimulation of the brain in toadfish (*Opsanus beta*). *Anim. Behav.* **20**, 507-513.

Demski, L. S. and Gerald, J. W. (1974). Sound production and other behavioral effects of midbrain stimulation in the free-swimming toadfish, *Opsanus beta. Brain Behav. Evol.* **9**, 41-59.

Dimitrieva, L. P. and Gottlieb, G. (1992). Development of brainstem auditory pathway in mallard duck embryos and hatchlings. *J. Comp. Physiol. A* **61**, 19-28.

Dimitrieva, L. P. and Gottlieb, G. (1994). Influence of auditory experience on the development of brainstem auditory-evoked potentials in mallard duck embryos and hatchlings. *Behav. Neurol. Biol.* **61**, 19-28.

Dos Santos, M. E., Modesto, T., Matos, R. J., Grober, M. S., Oliveira, R. F. and Canário, A. (2000). Sound production by the Lusitanian toadfish, *Halobatrachus didactylus*. *Bioacoustics* **10**, 309-321.

Egner, S. A. and Mann, D. A. (2005). Auditory sensitivity of sergeant major damselfish *Abudefduf saxatilis* from post-settlement juvenile to adult. *Mar. Ecol. Progr. Ser.* **285**, 213-222.

Fay, R. R. and Edds-Walton, P. L. (1997). Diversity in frequency properties of saccular afferents of the toadfish, *Opsanus tau*. *Hear. Res.* **113**, 235-246.

Fine, M. L. (1979). Sounds evoked by brain stimulation in the oyster toadfish *Opsanus tau*. *Exp. Brain Res.* **35**, 197-212.

Fine, M. L. (1989). Embryonic, larval and adult development of the sonic neuromuscular system in the oyster toadfish. *Brain Behav. Evol.* **34**, 13-24.

Fine, M. L. and Perini, M. A. (1994). Sound production evoked by electrical stimulation of the forebrain in the oyster toadfish. *J. Comp. Physiol. A* **174**, 173-185.

Fine, M. L., Economos, D., Radtke, R. and Mcclung, J. R. (1984). Ontogeny and sexual dimorphism of the sonic motor nucleus in the oyster toadfish. *J. Comp. Neurol.* **225**, 105-110.

Fine, M. L., Malloy, K. L., King, C. B., Mitchell, S. L. and Cameron, T. M. (2001). Movement and sound generation by the toadfish swimbladder. *J. Comp. Physiol. A* **187**, 371-379.

Fish, J. and Offutt, C. (1972). Hearing thresholds from toadfish, *Opsanus tau*, measured in the laboratory and field. *J. Acoust. Soc. Am.* **51**, 1318-1321.

Hawkins, A. D. and Myrberg, A. A. (1983). Hearing and sound communication under water. In *Bioacoustics: A Comparative Approach* (ed. B. Lewis), pp. 347-405. London: Academic Press.

Henglmüller, S. M. and Ladich, F. (1999). Development of agonistic behaviour and vocalization in croaking gouramis. *J. Fish Biol.* **54**, 380-395.

Higgs, D. M., Souza, M. J., Wilkins, H. R., Presson, J. C. and Popper, A. N. (2002). Age- and size-related changes in the inner ear and hearing ability of the adult zebrafish (*Danio rerio*). *J. Assoc. Res. Otolaryngol.* **3**, 174-184.

Higgs, D. M., Rollo, A. K., Souza, M. J. and Popper, A. N. (2003). Development of form and function in peripheral auditory structures of the zebrafish (*Danio rerio*). *J. Acoust. Soc. Am.* **113**, 1145-1154.

Kenyon, T. N. (1996). Ontogenetic changes in the auditory sensitivity of damselfish (Pomacentridae). *J. Comp. Physiol. A* **179**, 553-561.

Kenyon, T. N., Ladich, F. and Yan, H. Y. (1998). A comparative study of hearing ability in fishes: the auditory brainstem response approach. *J. Comp. Physiol. A* **182**, 307-318.

Ladich, F. (1998). Sound characteristics and outcome of contests in male croaking gouramis (Teleostei). *Ethology* **104**, 517-529.

Ladich, F. and Fine, M. L. (2006). Sound-generating mechanisms in fishes: a unique diversity in vertebrates. In *Communication in Fishes*. Vol. 1 (ed. F. Ladich, S. P. Collin, P. Moller and B. G. Kapoor), pp. 1-43. Enfield, NH: Science Publishers.

Ladich, F. and Myrberg, A. A. (2006). Agonistic behavior and acoustic communication. In *Communication in Fishes*. Vol. 1 (ed. F. Ladich, S. P. Collin, P. Moller and B. G. Kapoor), pp. 121-148. Enfield, NH: Science Publishers, Enfield.

Ladich, F. and Popper, A. N. (2004). Parallel evolution in fish hearing organs. In *Evolution of the Vertebrate Auditory System* (ed. G. Manley, A. N. Popper and R. R. Fay), pp. 95-127. New York: Springer-Verlag.

Ladich, F., Bischof, C., Schleinzer, G. and Fuchs, A. (1992). Intra- and interspecific differences in agonistic vocalization in croaking gouramis (genus: *Trichopsis*, Anabantoidei, Teleostei). *Bioacoustics* **4**, 131-141.

Lugli, M. and Fine, M. L. (2007). Stream ambient noise, spectrum and propagation of sounds in the goby *Padogobius martensii*: sound pressure and particle velocity. *J. Acoust. Soc. Am.* **122**, 2881-2892.

Lugli, M., Yan, H. Y. and Fine, M. L. (2003). Acoustic communication in two freshwater gobies: the relationship between ambient noise, hearing thresholds and sound spectrum. *J. Comp. Physiol. A* **189**, 309-320.

McKibben, J. R. and Bass, A. H. (1999). Peripheral encoding of behaviorally relevant acoustic signals in a vocal fish: single tones. *J. Comp. Physiol. A* **184**, 563-576.

Modesto, T. and Canário, A. V. M. (2003a). Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, *Halobatrachus didactylus*. *Gen. Comp. Endocrinol.* **131**, 220-231.

Modesto, T. and Canário, A. V. M. (2003b). Hormonal control of swimbladder sonic muscle dimorphism in the Lusitanian toadfish *Halobatrachus didactylus*. *J. Exp. Biol.* **206**, 3467-3477.

Moss, C. F., Redish, D., Gounden, C. and Kunz, T. H. (1997). Ontogeny of vocal signals in the little brown bat, *Myotis lucifugus*. *Anim. Behav.* **54**, 131-141.

Myrberg, A. A., Ha, S. J. and Shamblott, M. J. (1993). The sounds of the bicolor damselfish (*Pomacentrus partitus*): predictors of body size and a spectral basis for individual recognition and assessment. *J. Acoust. Soc. Am.* **94**, 3067-3070.

Noll, A. M. (1967). Cepstrum pitch detection. *J. Acoust. Soc. Am.* **41**, 293-309.

Palazón-Fernández, J. L., Arias, A. M. and Sarasquete, C. (2001). Aspects of the reproductive biology of the toadfish *Halobatrachus didactylus* (Schneider, 1801). *Sci. Mar.* **65**, 131-138.

Podos, J., Sherer, J. K., Peters, S. and Nowicki, S. (1995). Ontogeny of vocal tract movements during song production in song sparrows. *Anim. Behav.* **50**, 1287-1296.

Popper, A. N. (1971). The effects of fish size on auditory capacities of the goldfish. *J. Aud. Res.* **11**, 239-247.

Reimer, K. (1996). Ontogeny of hearing in the marsupial, *Monodelphis domestica,* as revealed by brainstem auditory evoked potentials. *Hear. Res.* **92**, 143-150.

Ruben, R. J. (1995). The ontogeny of human hearing. *Int. J. Pediatr. Otorhinolaryngol.* **32**, 199-204.

Schneider, H. (1964). Physiologische und morphologische Untersuchungen zur Bioakustik der Tigerfische (Pisces, Theraponidae). *Z. Vergl. Physiol.* **47**, 493-558.

Scholik, A. R. and Yan, H. Y. (2002). The effects of noise on the auditory sensitivity of the bluegill sunfish, *Lepomis macrochirus*. *Comp. Biochem. Physiol. A* **133**, 43-52.

Sisneros, J. A. and Bass, A. H. (2005). Ontogenetic changes in the response properties of individual, primary auditory afferents in the vocal plainfin midshipman fish *Porichthys notatus* Girard. *J. Exp. Biol.* **208**, 3121-3131.

Valinski, W. and Rigley, L. (1981). Function of sound production by the skunk loach *Botia horae* (Pisces Cobitidae). *Z. Tierpsychol.* **55**, 161-172.

Vasconcelos, R. O., Amorim, M. C. P. and Ladich, F. (2007). Effects of ship noise on the detectability of communication signals in the Lusitanian toadfish. *J. Exp. Biol.* **210**, 2104-2112.

Walsh, P. (1987). Lactate uptake by toadfish hepatocytes: passive diffusion is sufficient. *J. Exp. Biol.* **130**, 295-304.

Weeg, M., Fay, R. and Bass, A. (2002). Directionality and frequency tuning of primary saccular afferents of a vocal fish, the plainfin midshipman (*Porichthys notatus*). *J. Comp. Physiol. A* **188**, 631-641.

Wysocki, L. E. and Ladich, F. (2001). The ontogenetic development of auditory sensitivity, vocalization and acoustic communication in the labyrinth fish *Trichopsis vittata*. *J. Comp. Physiol. A* **187**, 177-187.

Wysocki, L. E. and Ladich, F. (2005a). Hearing in fishes under noise conditions. *J. Assoc. Res. Otolaryngol.* **6**, 28-36.

Wysocki, L. E. and Ladich, F. (2005b). Effects of noise exposure on click detection and the temporal resolution ability of the goldfish auditory system. *Hear. Res.* **201**, 27-36.

Yan, H. Y., Fine, M. L., Horn, N. S. and Colón, W. E. (2000). Variability in the role of gasbladder in fish audition. *J. Comp. Physiol. A* **186**, 435-445.

CHAPTER III

ONTOGENY OF ACOUSTIC COMMUNICATION

(ii) **Development of the auditory sense parallels vocal differentiation in a highly vocal fish, *Halobatrachus didactylus***

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(in prep.)

DEVELOPMENT OF THE AUDITORY SENSE PARALLELS VOCAL DIFFERENTIATION IN A HIGHLY VOCAL FISH, *HALOBATRACHUS DIDACTYLUS*

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SUMMARY

Development of hearing abilities is still poorly investigated in fish. Moreover, the relation between developmental changes of the auditory sense and vocal differentiation remains unknown in this taxon. The Lusitanian toadfish *Halobatrachus didactylus* is a highly vocal teleost that shows remarkable territoriality since early developmental stages. Our aims were: (1) investigate whether the saccule sensitivity in *H. didactylus* changes with growth and (2) determine if ontogenetic auditory modifications parallel the development of the vocal repertoire. We recorded evoked potentials from populations of saccular hair cells from juveniles (g1: 2-5 cm; g2: 5-9 cm standard length) and adult toadfish (g3: >19 cm) under 75-945 Hz tone stimuli. Vocal activity was recorded during social interactions within the same juvenile groups. Saccular hair cells were most sensitive at the lowest frequencies in all groups but significant differences in auditory thresholds were found between the smallest juveniles (g1) and the other two groups, i.e. circa 10 dB improvement with growth. Larger juveniles (g2) did not differ in sensitivity from adults. Both g1 and g2 fish produced sounds during agonistic interactions but considerable differences were found in respect to the vocal complexity. While g1 emitted mostly single grunts and less frequently other variations of grunt calls (grunt train and long grunt train), g2 exhibited the full vocal repertoire of adults with at least five different vocalizations, including double-croaks and boatwhistles. Our data indicate that modifications in the auditory periphery seem to parallel the development of the vocal system in the Lusitanian toadfish.

Keywords: *hearing, saccule, ontogeny, acoustic communication, Batrachoididae.*

INTRODUCTION

Species-specific behaviours are often originated via incomplete patterns that are gradually transformed into the final complete adult form. A classical example is the vocal behaviour in immature animals that generally evolves from unstructured or simple calls into more complex and stereotyped adult vocalizations. This process can be achieved by learning the adult behaviour (e.g. Brainard and Doupe 2002), although non-learner species also reveal considerable ontogenetic changes in their calls resulting from modifications in the vocal motor system, i.e. peripheral apparatus and central neural mechanisms (e.g. Jürgens 2002; Derégnaucourt et al. 2009).

The development of the vocal repertoire, or vocal differentiation, has been well documented in birds and mammals (e.g. Moss et al. 1997; Aronov et al. 2008). However, studies that examine vocal production in other vertebrates such as fish, with relative simplicity of central and peripheral vocal mechanisms, can ultimately provide valuable insight into the evolution of vocal communication systems.

Some studies reported ontogenetic changes in sound characteristics, such as amplitude, repetition rate, sound duration and dominant frequency, with increasing fish size, most likely resulting from changes in the size of the sound generating apparatus (e.g. Ladich et al. 1992; Myrberg et al. 1993; Crawford 1997; Amorim and Hawkins 2005). Nevertheless, whether in fish can also exhibit changes in the vocal repertoire throughout development, as documented in birds (Dmitriy et al. 2008), has never been investigated.

On the other hand, data on development of hearing abilities is again mainly available for anurans, birds and mammals and scarce for other vertebrates. These studies revealed common principles, namely improvement of auditory sensitivity (e.g. anurans, Boatright-Horowitz and Megela Simmons 1995; reptiles, Werner et al. 1998; birds, Dimitrieva and Gottlieb 1994; mammals, Reimer 1996), an extension of the frequency hearing range (e.g. birds, Golubeva and Tikhonov 1985; mammals, Rübsamen 1992) and a shift in the most sensitive frequency range (e.g. birds, Golubeva and Tikhonov

1985; frogs, Boatright-Horowitz and Megela Simmons 1995). Studies on the ontogeny of hearing in teleost fishes show varying results, ranging from no differences (Popper 1971; Zeddies and Fay 2005), expansion of the detectable frequency range (Higgs et al. 2001; Higgs et al. 2003), up to improvements of hearing sensitivities with size (Kenyon 1996; Wysocki and Ladich 2001; Sisneros and Bass 2005).

The onset of acoustic communication, which has been less documented, requires that the auditory system sensitivity and the main energy of the vocal output match within the same frequency range. In birds, Golubeva and Tikhonov (1985) showed that the major auditory improvement coincides with the onset of vocal behaviour. In fish, the onset of acoustic communication has been comparatively less investigated and the few existing studies indicate that sound detection develops prior to the ability to generate sounds and that acoustic communication might be absent in earlier developmental stages due to low hearing sensitivity (gourami, Wysocki and Ladich 2001) or may occur at all developmental stages (catfish, Lechner et al. 2010). Nerverthess, these studied species only produce broad-band pulsed sounds and, consequently, whether the vocal differentiation parallels the enhancement of hearing abilities throughout development in fish with more elaborate and diverse acoustic signals remains uninvestigated in this taxon.

Lusitanian toadfish *Halobatrachus didactylus* is a highly vocal teleost that exhibits a complex acoustic repertoire in the adult form (Amorim et al. 2008) and shows remarkable territoriality since early developmental stages. Vasconcelos and Ladich (2008), based on overall auditory evoked potentials (AEP), reported developmental auditory improvements both at the lowest (100 Hz) and highest tested frequencies (800-1000 Hz). This study indicated that acoustic communication in this species might be absent in early developmental stages and that it seemed to start when juveniles (above circa 5 cm standard length) are able to generate grunts of higher sound level and lower dominant frequency.

The aim of this study was twofold: (1) investigate whether the sensitivity of saccular hair cells changes with growth in *H. didactylus* and (2) determine if ontogenetic auditory modifications parallel the development of the vocal repertoire.

METHODS

Test subjects

Test subjects were juveniles collected in Tagus and Mira estuaries (Portugal) by local fishermen (trawl), during February-March, which were classified into two size groups (see Vasconcelos and Ladich 2008): (g1) 2.4-4.9 cm, standard length (SL), 0.60-3.10 g, body mass (BM); (g2), 5.0-8.69 cm SL, 3.17-15.02 g BM. These size groups were tested both for saccular sensitivity measurements (g1: N=25; g2: N=8) and sound recordings (g1: N=17; g2: N=13). Toadfish adults also caught by trawling in the Tagus estuary, during March and September, were tested for auditory sensitivity: 27 type I males, with 19.1-31.1 cm, SL, 180-795 mg, BM; 5 females, 26.5-30 cm SL, 435-811 g BM. Fish were transported during the same day to stock tanks. Juveniles and adult fish used for saccular potential recordings were kept at 21 ± 1 ºC in 40 l, and 80-250 l tanks, respectively, up to 15 days prior to testing, in order to avoid effects of stress and captivity that may affect saccular sensitivity (i.e. possible reduction in sensitivity, Sisneros and Bass 2003).

After auditory recordings fish were sacrificed by immersion in a 0.025% ethyl p-aminobenzoate saltwater bath. Both sex and adult male type (nest-guarding type I vs. type II sneakers, see Modesto and Canário 2003) were always confirmed by dissection of each specimen.

All electrophysiological experiments were perform at the University of Washington and followed National Institutes of Health guidelines for the care and use of animals and were approved by the University of Washington Institutional Animal Care and Use Committee. The animals used in this study, namely those that were transported from Portugal to USA, rapidly recovered from transportation and started to behave normally in the stock tanks and eating mostly within 24-48 h.

Saccular potential recording setup

The method for recording the saccular potentials was based on the experimental procedure adopted by Sisneros (2007, 2009) and Alderks and Sisneros 2011. Surgical procedures for exposing the inner ear saccule followed those in previous studies (e.g. Sisneros 2007). Briefly, fish were firstly anesthetized in a 0.025 % ethyl p-aminobenzoate saltwater bath and then immobilized by an intramuscular injection of pancuronium bromide: juveniles, circa 0.5 mg/Kg; adults, 2-4 mg/Kg. The saccule was then exposed by dorsal craniotomy and a barrier of denture cream was built up around the cranial cavity to allow the fish to be lowered below the water surface. A saline solution was used to prevent the cranial cavity from drying out and to clean eventual bleeding.

Test fish were placed in a Nalgene tank (30 cm diameter, 24 cm high) similar to Fay (1990) and positioned 10 cm above the surface of an underwater speaker that was embedded in gravel. During the experiment fresh seawater (21 ± 1ºC) was pumped into the mouth and over the gills. We monitored blood flow in the dorsal vasculature of the brain to ensure that especially the juveniles remained alive. The recording setup was located on a vibration isolation table housed inside an acoustic isolation chamber (Industrial Acoustics, New York, NY). All of the recording and stimulus generation equipment was located outside the isolation chamber.

Acoustic stimuli was generated via the reference output signal of a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA) that passed the stimulus signal through an audio amplifier to an underwater loud speaker (UW-30, Telex Communications, Burnsville, MN). Prior to each experiment we tested the speaker's frequency response characteristics by placing a mini-hydrophone (Bruel and Kjaer model 8103) 10 cm above the underwater speaker, in the position normally occupied by the fish's head during an experiment, and then measured the peak-to-peak voltage on an oscilloscope. This peak-to-peak voltage was then used by custom Matlab software to control an automated compensation script to calibrate the speaker so that pressure level at all test frequencies (75-945 Hz) was of equal amplitude within ±2 dB re 1µPa. In order to calibrate the stimulation system, sound pressure measurements of the stimulus frequencies were controlled using a spectrum analyzer (Stanford Research Systems SR780). Auditory stimuli consisted of eight repetitions of single 500 ms tones from 75 Hz to 945 Hz (in 10-80 Hz increments) presented randomly at a rate of one every 1.5 s.

Although toadfishes possess no hearing specializations and thus are primarily sensitive to particle motion (Fay and Edds-Walton 1997), we report in this study hearing thresholds based on pressure measurements for both technical reasons and comparison purposes with previous studies using batrachoidid fish (e.g. Sisneros 2007, 2009; Alderks and Sisneros 2011). Our aim was to compare the saccular sensitivity of *H. didactylus* between different size groups under identical experimental conditions. The data presented in sound pressure levels (SPL) to describe hearing sensitivities should not be considered as absolute values but instead should be considered to perform quantifiable comparisons of relative differences between the different groups.

Saccular potentials were recorded with glass electrodes filled with 3 M KCl (0.5-6 MΩ). Electrodes were visually guided and placed in the middle region of the saccular macula in either the left or right saccule. All recordings were from the middle recording region of the saccule in order to obtain data that could be comparable between different specimens. Analog saccular potentials were preamplified (109, Getting 5A), input into a lock-in amplifier (109, SR830, Stanford Research Systems) and then stored on a computer running a custom data acquisition Matlab script. The lock-in amplifier yields a DC voltage output that is proportional to the component of the signal whose frequency is locked to the reference frequency. The reference frequency was set to the second harmonic of the stimulation frequency (i.e., twice the stimulation frequency) while the sensitivity of the lock-in amplifier was set to 50 mV with a time constant of 100 ms. We used the second harmonic of the stimulus frequency as the reference frequency because the greatest evoked potential from the saccule of teleost fishes typically occurs at twice the stimulus frequency due to the nonlinear response and opposite orientation of hair cell populations within the saccule (Cohen and Winn 1967). To estimate auditory thresholds, the saccular potentials were recorded in response to single tone stimuli that were reduced in 3 dB steps until the saccular response (mean voltage of eight evoked saccular potential measurements) was no longer above background noise (mean voltage measured without acoustic stimulation) ± 2 SD (standard deviation).

Background noise measurements were performed prior to recording each threshold tuning curve. Noise measurements were similar to that of the saccular potentials recordings with sound but in this the loud speaker was turned off so that no auditory stimulus was present. The background noise levels were consistently between 2-5 μV.

Sound recording setup and acoustic analysis

Both g1 and g2 juveniles were placed separately in two 50-60l saltwater observation tanks that were provided with several shelters (halved small flower pots), sand substrate, and maintained at 22 ± 2 º C. Two hydrophones (High Tech 94 SSQ, Gulfport, MS, USA; frequency range: 30 Hz–6 kHz, ±1 dB; voltage sensitivity: –165 dB re. 1 V/μPa) were placed in the middle of the tank (circa 20 cm apart) at about 5 cm from the bottom. These hydrophones were connected to an audiocapture device

Edirol UA-25 (Roland, 16 bit, 6 kHz acquisition rate per channel) and then to a laptop, to perform double-channel recordings controlled with Adobe Audition 2.0 (Adobe Systems Inc., 2005). Behavioural observations of the fish behaviour and sound recordings were performed during 60 min. In the middle of each session food was provided to stimulate social interactions. A total of 10 sessions were done for each size group, at most one per day.

Sound recordings (sampling frequency 6 kHz) were analyzed using Raven 1.2 for Windows (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, NY, USA). Toadfish vocalizations were identified based on Amorim et al. (2008), Vasconcelos and Ladich (2008) and Vasconcelos et al. (2010). Sounds were classified as follows: single grunt (SG); grunt train, when more than one grunt is emitted in series (GT); long grunt train (LGT); double-croak (DC); boatwhistle (BW). The calling rate, i.e. number of sounds emitted per min per fish, was determined for each sound type and recording session. As sometimes it was not obvious which fish was calling, the calling rate observed in a recording session was divided by the number of fish in the tank.

For comparison purposes, the following sound characteristics were determined from 10 single grunts chosen randomly (with good SNR) from g1 and g2: total duration of single grunts (ms), from the start of the first pulse to the end of the last pulse; number of pulses within a single grunt; pulse period (ms), as the average period between two up to six consecutive pulse peaks (depending on number of pulses within a grunt); and dominant frequency (Hz), as the frequency with the highest amplitude within the sound power spectrum (Blackman-Harris window, filter bandwidth 10 Hz).

Statistical analysis

Comparisons of sound characteristics between the two juvenile groups were performed with Mann-Whitney U-tests.

Saccular sensitivity threshold curves from different size groups were compared by a repeated-measures ANOVA, which analyzed responses (auditory thresholds) to several frequencies in each subject fish (within subject factor) of different size groups (between-subject factor). This analysis was followed by a LSD *post-hoc* test, in order to verify pairwise group-specific differences.

Parametric tests were used only when data was normally distributed and variances were homogeneous. The statistical analysis was performed with Statistica 7.1 for Windows (StatSoft, Inc., 2005).

RESULTS

Saccular sensitivity

All different size groups revealed best saccular sensitivity at the lowest tested frequencies 75-85 Hz and a gradually decrease in sensitivity towards 945 Hz (Figure 1). The mean auditory thresholds increased from 110 dB re. 1µPa at 85 Hz (g3) up to 151 dB at 785-945 Hz (g1-g3).

Comparisons between auditory threshold curves obtained from the three different size groups (at the frequency range 75–425·Hz) showed significant overall differences (repeated-measures ANOVA, $F_{2,51}$=5.80, P=0.005) and a significant interaction between size and frequency ($F_{34,867}$=2.11, P<0.001). LSD pot-hoc tests showed significant group-specific differences – see Figure 1, namely between g1 and g3 at all frequencies (with exception of 385 Hz), and between g1 and g2 at most frequencies within the frequency range 105-385 Hz. Fish from g2 group did not differ from g3 at any frequency.

Vocal differentiation and behavioural context of acoustic signalling

Behavioural observation showed that Lusitanian toadfish juveniles start early to defend territories. In both test groups (g1 and g2), most of the shelters available were occupied by territorial juveniles that were clearly defending the nest by visual displays and acoustic signalling. Visual displays included mouth opening and spreading of pectoral fins and opercula, but also more aggressive behaviours such as attacks (bites or bite attempts) towards the opponents. Nesting juveniles were typically positioned at the entrance of the shelters and dominant males, usually the biggest specimens, exhibited darker skin colouration. During feeding, animals became more aggressive and attacks accompanied by sound production (grunts and grunt trains) were often observed while competing for food.

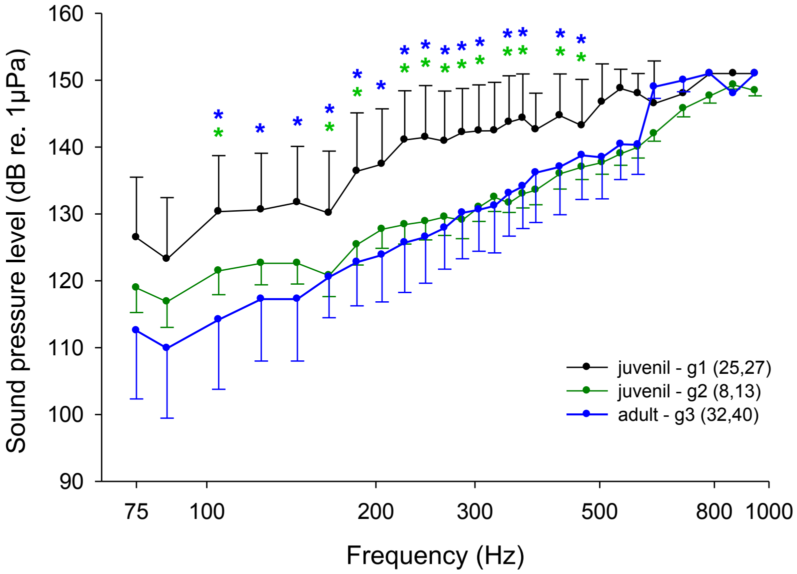

Figure 1 - Comparison between mean (± standard deviation) auditory threshold curves from g1 and g2 juveniles and adult Lusitanian toadfish. Number of animals and records per group are indicated in parentheses. Blue and greed asterisks indicate statistically significant differences (LSD post-hoc test) between g1 vs. g2 and g1 vs. g3, respectively. g1 and g3 did not differ at any frequency.

Both g1 and g2 fish produced single grunts (SG); grunt trains (GT) and long grunt trains (LGT) – see Figure 2, but differences in the calling rates were observed - Figure 3. While g1 specimens emitted mostly SGs (1.61 ± 0.06 calls hour^{-1} per fish) and occasionally GTs (0.28 ± 0.01 calls min^{-1}) and LGTs (0.08 ± 0.00 calls min^{-1}); g2 presented a larger vocal repertoire composed of up to five different calls: SG, GT, LGT, DC and BW. In these larger juveniles, LGTs were the most frequently signals (0.55 ± 0.31 calls min^{-1} per fish) in comparison to SGs (0.25 ± 0.42 calls min^{-1}), DCs (0.05 ± 0.08 calls min^{-1}) and BWs (0.02 ± 0.05 calls min^{-1}).

Grunts and grunt trains were typically registered during agonistic interactions, either during food or space competition in g1 and g2. Long grunt trains (LGTs) and double croaks (DCs) were generally produced when fish were inside the shelters possibly for nest signalling. Boatwhistles (BWs), only detected in the g2, were uttered during active nest defence.

A comparison of the acoustic parameters of single grunts of both test groups did not reveal significant differences in sound duration, dominant frequency and pulse period with increasing fish size (Mann-Whitney U tests: U=13-19, $n_{g1}=n_{g2}=10$, p>0.05). Single grunts presented sound duration of 80.27 ± 25.88 ms, pulse period of 8.0 ± 1.0 ms and dominant frequency of 386 ± 106 Hz.

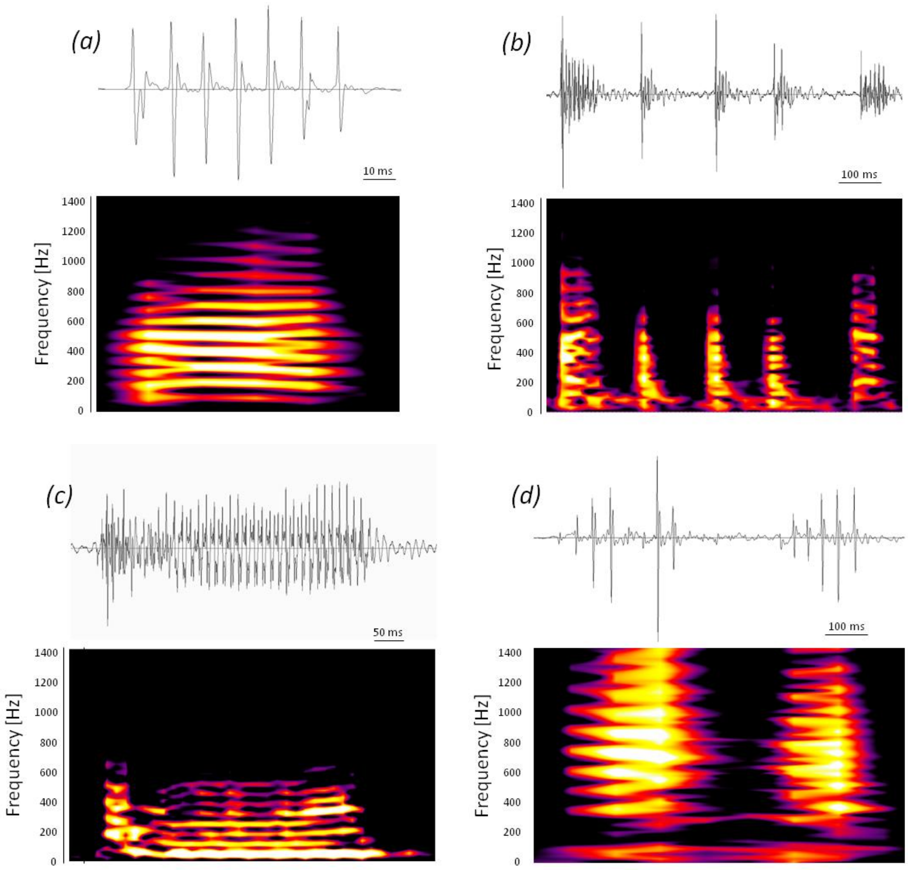

Figure 2 – Oscilograms (in relative amplitude) and respective spectrograms (below) of vocalizations produced by g2 toadfish juveniles (> 5 cm, standard length): (a) single grunt, (b) grunt train, (c) boatwhistle, and (d) double-croak. Sampling frequency 8 kHz, Hamming window, 30 Hz filter bandwidth.

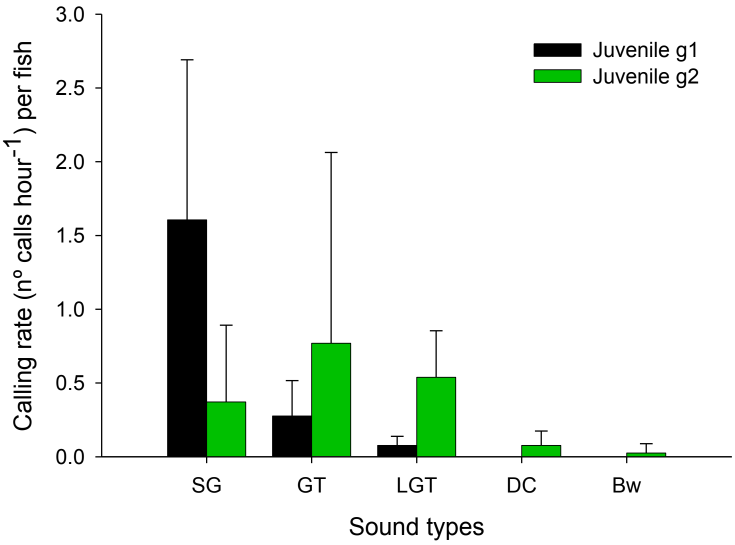

Figure 3 - Mean calling rate (± standard deviation) of the several calls by the two different size toadfish juvenile groups. SG, single grunt; GT, grunt train; LGT, long grunt train; DC, double croak; Bw, boatwhistle.

DISCUSSION

The aim of this study was to characterize the frequency response and auditory thresholds of saccular hair cells to behaviourally relevant stimuli throughout development in the Lusitanian toadfish. Moreover, the differentiation of the vocal repertoire with growth was also investigated in this highly vocal fish species. Our results indicate an enhancement of auditory sensitivity and concurrent enlargement of the vocal repertoire and call complexity with ontogenetic development.

Development of peripheral auditory sensitivity

Our data showed an increment in auditory saccular sensitivity with increasing fish size. The smallest juveniles presented a decrement in circa 10 dB in auditory sensitivity at most frequencies tested, comparing to larger juveniles and adults. Surprisely, larger juveniles exhibited similar auditory thresholds to adults. However, as these juveniles already produced the full adult vocal repertoire, we suggest that in this species the auditory sense parallels vocal development to enhance sound detection and acoustic communication.

Sensitivity changes in the peripheral auditory system probably resulted from age-related differences in the number of hair cells within the saccular macula and from differences in the size of the otolithic structure (Popper et al. 1988; Rogers et al. 1988). Vasconcelos and Ladich (2008), using the AEP recording technique, also reported lower hearing sensitivity in the smallest juveniles tested (with 2.8–3.8 cm SL, corresponding to g1), but only at 100 Hz and higher frequencies (800 and 1000 Hz). AEP recording technique records the overall synchronous neural electric activity induced by acoustic stimulation and includes responses from potentially more than one endorgan (i.e., saccule, lagena and utricle), VIII nerve and CNN auditory nuclei. Therefore, these summed neural responses become difficult to compare with the specific saccular responses analysed in this study. Saccular potential recordings reflect only the receptor potentials from a limited population of hair cells within the saccule closest to the recording electrode.

Our findings contrast to data reported in another batrachoidid, *Porichthys notatus*, which shows that the frequency response and auditory sensitivity is established early in development and does not change throughout ontogeny (Alderks and Sisneros 2011). Such different results found in these two batrachoidids can be explained by species-specific differences related to their life histories. While in *P. notatus* saccular sensitivity increases during the breeding season when they are mostly vocal (Sisneros 2009), in *H. didactylus* sensitivity is retained to allow acoustic communication throughout the year (Vasconcelos et al. 2011). On the other hand, *P. notatus* juveniles are clearly non-territorial and vocal activity in this species seems to start later in life probably associated with sexual maturity and reproduction behaviours (personal observations). This contrasts to the highly vocal juveniles *H. didactylus* that start early to vocalize and defend territories (present study).

Although *H. didactylus* and *P. notatus* belong to the family Batrachoididae, they are classified in different subfamilies (Halophryninae and Porichthinae). The clearly distinct species-specific behaviours suggests that the relation between the vocal system and the auditory sense might have evolved in completely different ways in these two subfamilies, although more data is needed for other members of these subfamilies.

Future ontogenetic studies that investigate the eventual increment in the number of hair cells coupled with anatomical studies on the auditory periphery in the Lusitanian toadfish should be performed to provide valuable insight into the mechanisms that allow enhancement of saccular sensitivity throughout development.

Development of the vocal motor system

Both Lusitanian toadfish juvenile groups (in total ranging from 2 cm up to 9 cm SL) were extremely territorial and exhibited agonistic displays during nest defence, including extension of pectoral fins and opercula at the entrance of the shelters and attacks towards similar-sized conspecifics. Moreover, acoustic activity was detected in both groups. Vasconcelos and Ladich (2008) reported that in the smallest tested group (with 2.8–3.8 cm SL, corresponding to g1), most of the specimens did not exhibit vocal activity, and suggested that either in this early stage the sound-producing apparatus was not sufficiently developed or it could be too risky to make sounds as it would increase vulnerability for example to potential predators. Our data showed that this size group is capable of producing sounds during conspecific agonistic interactions, although the vocal repertoire is not fully developed.

While the smallest fish (g1: 2.4-4.9 cm SL) emitted mostly single grunts and less frequently other variations of grunt calls (grunt train and long grunt train), larger juveniles (g2: 5.0-8.7 cm SL) exhibited the full vocal repertoire of adults with at least five different vocalizations, including double-croaks and boatwhistles. These changes are perhaps associated with the development the sonic neuromuscular system (Fine et al. 1989; Fine et al. 1984), as at least the peripheral sound producing apparatus (swimbladder and intrinsic sonic muscles) is known to increase in size throughout life in *H. didactylus* (Modesto and Canário 2003).

One of the most surprising results was the fact that juveniles with 5-9 cm SL (g2) were capable of producing long harmonic vocalizations such as the boatwhistles. This clearly indicates that the vocal motor system was probably completely developed to allow production of more complex vocal signals important for acoustic communication at this stage of development. Boatwhistles are described as a mating call that is used by nesting males of different batrachoidid species to attract females for spawning (Bass and Mckibben 2003). Vasconcelos et al. (2010) reported that the boatwhistles produced by *H. didactylus* also occur during agonistic contexts such as active nest defence. Boatwhistle production by *H. didactylus* juveniles further supports the agonistic role of boatwhistles in this species.

We provide first evidence that the development of the auditory sense parallels differentiation of the vocal repertoire in fish. It would be useful to determine exactly the age of each specimen based on analysis of otoliths. Future studies using juveniles in earlier developmental stages (with yolk sacs attached) will provide valuable information regarding the onset of sound production in this remarkably vocal species. In particular, it would be very interesting to test to what extent fry can perceive parental calls and its eventual influence in the individual vocal development.

Note that this manuscript is in preparation. Future steps will include recording adult vocal repertoire in captivity using the same protocol for comparison purposes. Moreover, data on temporal resolution, based on auditory evoked potentials (AEP), is under analysis, and will certainly strength our findings in terms of the development of the auditory abilities throughout development in this species.

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REFERENCES

Alderks, P.W. and Sisneros, J.A. (2011). Ontogeny of auditory saccular sensitivity in the plainfin midshipman fish, *Porichthys notatus*. J. Comp. Physiol. *A* 197, 387-398.

Amorim, M.C.P. and Hawkins, A.D. (2005). Ontogeny of acoustic and feeding behaviour in the Grey Gurnard, *Eutrigla gurnardus*. Ethology 111, 255-269.

Amorim, M.C.P., Simões, J.M. and Fonseca, P.J. (2008). Acoustic communication in the Lusitanian toadfish, *Halobatrachus didactylus*: evidence for an unusual large vocal repertoire. J. Mar. Biol. Assoc. UK 88, 1069-1073.

Aronov, D., Andalman, A.S. and Fee, M.S.A. (2008). Specialized Forebrain Circuit for Vocal Babbling in the Juvenile Songbird. Science 320, 630.

Bass, A.H. and McKibben, J.R. (2003). Neural mechanisms and behaviors for acoustic communication in teleost fish. Prog. Neurobiol. 69, 1-26.

Boatright-Horowitz, S.S. and Megela Simmons, A. (1995). Postmetamor-phic changes in auditory sensitivity of the bullfrog midbrain. J. Comp. Physiol. A 177, 577-590.

Brainard, M.S. and Doupe, A.J. (2002). What songbirds teach us about learning. Nature 417, 351-358.

Cohen, M.J. and Winn, H.E. (1967). Electrophysiological observation on hearing and sound production in the oyster toadfish *Porichthys notatus*. J. Exp. Zool. 165, 355-370.

Crawford, J.D. (1997). Hearing and acoustic communication in mormyrid electric fishes. Mar. Fresh. Beh. Phy. 29, 65-86.

Derégnaucourt, S., Saar, S. and Gahr, M. (2009). Dynamics of crowing development in the domestic Japanese quail (*Coturnix coturnix japonica*). Proc. R. Soc. B 276, 2153–2162.

Dimitrieva, L.P. and Gottlieb, G. (1994). Influence of auditory experience on the development of brain stem auditory-evoked potentials in mallard duck embryos and hatchlings. Behav. Neurol. Biol. 61, 19-28.

Fay, R.R. and Edds-Walton, P.L. (1997). Diversity in frequency properties of saccular afferents of the toadfish, *Opsanus tau*. Hear. Res. 113, 235-246.

Fine, M.L. (1989). Embryonic, larval and adult development of the sonic neuromuscular system in the oyster toadfish. Brain Behav. Evol. 34, 13-24.

Fine, M.L., Economos, D., Radtke, R. and Mcclung, J.R. (1984). Ontogeny and sexual dimorphism of the sonic motor nucleus in the oyster toadfish. J. Comp. Neurol. 225, 105-110.

Golubeva, T.B. and Tikhonov, A.V. (1985). The voice and hearing of birds in ontogeny, in: Acta XVIII Internat Ornithol Congr Nauka, Moscow, Russia, 259-274.

Higgs, D.M., Souza, M.J., Wilkins, H.R., Presson, J.C. and Popper, A.N. (2001). Age- and size related changes in the inner ear and hearing ability of the adult zebrafish (*Danio rerio*). JARO 3, 174-184.

Higgs, D.M., Rollo, A.K., Souza, M.J. and Popper, A.N. (2003). Development of form and function in peripheral auditory structures of the zebrafish (*Danio rerio*). J. Acoust. Soc. Am. 113, 1145-1154.

Jürgens, U. (2002). Neural pathways underlying vocal control. Neurosci. Biobehav. Rev. 235-258.

Kenyon, T.N. (1996). Ontogenetic changes in the auditory sensitivity of damselfish (Pomacentridae). J. Comp. Physiol. A 179, 553-561.

Ladich, F., Bischof, C., Schleinzer, G. and Fuchs, A. (1992). Intra- and interspecific differences in agonistic vocalization in croaking gouramis (Genus: *Trichopsis*, Anabantoidei, Teleostei). Bioacoustics 4, 131-141.

Lechner, W., Wysocki, L.E. and Ladich, F. (2010). Ontogenetic development of auditory sensitivity and sound production in the squeaker catfish *Synodontis schoutedeni*. BMC Biology 8, 10.

Modesto, T. and Canário, A.V.M. (2003). Hormonal control of swimbladder sonic muscle dimorphism in the Lusitanian toadfish Halobatrachus didactylus. J. Exp. Biol. 206, 3467-3477.

Moss, C.F., Redish, D., Gounden, C. and Kunz, T.H. (1997). Ontogeny of vocal signals in the little brown bat, *Myotis lucifugus*. Anim. Behav. 54, 131-141.

Myrberg, A.A., Ha, S.J. and Shamblott, M.J. (1993). The sounds of the bicolor damselfish (*Pomacentrus partitus*): predictors of body size and a spectral basis for individual recognition and assessment. J. Acous. Soc. Am. 94, 3067-3070.

Popper, A. (1971). The effects of size on auditory capacities of the goldfish. J. Aud. Res. 11, 239-247.

Popper, A.N., Roger, P.H., Saidel, W.M. and Cox, M. (1988). The role of the fish ear in sound processing, in: Atema, J., Fay, R.R., Popper, A.N., Tavolga, W.N. (Eds.) Sensory biology of aquatic animals. Springer, New York, U.S.A. pp. 687-710.

Reimer, K. (1996). Ontogeny of hearing in the marsupial, *Monodelphis domestica*, as revealed by brainstem auditory evoked potentials. Hear. Res. 92, 143-150.

Rogers, P.H., Popper, A.N., Hasting, M.C. and Saidel, W.M. (1988). Processing of acoustic signals in the auditory system of bony fish. J. Acoust. Soc. Am. 83, 338-349.

Rübsamen, R. (1992). Posnatal development of central auditory frequency maps. J. Comp. Physiol. A 170, 129-143.

Sisneros, J.A. (2007). Saccular potentials of the vocal plainfin midshipman fish, *Porichthys notatus*. J. Comp. Physiol. A 193, 413-424.

Sisneros, J.A. (2009). Seasonal plasticity of auditory saccular sensitivity in the vocal plainfin midshipman fish, *Porichthys notatus*. J. Neurophysiol. 102, 1121-1131.

Sisneros, J.A. and Bass, A.H. (2003). Seasonal plasticity of peripheral auditory frequency sensitivity. J. Neurosci. 23, 1049-1058.

Sisneros, J.A. and Bass, A.H. (2005). Ontogenetic changes in the response properties of individual, primary auditory afferents in the vocal plainfin midshipman fish *Porichthys notatus* Girard. J. Exp. Biol. 208, 3121-3131.

Vasconcelos, R.O. and Ladich, F. (2008). Development of vocalization, auditory sensitivity and acoustic communication in the Lusitanian toadfish *Halobatrachus didactylus*. J. Exp. Biol. 211, 502-509.

Vasconcelos, R.O., Simões, J.M., Almada, V.C., Fonseca, P.J. and Amorim, M.C.P. (2010). Vocal behaviour during territorial intrusions in the Lusitanian toadfish: boatwhistles also function as territorial ‘keep-out’ signals. Ethology 116, 155-165.

Vasconcelos, R.O., Sisneros J.A., Amorim, M.C.P. and Fonseca, P.J. (2011). Auditory saccular sensitivity of the vocal Lusitanian toadfish: low frequency tuning allows acoustic communication throughout the year. J. Comp. Physiol. A. (online first)

Werner, Y.L., Montgomery, L.G., Safford, S.D., Igic, P., Saunders, J.C. (1998). How body size affects middle-ear structure and function and auditory sensitivity in gekkonoid lizards. J. Exp. Biol. 201, 487-502.

Wysocki, L.E. and Ladich, F. (2001). The ontogenetic development of auditory sensitivity, vocalization and acoustic communication in the labyrinth fish *Trichopsis vittata*. J. Comp. Physiol. A 187, 177-187.

Zeddies, D.G. and Fay, R.R. (2005). Development of the acoustically evoked behavioral response in zebrafish to pure tones. J. Exp. Biol. 208, 1363-1372.

One of the major goals of this thesis was to investigate the functional role of acoustic signalling in a highly vocal teleost fish, the Lusitanian toadfish *H. didactylus*. Previous literature described that vocal activity is considerably conspicuous during the reproductive season in this species (Amorim et al. 2006) and that, in other members of the Batrachoididae family, the long and tonal calls emitted by males (i.e. boatwhistles or hums) seem to induce females´ phonotaxis (Fine 1972). Based on these evidences, it was hypothesized that sound production may play role on mate attraction in *H. didactylus*. Besides, behavioural evidences suggest the possible existence of individual acoustic differences that may provide information about singing males´ quality. However, this has never been investigated in detail in Batrachoididae and in fish in general.

The second major goal of this thesis was to investigate the auditory sensitivity and evaluate whether vocal complexity can be perceived by the toadfish auditory system. This species exhibits a rich acoustic repertoire rare among fishes, which suggests that the sensory system is probably adapted to encode different sound characteristics of communication signals. The representation of natural calls in the fish auditory system remains almost unknown.

In this discussion, I will highlight the most important findings of this thesis and discuss results as they relate to the existing literature on sensory-vocal systems in fish and in other vertebrates. Future perspectives are also given.

Vocal signatures and function of acoustic signalling

The existence of individual vocal signatures that can provide scope for mate choice has been scarcely investigated in fish. Acoustic recognition has only been demonstrated in a coral reef species that breeds in dense colonies, the bicolour damselfish *Stegastes partitus*. Myrberg and Riggio (1985) tested the ‘dear enemy effect’ in this species and verified that males can recognize territorial neighbours based on acoustic cues, likely the dominant frequency of their courtship chirp sounds.

The work presented in Chapter I (i) showed that, in the Lusitanian toadfish, male advertising acoustic signals are more variable between individuals than within individuals and that the dominant frequency and frequency modulation, followed by pulse period, amplitude modulation and total duration, were the parameters that best discriminated individuals. Individual differences in acoustic signals have been mentioned for other batrachoidids (Fine and Thorson, 2008) and mormyrids (Crawford et al. 1997) but a detailed characterization of individual differences in acoustic signals produced by fish is provided here for the first time. These results support the potential existence of individual recognition based on acoustic cues in this species. Lusitanian toadfish males establish long-term territories forming dense breeding aggregations and often inhabit turbid environments where vision is considerably impaired. Consequently, being able to discriminate different individuals would be beneficial both for male-male assessment and mate choice. A comparable social system where individual recognition has been demonstrated is found in anurans that also form breeding choruses and learn about neighbours' calls and position by repeatedly hearing the call from a particular location (Bee and Gerhardt 2001).

The ability to discriminate individuals based on acoustic cues is useful especially if the vocal traits can inform about the senders' quality. Hence, the aim of Chapter I (ii) was to test whether the sonic muscle mass, which relates to the vocal output, reflects morphological features of the vocal fish. Sonic muscle variability was best explained by the body length and condition, suggesting that vocal performance can inform about the sender's quality, which is important for social interactions, including the evaluation of potential mates. Consistently in other taxa, acoustic cues advertise body size and condition (e.g. Mager et al. 2007).

In order to provide clear evidence that certain acoustic features could be more attractive and induce phonotaxis in females *H. didactylus*, playback experiments were performed in tanks similar to those used for the intrusion experiments in Chapter I (iv) – this preliminary data is not presented in the thesis due to the uncertain data obtained. In contrary to McKibbern and Bass (1998) that tested phonotaxis in the batrachoidid *P. notatus*, in our study species *H. didactylus* (both females and type I males) did not reveal a clear attraction or repulsive response towards the sound source, i.e. the underwater speaker that played back boatwhistles, agonistic sounds or control stimulus (white noise). These observations suggest that this species may require more space conditions to carry out such experiments, as adult specimens can reach up to 50 cm total length. Also, it might be necessary to use an underwater speaker with enhanced performance at lower frequencies, where most of the sound energy of toadfish natural call is concentrated. We adopted the same speaker used by McKibbern and Bass (1998), but *H. didactylus* calls have lower dominant frequencies (down to 40 Hz), in contrast to *P. notatus* (circa 100 Hz), and such low frequency sounds are more difficult to generate with the commercially available systems. In this vein, future studies should involve playback experiments using an appropriate sound-generating device. The laboratory of Prof. Paulo Fonseca (FCUL) is currently working on a new underwater speaker capable of generating low frequency signals with a very accurate performance (good response to fast transients) independent of the water depth. Such playback experiments could be performed in tanks, but also in the field (toadfish breeding area), by placing the speaker inside a fish trap resembling a shelter.

To further investigate whether acoustic signalling has indeed a role in mate choice and affects reproductive success in the Lusitanian toadfish, several males were confined in artificial nests in the peak of the breeding season and their vocal behaviour was monitored for up to 2 weeks - Chapter I (iii). The emitted boatwhistles were registered and quantified for each male, as well as, the number of eggs present in the nests (reproductive success). Maximum calling rate and calling effort (percentage of time spent calling) were the best predictors of the number of eggs and these vocal parameters were in turn related with the male length and condition. The relation between acoustic signalling and reproductive success is important to understand the evolution of vocal communication systems and has been well studied in various vertebrates (e.g. White et al. 2010), but never clearly shown in fish. These data represent the first clear evidence that the vocal behaviour can affect reproductive success in a teleost fish and showed that acoustic signaling at higher rates and in a regular fashion can operate in mate choice.

Future work should include testing mute fish (with impairment of the swimbladder) in the same experimental conditions (enclosed nests), in order to verify the impact of the total absence of vocal activity in the reproductive success. Also, as the recorded specimens used in this study presented elevated cortisol and low androgens levels (at least when blood was collected in low tide) probably due to effects of captivity, further hormonal analysis should be performed in free-swimming fish captured from the respective nest. The number of eggs should be quantified and related to the androgen profiles in non-manipulated nesting males, in order to investigate the specific role of sex steroids in the reproductive success in this species.

Although the aforementioned results point to an undoubtedly role of the boatwhistle in reproduction, previous observations indicated that the emission of this acoustic signals is not restricted to the breeding period, which suggests a function in other behavioral contexts such as agonistic territorial interactions. Therefore, in Chapter I (iv), the social context of toadfish males was manipulated to investigate whether boatwhistles could be produced during territorial defence, by introducing ‘intruders’ in an experimental tank containing nesting ‘resident’ males. Interestingly, resident males defended their shelters producing mostly agonistic boatwhistles towards intruders, and fewer other sound types previously described as agonistic. The boatwhistles registered were similar in duration and frequency harmonic structure 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. This work provided first evidence that toadfish boatwhistles also function as active ‘keep-out’ signals during territorial defence. In fact, recent observation with Lusitanian toadfish juveniles (> 5 cm standard length) in observation tanks indicated that these animals emit boatwhistles during territorial defence – see Chapter III (ii), which further corroborates the agonistic function of this signal. The use of boatwhistles in an agonistic context also suggests that males can assess the opponents’ size and condition based on these acoustic signals. Example of such male dual-function signals that are used both as ornaments and armaments have been broadly described in various taxa such as mammals, birds, anurans and arthropods, and may include visual as well as acoustic signals (reviewed in Chapter I (iv)).

Hearing sensitivity and encoding of natural signals

Comparative studies that examine mechanisms of vocal production and auditory reception across closely-related species can provide valuable insights into the diversity and evolution of communication systems. A novel form of auditory plasticity that enhances the coupling between sender and receiver has been reported in the batrachoidid *P. notatus* and revealed that females become more sensitive to the dominant frequency components of male advertising calls during the breeding season

(Sisneros et al. 2004; Sisneros 2009). However, it is not known whether other seasonally-reproductive teleost species that rely heavily on acoustic signalling during social life show similar auditory plasticity to optimize vocal communication.

In Chapter II (i), peripheral auditory sensitivity was measured through evoked potentials from populations of saccular hair cells from non-reproductive and reproductive males and females. Saccular hair cells were most sensitive at 15-205 Hz and, therefore, were well suited to detect conspecific vocalizations and low frequencies that overlapped with lateral line sensitivity. Both sexes showed identical hearing sensitivity and no differences were found across seasons. These results contrasted with previous literature on seasonal plastic hearing in *P. notatus* (Sisneros et al. 2004; Sisneros 2009), possibly because *H. didactylus* uses acoustic communication throughout the year and significant sensory differences may exist between the two batrachoidid subfamilies (Porichthinae and Halophryninae). Also, the lack of differences in hearing abilities between sexes corroborates the findings presented in this thesis showing that detection of acoustic signals is equally relevant for both males (assess quality/fighting ability of opponents) and females (mate choice).

Most studies on fish audition have used artificial stimuli to test hearing abilities and, consequently, the representation of complex conspecific sounds in fish auditory system remains almost uninvestigated. Only two studies have used natural sounds to test sound detection/encoding in fish species that produce mostly simple pulsed sounds (Wysocki and Ladich 2003; Maruska and Tricas 2009). Using a technique to record the overall auditory neural activity (AEP, auditory evoked potentials) in both toadfish males and females (Chapter II (ii)) revealed a fine representation of duration and pulsed structure of conspecific calls and also encoding of the spectral content (harmonic structure) and amplitude modulation of the boatwhistles. Additionally, Lusitanian toadfish were able to detect ecologically-relevant stimuli from the natural acoustic environment, such as foraging sounds produced by a potential predator (bottle-nose dolphin) and advertising calls from a sympatric highly vocal fish (meagre). This work provides strong evidence that the auditory system of a vocal fish, lacking accessory hearing structures, is capable of resolving fine features of complex natural vocalizations. It further supports the suggestion that temporal, amplitude modulation and spectral information present in acoustic signals, such as boatwhistles, is used both in male-male interactions and in mate choice by females.

The hearing sensitivity measurements shown in Chapter II (i) and Chapter II (ii), as well as in Chapter III (ii), were performed in sound pressure. These works presented data in a comparative perspective between test groups (different sex, season and size), which perfectly justifies such approach. However, the sensitivity to particle motion, using a shaker table system (Edds-Walton and Fay 2009), should be measured in order to address other interesting topics such as directional hearing and the contribution of the auditory system and the lateral line for sound detection.

Ontogeny of acoustic communication

Studying the ontogeny of acoustic communication, namely the combined development of sound production and hearing sensitivity, is important to understand the vocal communication systems and remains poorly investigated in fish. In fact, the ontogenetic development of acoustic communication has only been previously investigated in one species, the croaking gourami *Trichopsis vitttata* (Wysocki and Ladich 2001). Personal observations indicated that the Lusitanian toadfish juveniles, contrary to other batrachoidids (e.g. *P. notatus*), are very territorial and start to emit sounds in early developmental stages, within just a few months age. In Chapter III (i) the relationship between the development of hearing and sound production was studied. Agonistic vocalizations were recorded in several size groups from 3 to 32 cm standard length. Using the same technique as in Chapter II (ii) (auditory evoked potentials) hearing sensitivity was also measured in the same size classes. Results showed that several vocal parameters such as dominant frequency, sound duration and number of pulses decreased, whereas pulse period and sound level increased, with increasing fish size. The best hearing was below 300 Hz, independently of the size, but the smallest juveniles showed lower sensitivity at both lowest and highest tested frequencies (100 Hz and 800-1000Hz). Comparisons between audiograms and sound spectra within the same-sized fish revealed that smaller juveniles would be barely able to detect their agonistic sounds. This study demonstrated that acoustic communication in the Lusitanian toadfish seems to start when juveniles (above 5 cm standard length) are able to generate grunts of higher sound level and lower dominant frequency.

Finally, in order to verify at which level within the auditory system (more peripheral or more central) the hearing sensitivity undergoes improvements throughout development, auditory potentials were measured from populations of saccular hair cells in the smallest juveniles groups tested in Chapter III (i) and compared to adults. Sound recording were also performed in juveniles, in order to compare the vocal repertoire between different size classes. Results point to a parallel development of both peripheral auditory sensitivity and vocal differentiation. Juveniles with more than 5 cm length, contrary to the smallest fish, exhibited already the full vocal repertoire as well as similar auditory sensitivity to adults and, consequently, are already able to communicate in an sophisticated fashion.

This last manuscript is still in preparation. Behavioural observations and sound recordings will be made using adult toadfish in large tanks, in order to compare the calling rate of different vocalizations with the juvenile groups. Specifically, acoustic parameters of agonistic grunts will be measured and compared with the juvenile grunts. Moreover, data on temporal resolution, based on auditory evoked potentials, is being recorded in both juveniles (the same size groups) and adult fish for comparison purposes. The temporal resolution abilities of *H. didactylus* will be described, using both a gap-detection paradigm and the analysis of auditory responses to playbacks of grunts with manipulated temporal structure. Preliminary results suggest that the ability to discriminate sound temporal features show improvements throughout development and that this species may detect silence gaps as short as 2 ms in continuous noise.

The communication system of the Lusitanian toadfish is more complex that initially thought, being comparable to some extent to the complexity of vocal systems shown in other vertebrates such as anurans and birds.

The demonstration of individual vocal signatures in this species, the fundamental role of acoustic signalling for reproductive success and during territorial defence, together with the ability to encode fine features of complex conspecific calls and the parallelism between the development of the auditory sense and vocal differentiation, constitute important findings that provide novel insights into the diversity and evolution of vocal communication systems in vertebrates. The present thesis also raised a number of questions that should be address in the future. A few have already been pinpointed throughout this section but others include:

Are there complex acoustic interactions within a toadfish chorus?

What is the minimum sound amplitude difference allowing the detection of a boatwhistle over a toadfish chorus?

What is the role of each otolithic endorgans for sound detection? Which endorgan is responsible for auditory feedback?

What is the contribution of the auditory system versus lateral line in intraspecific communication?

Which vocal parameters are encoded by which nuclei in the central auditory pathways?

To which extent is the toadfish hearing and communication impaired by anthropogenic noise typically detected in estuarine habitats?

References

Amorim, M.C.P., Vasconcelos, R.O., Marques, J.F. and Almada, F. (2006). Seasonal variation of sound production in the Lusitanian toadfish, *Halobatrachus didactylus*. J. Fish Biol. 69, 1892-1899.

Bee, M.A. and Gerhardt, H.C. (2001). Neighbour–stranger discrimination by territorialmale bullfrogs (*Rana catesbeiana*): II. Perceptual basis. Anim. Behav. 62, 1141-1150.

Crawford, J.D., Cook, A.P. and Heberlein, A.S. (1997). Bioacoustic behavior of African fishes (Mormyridae): potential cues for species and individual recognition in Pollimyrus. J. Acoust. Soc. Am. 102, 1200-1212.

Edds-Walton, P.L. and Fay, R.R. (2009). Physiological evidence for binaural directional computations in the brainstem of the oyster toadfish, *Opsanus tau* (L.). J. Exp. Biol. 212, 1483-1493.

Fine, M.L. and Thorson, R.F. (2008). Effects of passive acoustics for assessing behavioural interactions in individual toadfish. Trans. Am. Fish. Soc. 137, 627-637.

Fish, J.F. (1972). The effect of sound playback on the toadfish, in: Behavior of Marine Animals. Winn, H.E. and Olla, B. (eds.) Vol. 2, Plenum Press, New York, pp. 386-434.

Mager, J.N., Walcott, C., and Piper, W.H. (2007). Male common loons, *Gavia immer*, communicate body mass and condition through dominant frequencies of territorial yodels. Anim. Behav. 73, 683-690.

Maruska, K.P. and Tricas, T.C. (2009). Encoding properties of auditory neurons in the brain of a soniferous damselfish: reponse to simple tones and complex conspecific signals. J. Comp. Physiol. A 195, 1071-1088.

McKibben, J.R. and Bass, A.H. (1998). Behavioral assessment of acoustic parameters relevant to signal recognition and preference in a vocal fish. J. Acoust. Soc. Am. 104, 3520-3533.

Myrberg, A.A. and Riggio, R.J. (1985). Acoustically mediated individual recognition by a coral reef fish (*Pomacentrus partitus*). Anim. Behav. 33, 411-416.

Sisneros, J.A., Forlano P.M., Deitcher D.L. and Bass, A.H. **(**2004). Steroid-dependent auditory plasticity leads to adaptive coupling of sender and receiver. Science 305, 404-407.

Sisneros, J.A. (2009). Seasonal Plasticity of Auditory Saccular Sensitivity in the Vocal Plainfin Midshipman Fish, *Porichthys notatus.* J. Neurophysiol. 102, 1121-1131.

White, D.J., King, A.P., West, M.J., Gros-Louis, J., Tuttled, E.M. (2010). Effects of singing on copulation success and egg production in brown-headed cowbirds, *Molothrus ater*. Behav. Ecol. 21:211-218.

Wysocki, L.E. and Ladich, F. (2001). The ontogenetic development of auditory sensitivity, vocalization and acoustic communication in the labyrinth fish *Trichopsis vittata*. J. Comp. Physiol. A 187, 177-187.

Wysocki, L.E. and Ladich, F. (2003). The representation of conspecific sounds in the auditory brainstem of teleost fishes. J. Exp. Biol. 206, 2229-2240.