Novel vocal repertoire and paired swimbladders of the three-spined toadfish, *Batrachomoeus trispinosus*: insights into the diversity of the Batrachoididae

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SUMMARY

Toadfishes (Teleostei: Batrachoididae) are one of the best-studied groups for understanding vocal communication in fishes. However, sounds have only been recorded from a low proportion of taxa within the family. Here, we used quantitative bioacoustic, morphological and phylogenetic methods to characterize vocal behavior and mechanisms in the three-spined toadfish, *Batrachomoeus trispinosus*. *B. trispinosus* produced two types of sound: long-duration 'hoots' and short-duration 'grunts' that were multiharmonic, amplitude and frequency modulated, with a dominant frequency below 1 kHz. Grunts and hoots formed four major classes of calls. Hoots were typically produced in succession as trains, while grunts occurred either singly or as grunt trains. Aside from hoot trains, grunts and grunt trains, a fourth class of calls consisted of single grunts with acoustic beats, apparently not previously reported for individuals from any teleost taxon. Beats typically had a predominant frequency around 2 kHz with a beat frequency around 300 Hz. Vocalizations also exhibited diel and lunar periodicities. Spectrographic cross-correlation and principal coordinates analysis of hoots from five other toadfish species revealed that *B. trispinosus* hoots were distinct. Unlike any other reported fish, *B. trispinosus* had a bilaterally divided swimbladder, forming two separate swimbladders. Phylogenetic analysis suggested *B. trispinosus* was a relatively basal batrachoidid, and the swimbladder and acoustic beats were independently derived. The swimbladder in *B. trispinosus* demonstrates that toadfishes have undergone a diversification of peripheral sonic mechanisms, which may be responsible for the concomitant innovations in vocal communication, namely the individual production of acoustic beats as reported in some tetrapods.

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Key words: acoustic communication, courtship, functional morphology, ontogeny, phylogeny, sound production, swimbladder, vocalization.

INTRODUCTION

Of the many species of fishes that are known to acoustically communicate, perhaps the group most intensively studied and best understood is the Batrachoididae, which are generally referred to as toadfishes, but also include several genera referred to more specifically as toadfish and midshipman (see Nelson, 2006). Toadfishes are best known for their distinctive multiharmonic, male advertisement vocalizations used in courtship to attract females to male nest sites (reviewed by Bass and McKibben, 2003). Within the family, there is considerable variation in the temporal properties of courtship vocalizations. For example, the 'boatwhistle' of the toadfish *Opsanus beta* typically includes an initial broad-band, brief (~50 ms) 'grunt' that continues as a longer duration (~200–300 ms) multiharmonic 'hoot' (Tavolga, 1958; Tavolga, 1960). In contrast, the multiharmonic 'hum' of the plainfin midshipman (*Porichthys notatus*) is only hoot like with little to no amplitude modulation and can last from minutes to upwards of 1 h (Brantley and Bass, 1994; Hubbs, 1920; Ibara et al., 1983).

The batrachoidid vocal repertoire also includes sounds that function in aggression. Aggressive vocalizations are usually emitted during nest defense and are distinct from courtship calls in two specific temporal properties: duration and call rate. In agonistic interactions, both male and female toadfishes produce 'grunts': short duration, typically non-harmonic calls. In some cases, grunts can be rapidly repeated to form a 'grunt train' that may last for several seconds (e.g. see Amorim et al., 2008; Brantley and Bass, 1994; Gray and Winn, 1961). Unlike courtship sounds, agonistic grunts

are not seasonally dependent. Midshipman also produce an amplitude- and frequency-modulated call known as a 'growl' that has grunt and hoot-like portions like the boatwhistle of some toadfishes (see above), can last for several seconds, and probably functions in agonistic contexts (Bass et al., 1999).

The role of the swimbladder in toadfish sound production has been investigated for over a century (Tower, 1908), and it remains the best-studied system for peripheral sonic mechanisms in fishes (for a review, see Ladich and Fine, 2006). Vocalizations are produced by the rapid contraction of paired striated muscles attached to the walls of the swimbladder (Fine et al., 2001; Fine et al., 2002), with ultrastructural traits divergent from trunk skeletal muscles (e.g. Bass and Marchaterre, 1989; Fawcett and Revel, 1961), that are adapted to contraction frequencies which are among the fastest of vertebrate skeletal muscles (Rome, 2006; Rome et al., 1996; Skoglund, 1961).

While broad comparisons have been made across species between the different toadfish sounds and divergent neural and peripheral vocal mechanisms (Amorim et al., 2008; Bass and Marchaterre, 1989; Bass and Baker, 1991; Bass and McKibben, 2003; dos Santos et al., 2000; Mann et al., 2002; Tavolga, 1958; Tavolga, 1965), no robust quantitative methods have been used to rigorously investigate the species-level diversity of vocalizations in the family, as have been used in studying acoustics in other vertebrate taxa (e.g. Buck and Tyack, 1993; Clark et al., 1987; Mitani and Marler, 1989; Nowicki and Nelson, 1990; Rendell and Whitehead, 2003; Young et al., 1999). Additionally, the majority of this work has focused

only on three species within the Batrachoididae (*O. beta*, *O. tau* and *P. notatus*) (Amorim, 2006; Bass and McKibben, 2003), despite the moderate diversity of the family, which contains 25 genera and 78 species (Greenfield et al., 2008) (see also Nelson, 2006).

Little is known about the natural history, internal anatomy, or behavior of most toadfishes. An example of one such species is the three-spined toadfish, *Batrachomoeus trispinosus*. Outside of some taxonomic and systematic work (e.g. Hutchins, 1976; Miya et al., 2005), little is known about the behavioral biology of this taxon. *Batrachomoeus trispinosus* is a tropical euryhaline species, ranging from 0 to 36 m in depth in fresh and saltwater habitats throughout the tropical western Pacific (Greenfield, 1999). Based on specimens collected in trawls, individuals appear to occur in low densities within estuarine environments (Hajisamae et al., 2006; Tonks et al., 2008). Despite this lack of knowledge of *B. trispinosus* in the wild, they have become popular in the aquarium trade, appearing under a variety of (often misleading) names such as frogfish, lionfish or *Halophryne trispinosus* (Norman, 1976). Though disturbance/agonistic sounds have been anecdotally reported from a congeneric species, *B. dubius* (Graham, 1992; Grant, 1987), no quantitative or in-depth analyses have been conducted for sounds of the genus.

This investigation of *B. trispinosus* has two main goals. The first is to characterize the vocal repertoire and quantitatively compare it with that of other toadfishes. The second goal is to describe the unique morphology of the sonic swimbladder and its functional implications. By generating a molecular phylogeny of the toadfishes, we demonstrate that *B. trispinosus* has evolved both a novel vocal signal (acoustic beat) and a novel swimbladder (bilaterally divided). The diversity in vocal behaviors and supporting biomechanical mechanisms in this family broadens our understanding of the evolution of acoustic communication in toadfishes, and among closely related groups of fishes in general (Malavasi et al., 2008).

MATERIALS AND METHODS

Batrachomoeus trispinosus (Günther) were acquired through the aquarium trade and maintained in freshwater aquaria (150 and 380 l) at 26°C. The room was maintained on a 13 h:11 h light:dark cycle along with incandescent moonlight timer replicating the lunar cycle (Solar 1000 dimmer, Blueline Aquatics, Ambler, PA, USA). The overhead lights in the aquarium room were off from 20:00 h to 07:00 h. Fish were fed live minnows and small goldfish weekly. All procedures were approved by the Institutional Animal Care and Use Committee at Cornell University.

Recordings were made in aquaria using a hydrophone (Aquarian AQ-6, Aquarian Audio Products, Anacortes, WA, USA, frequency response 20 Hz to 100 kHz; or 171/1/5 hydrophone, High Tech, Gulfport, MS, USA, sensitivity –168.2 dB re: 1 V μPa^{-1}, frequency response 2 Hz to 30 kHz) suspended 20–30 cm above fish nests. Sounds were recorded either through the Audacity 1.2.5 software package (http://audacity.sourceforge.net/) on a MacBook Pro or on an Olympus LS-10 digital recorder (Olympus Imaging America, Center Valley, PA, USA), at a sampling rate of 44.1 kHz as 16-bit WAV files. The hydrophone passively recorded in aquaria for 12–15 h periods to detect mainly undisturbed conspecific sounds; as reported, a few sounds were recorded from individual fish while they were chased by an observer. Sounds were then analyzed using Raven 1.3 (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Cornell University, Ithaca, NY, USA).

The physical parameters of the aquaria were taken into account when positioning the hydrophones and analyzing the fish sounds, as fish sound recordings in captivity are known to be affected by the dimensions of aquaria (Akamatsu et al., 2002; Okumura et al., 2002; Parvulescu, 1967). The speed of sound in tanks was calculated to be 1547 cm s^{-1} following the equation of Medwin (Medwin, 1975). This calculated sound velocity was then used to determine the minimum resonant frequency (150 l tank: 2761 Hz, 380 l tank: 2482 Hz) and attenuation distance (20–30 cm) of the sounds in aquaria (Akamatsu et al., 2002; Okumura et al., 2002). Attenuation distance is the length over which the sound pressure decreases by 20 dB (Akamatsu et al., 2002; Okumura et al., 2002). Because of the small aquarium size, the amplitude of upper frequency components of the sounds near the resonant frequency of the tank may be distorted (Akamatsu et al., 2002). Additionally, the size constraints of the aquarium caused all sounds to be recorded in the near field and, combined with the fact that the distance between the vocalizing fish and the hydrophone could not be exactly quantified, the absolute sound pressure level was not measured.

Sound analysis

Seven variables were measured for the two basic types of sound, hoots and grunts: total sound duration, interval between successive sounds, fundamental frequency (the lowest frequency component in a harmonic sound), dominant frequency (the highest amplitude frequency component in either a broad-band or harmonic sound), relative amplitude, number of pulses and inter-pulse interval for each sound (see Bradbury and Vehrencamp, 1998; Fine et al., 1977; Winn, 1964). The temporal properties of sounds were determined from oscillograms, while the frequency properties were determined from both spectrograms and power spectra (Hann filter, 3 dB filter bandwidth 9 Hz, FFT 3524 samples, 50% overlap). Only sounds that had a clear structure and minimal background noise were included in quantitative analyses. Sounds were classified as a particular call type based on their relative duration, pattern of amplitude modulation and frequency content.

Quantitative comparisons of toadfish sounds

Spectrographic cross-correlation (Clark et al., 1987; Nowicki and Nelson, 1990) combined with principal coordinates analysis (SPCC–PCo) (Cortopassi and Bradbury, 2000) was used to quantitatively compare the multiharmonic, boatwhistle-like calls of different species of toadfish. While the boatwhistles of some toadfish include an initial grunt-like segment (Remage-Healey and Bass, 2005; Tavolga, 1958; Thorson and Fine, 2002a), only the otherwise subsequent multiharmonic hoot portion of the call was analyzed here as the putative boatwhistles of *Batrachomoeus*, like those of some other batrachoidids (e.g. midshipman), lack an initial grunt. Fifty-nine representative hoots from *B. trispinosus* were quantitatively compared with hoot-like sounds from five other toadfish species: *Halobatrachus didactylus* (*N*=9), *O. beta* (*N*=29), *O. phobetron* (*N*=5), *O. tau* (*N*=30) and *P. notatus* (*N*=18). Additionally, because the growl of *P. notatus* acoustically resembles the *Opsanus* boatwhistle in having both broad-band and multiharmonic portions (Bass et al., 1999), examples of these growls (*N*=18) were also included in the analysis. *Halobatrachus didactylus* sounds were recorded by M. C. P. Amorim (July 2001 and 2002, Tagus Estuary, Portugal), *O. beta* sounds were recorded by L. Remage-Healey (June 2002 at the Florida State University Coastal and Marine Laboratory, St Teresa, FL, USA), *O. tau* sounds were recorded by J. R. McKibben (July 1994, Point Pleasant, NJ, USA), and *P. notatus* sounds were recorded by M. A. Marchaterre (July 1998, Bodega Marine Laboratory, Bodega Bay, CA, USA). *Opsanus phobetron* sounds were obtained from the Macaulay Library of Animal Sounds at the Cornell Laboratory of Ornithology (ML

catalog number 112910, recorded by M. P. Fish and W. H. Mowbray, Bimini, Bahamas). All sounds were down-sampled to 22.05 kHz. Because the *P. notatus* hum is substantially longer in duration than any of the other vocalizations in the analysis, 10 long-duration hums (greater than 20 min) were sub-sampled to create 10 s hums to avoid biasing the correlation due to the extreme disparity in sound duration.

Sounds were cross-correlated in Raven 1.3 using the batch correlator function. Sounds were normalized and bandpass filtered between 0 and 5000 Hz to reduce the effects of any incidental background noise (Cortopassi and Bradbury, 2000). Both spectrograms (Hann Window, 3 dB bandwidth 49.6 Hz, FFT 1280 samples) and waveforms were cross-correlated. A total of 166 sounds were included in the SPCC analysis resulting in 27,556 sound comparisons. The resulting output is a similarity matrix, consisting of the similarity score between all possible pair-wise comparisons of sounds. This matrix was converted to a distance matrix (distance=1–similarity) and analyzed with a PCo analysis using the PCoord script in the R Package (Casgrain and Legendre, 2004) following the method used by Cortopassi and Bradbury (Cortopassi and Bradbury, 2000).

The relative temporal position of the maximum amplitude of each batrachoidid sound was calculated as a function of its occurrence within the sound (time of maximum amplitude/duration=relative temporal position of maximum amplitude), and differences among species were analyzed with an ANOVA.

Morphological measurements

Specimens preserved in 70% ethanol (N=28) were dissected to examine the swimbladder morphology. Standard length, body mass and sex were recorded. Two specimens were cleared and stained [following the protocol of Song and Parenti (Song and Parenti, 1995)] and the swimbladder was left in place to allow its morphology to be visualized in relation to the rest of the body. The length and width of swimbladders were measured *in situ* with calipers and photographed under a dissecting microscope. Sexual dimorphism of the swimbladder characteristics was tested with an ANCOVA using JMP 5.0.1.2 (SAS Institute, Cary, NC, USA) with standard length as the covariate.

Swimbladders were dissected out from the peritoneal cavity (N=11) and the muscles attached to the walls were dissected free, blotted twice with filter paper, and then weighed. Differences in mass between the left and right swimbladder muscles were tested with Student's paired *t*-test using the residuals of the swimbladder muscle mass from a regression against swimbladder length (to account for differences in swimbladder size). Differences in muscle mass between the sexes were tested using an ANOVA on the muscle residuals.

To visualize the light microscopic structure of the swimbladder and muscles, swimbladders were sectioned at 70 μm on a sledge microtome (Microm HM440E, Neuss, Germany). Sections were mounted on glass slides, stained with Methylene Blue, dehydrated and coverslipped, and then photographed under a microscope at ×4 and ×10 magnification.

Molecular systematics

To understand the evolutionary relationships of the included toadfish taxa, a molecular phylogeny was constructed based on four genes from available toadfish taxa with sequences previously deposited in GenBank: *16S*, *28S*, cytochrome oxidase subunit I (*COI*) and cytochrome b (*CytB*). A list of accession numbers of the sequences used in the phylogenetic analysis is given in supplementary material Table S1. *Gadus morhua* was used as an outgroup (see Nelson, 2006). As secondary structural information is critical for the determination of ribosomal DNA homology in multiple sequence alignment (Kjer, 1995), *16S* and *28S* genes were aligned using the alignment program Expresso (Armougom et al., 2006), which uses structural information to calculate alignments. *COI* and *CytB* sequences were aligned using the program MCoffee (Moretti et al., 2007), which outputs a consensus alignment using eight different alignment algorithms. The most appropriate nucleotide substitution model, TrN+G (rmat=1.0000 2.4742 1.0000 1.0000 4.5176, pinvar=0), was selected using the hierarchical likelihood test in ModelTest 3.7 (Posada and Crandall, 1998). Using the aligned sequences and the nucleotide substitution model, a maximum likelihood analysis was conducted in PAUP* 4.10b (Swofford, 1998) using a heuristic search with 10 random sequence additions [following Westneat and Alfaro (Westneat and Alfaro, 2005)]. To determine statistical support of each node, a bootstrap analysis was performed using tree bisection–reconnection branch swapping [following Westneat and Alfaro (Westneat and Alfaro, 2005)].

RESULTS

Over a period of 6 months in captivity (January to June 2008), a total of 1094 sounds produced by *B. trispinosus* were recorded. The sounds were initially classified mainly on the basis of the similarity of two temporal characters, duration and repetition rate, that apply to other toadfish sounds (see Introduction): short duration grunts and longer duration hoots produced either alone or in succession as grunt trains. Together with additional analyses of spectral (e.g. either broad-band or multiharmonic) and temporal (e.g. sound pulse number and inter-pulse intervals) characters, four major classes of calls were identified: hoot trains, single broad-band grunts, multiharmonic grunt trains, and single broad-band grunts with acoustic beats. Of the total calls recorded (N=198 calls, comprising 1094 individual sounds), 34.8% were hoot trains (N=69 trains, composed of 592 individual hoots), 48.0% were single grunts (N=95), 7.1% were grunt trains (N=14 trains, composed of 392 grunts within the train) and 10.1% were grunts with acoustic beats (N=20). There were only three occurrences of single hoots and hence these were not considered a separate class of calls.

Hoot trains

The hoots of *B. trispinosus* were the longest duration sounds, typically heard in succession (Fig. 1A). Individual hoot duration ranged from 0.285 to 6.077 s (1.15±0.04 s mean±s.e.m., N=295 hoots analyzed). The mean fundamental frequency of hoots was 151.35±0.39 Hz, while the mean dominant frequency (either the second or third harmonics) was 426.40±10.10 Hz. Hoots typically had around 10 pronounced harmonics, with the strongest power contained in the fundamental and the first three to four harmonics (Fig. 1B,C). Though not as strong as in frequencies below 1000 Hz, energy in the hoot extended into the 2000 Hz range (Fig. 1B,C). The fine temporal structure of the hoot shows a highly regular pattern, with the largest peaks in the wave corresponding to the fundamental frequency (~150 Hz), and the smaller peaks corresponding to upper harmonics (Fig. 1D). Many hoots exhibited frequency modulation that was present at all harmonics, but most prevalent through the tenth harmonics – a shift of around 10 Hz in lower harmonics, and as much as 20–40 Hz in upper harmonics (Fig. 1B). Hoots also exhibited amplitude modulation, typically showing a gradual increase in overall amplitude throughout the sound (Fig. 1A; also see single hoot on an expanded time scale as an inset in Fig. 1C).

As mentioned above, hoots mainly occurred as trains. The mean number of hoots in a train was 8±1, and the mean interval between hoots in the train was 1.95±0.08 s (N=32 trains). Over the course of trains, hoots in immediate succession within a sequence significantly decreased in duration (one-way ANOVA: d.f.=292; F=39.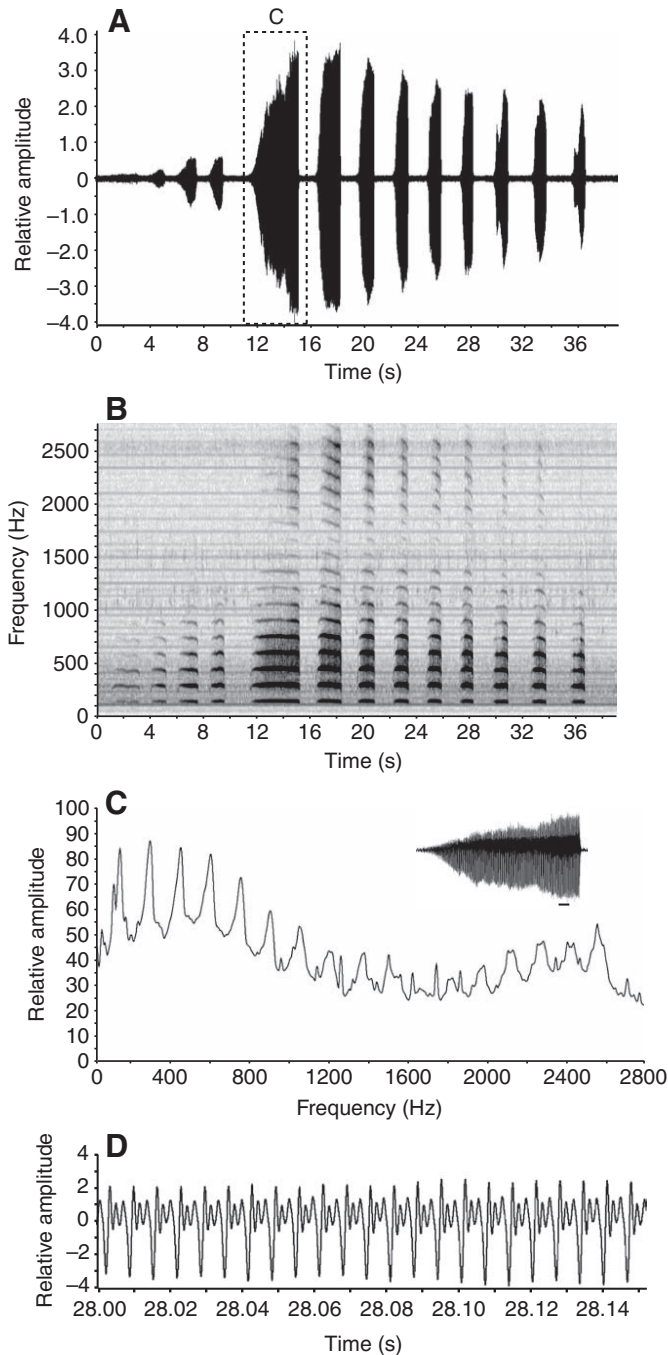8339, P<0.0001; Fig. 2A). However, there was no relationship between the duration of the inter-hoot interval as a function of the temporal position in the train.

Single grunts and grunt trains

Batrachomoeus trispinosus also produced short duration grunts, which were typically performed singly (N=65), but also occurred as groups of two (N=10), three (N=4), four (N=2) or five (N=1). We refer to the latter collectively as single grunts because they had an irregular periodicity when occurring in groups of two to five, unlike grunt trains (see below). Singly produced grunts were often coincident with gravel-like movement sounds suggestive of a rapid body movement and an agonistic role as observed in other toadfishes (e.g. Brantley and Bass, 1994; Gray and Winn, 1961). On average, single grunts were much shorter in duration than single hoots within a train, with a mean of 0.276±0.035 s (range: 0.029–0.775 s, N=37 grunts analyzed; Fig. 3A). Like the grunts of other toadfishes (e.g. Amorim et al., 2008; Bass et al., 1999; Thorson and Fine, 2002a), almost all *B. trispinosus* single grunts were broad-band (Fig. 3B,C), in this case with a mean dominant frequency of 968±172 Hz.

Fish also produced grunt trains (Fig. 4), with a mean number of 28±3 grunts in the train (range 8–48, N=330 grunts analyzed from 12 grunt trains). The mean fundamental frequency of grunts within the train was 182±1 Hz, with a dominant frequency of 1002±53 Hz. The mean interval between grunts was 0.353±0.014 s. Different from single grunts, grunts within trains had a clear and well-defined harmonic structure (Fig. 4B,D), though the harmonics were broader than those in hoots (Fig. 1B,C). The fine temporal structure did not appear to change during the course of a train (Fig. 4E).

The first grunt in a train was significantly longer in duration than subsequent ones (0.529±0.084 *versus* 0.135±0.004 s, d.f.=329, F=261.0041, P<0.0001; Fig. 2B). Grunts in immediate succession significantly decreased in duration over the course of a train (R^2=0.18, d.f.=329, F=69.5554, P<0.0001; Fig. 2B), with no relationship between the duration of the inter-grunt interval and the temporal position in the train.

The fundamental frequency was significantly different between the component sounds within hoot and grunt trains (one-way ANOVA: d.f.=520, F=1230.50, P<0.0001), with the fundamental frequency of hoots about 30 Hz lower than that of grunts (see above). The dominant frequency of hoots was also significantly lower than that of grunts either alone or in trains (one-way ANOVA: d.f.=571, F=63.06, P<0.0001, Tukey's HSD *post-hoc* test: q=2.35, α=0.05, P<0.05), but grunts and grunt trains were not different from each other (Tukey's HSD *post-hoc* test: q=2.35, α=0.05, P>0.05).

Acoustic beats

A small proportion of vocalizations exhibited acoustic beats (Fig. 5), to our knowledge previously undescribed in individual fishes. Although beats are known for the plainfin midshipman fish, they are formed by the temporal overlap in the advertisement hums of neighboring males (see Bass et al., 1999). The most common beat sounds for *B. trispinosus* were classified as single grunts (N=20) because of their overall duration (mean 0.147±0.026 s, range: 0.024–0.371 s) and lack of repetition as trains (Fig. 5A,E). Grunts with beats had a dominant frequency greater than 2000 Hz (2298±262 Hz) and a distinct beat frequency (303±75 Hz; Fig. 5C,G). However, some of these grunts had a clearer harmonic structure than others (compare Fig. 5B,C with 5F,G). Grunts with beats were recorded from fish in the different sized aquaria (see Materials and methods). The short duration of these calls, along with their stable temporal (Fig. 5D,H) and harmonic structure (e.g. Fig. 5B–G), support the conclusion that they were produced by a single individual
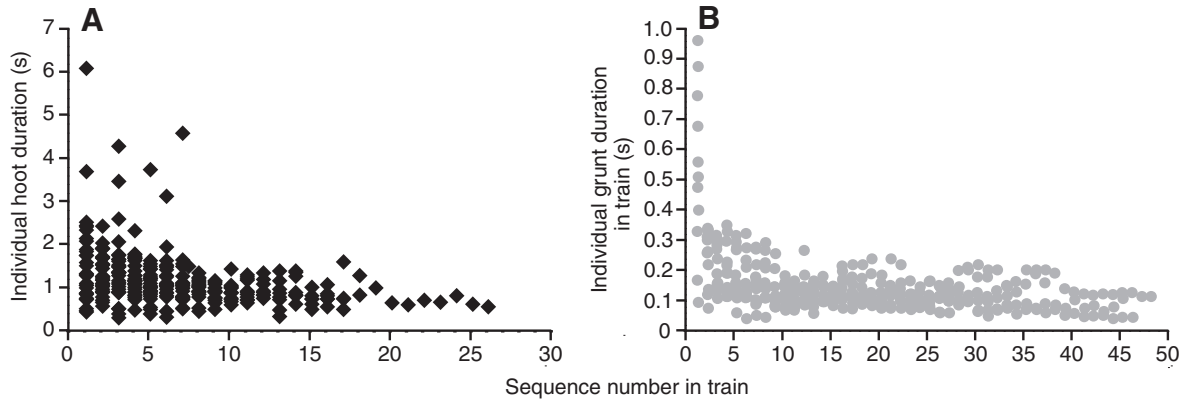

Fig. 1. Representative hoot train of *Batrachomoeus trispinosus*. (A) Waveform (amplitude is in dimensionless arbitrary units) and (B) spectrogram (Hz) of 13 boatwhistle calls in a train of 36 s in duration. Box indicates the position of the individual call represented in C. (C) Power spectrum of an individual, representative hoot on an expanded time base, shown in the inset (scale bar represents 250 ms). (D) Close-up of boatwhistle waveform, showing fine-scale call structure. Sounds were recorded at 44.1 kHz.

Fig. 2. Temporal patterns in different *B. trispinosus* vocalizations. (A) Duration of individual boatwhistle hoots within a train *versus* the order in which they occur within the train. (B) Duration of individual grunts within a grunt train *versus* the order in which they occur within the train. The duration of calls decreases with increasing order within the train (i.e. later calls are shorter), while there is no correlation with interval duration within the train.

[see Thorson and Fine (Thorson and Fine, 2002a) for stability of call structure for individual toadfish]. Moreover, individual fish produced grunts with beats when physically chased around the aquarium with a small net by an observer (N=4 grunts from two individuals, not shown). These grunts had a longer duration (0.39±0.04 s, range: 0.31–0.482 s) and much lower dominant and beat frequencies (176±1 and 21±2 Hz, respectively) than the conspecifically elicited ones.
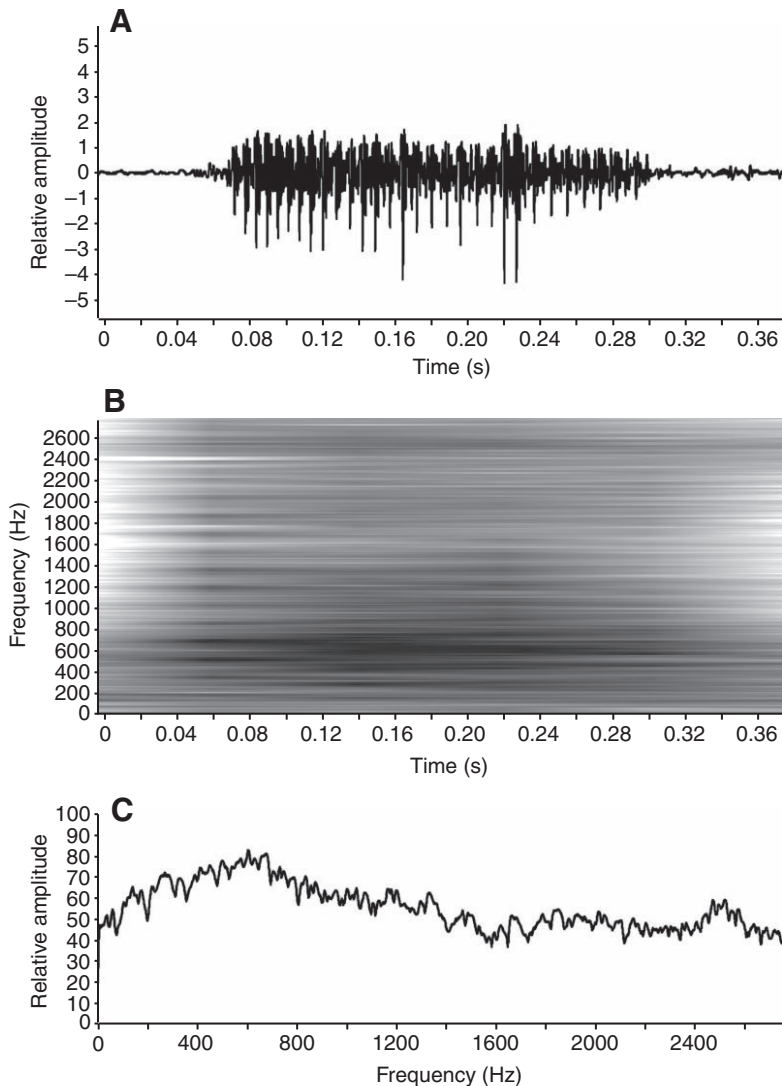

Fig. 3. (A) Waveform, (B) spectrogram and (C) power spectrum of a representative individual grunt of *B. trispinosus*. Sounds were recorded at 44.1 kHz.

Diel and lunar periodicity of calls

Vocalizations exhibited both a diel and lunar periodicity (Fig. 6). The number of hoot and grunt trains were both highest between 21:00h and 05:00h (Fig. 6A). The number of hoot trains declined in occurrence during the early morning hours until 09:00h. Single grunts were produced at higher levels during more hours of the day,

and were the most common call type throughout the day, with an elevated number of calls between 19:00 and 07:00h (Fig. 6A). Grunt trains were the most infrequently produced call type, produced only between 21:00h and 05:00h (Fig. 6A). The occurrence of all three call types increased leading up to the full moon (Fig. 6B). Both single grunts and hoots showed an increase in number following the three-
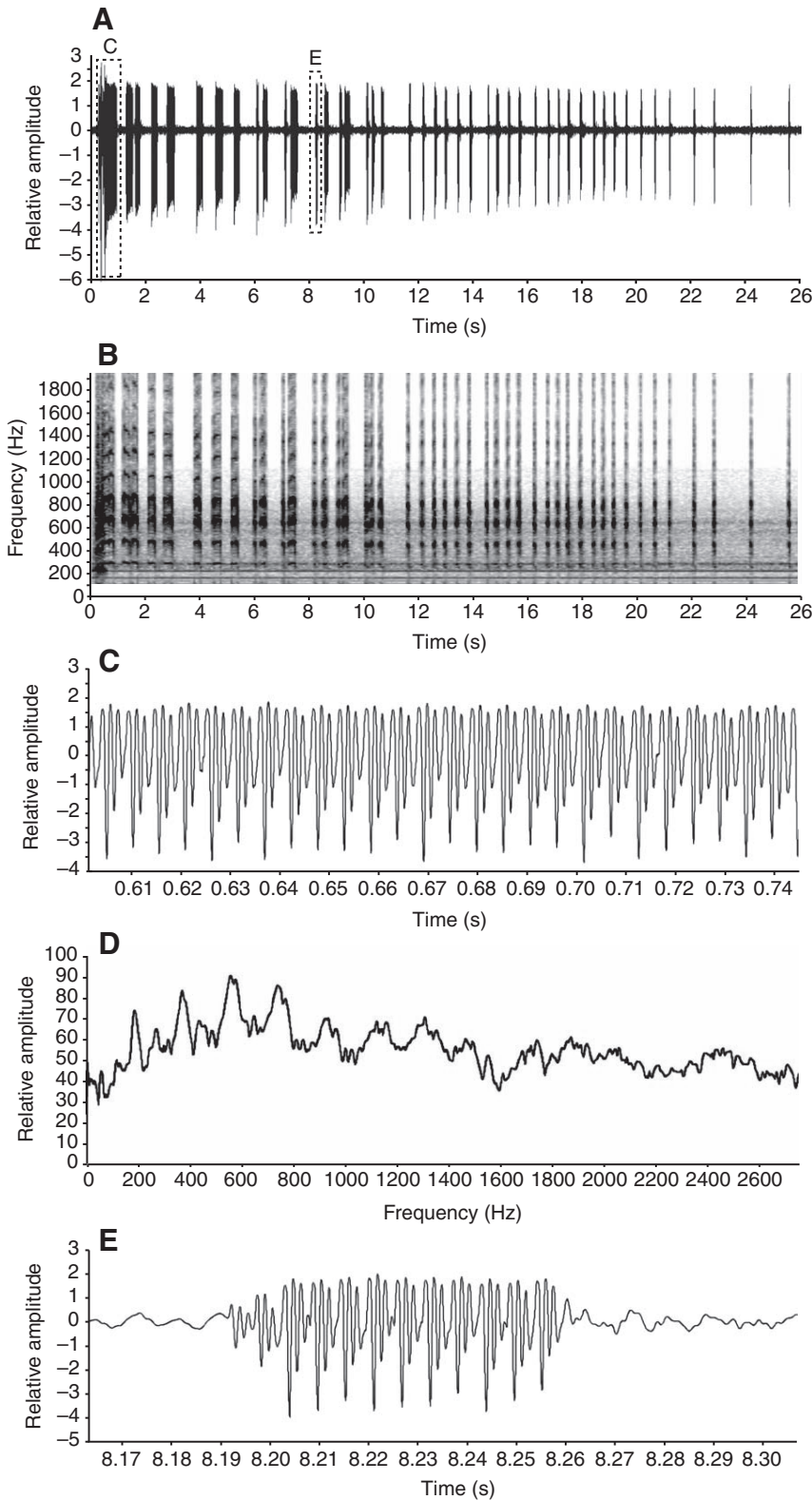

Fig. 4. (A) Waveform and (B) spectrogram of a representative grunt train (composed of 45 individual grunts) of *B. trispinosus*. Boxes in A represent individual grunts within the train in C and E. (C) The waveform and (D) corresponding power spectrum of an individual grunt at the beginning of the grunt train. (E) Waveform of an individual grunt in the middle of the grunt train. Sounds were recorded at 44.1 kHz.
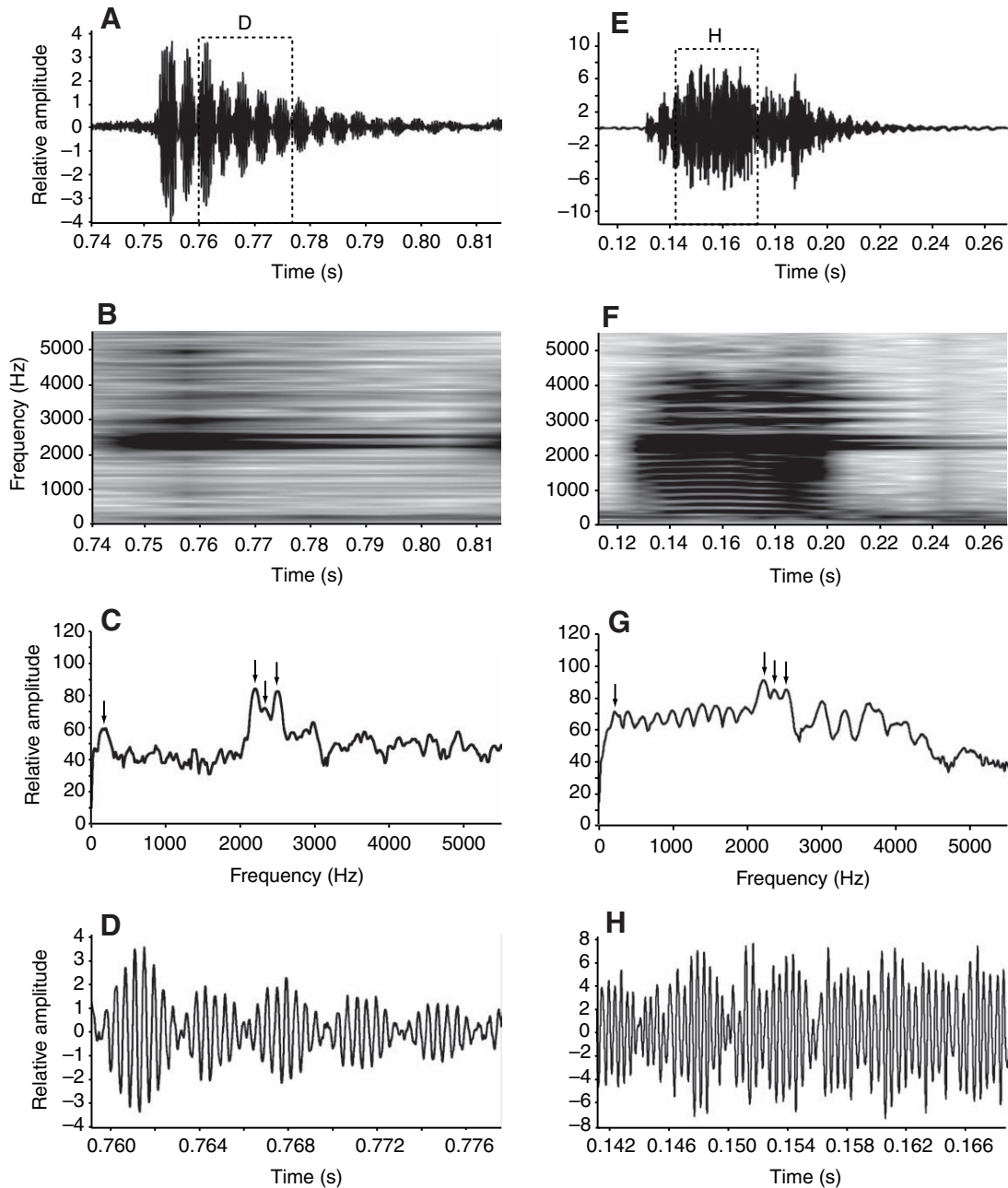
Fig. 5. Examples of two representative grunts exhibiting acoustic beats from *B. trispinosus*. (A) Waveform, (B) spectrogram, (C) power spectrum, (D) and waveform with expanded time base of a individual grunt. Box in A indicates enlarged section in D. Arrows in C represent peaks at 194, 2196, 2326 and 2498 Hz. (D) Waveform, (E) spectrogram, (F) power spectrum and (G) waveform with expanded time base of a second grunt, showing beat structure and clear harmonics. Box in E indicates enlarged section in H. Arrows in G represent peaks at 194, 2196, 2348 and 2520 Hz. For spectrograms (B,F) and power spectra (D,H), FFT size=1028 samples.

quarter waxing gibbous moon (Fig. 6B). The number of hoot trains dramatically increased during the full moon, and then proceeded to drop to lower levels afterwards, while the number of single grunts increased during the waning phase of the lunar cycle, becoming the predominant call type during this period (Fig. 6B).

Comparison of toadfish sounds

The hoot portion of the calls of all species considered exhibited different patterns of amplitude modulation. The time point at which the maximum amplitude occurred within the harmonic, hoot portion of all calls was significantly different among the batrachoidid species

sampled (Fig. 7A; one-way ANOVA: d.f.=165, F=149.82, $P<0.0001$). An *a posteriori* Tukey's HSD test (q=2.99, α=0.05) revealed three significantly different groups: *B. trispinosus* and *H. didactylus* had the latest position of maximum amplitude in their calls, *Opsanus* spp. boatwhistles and *P. notatus* growls had the earliest time of maximum amplitude, and *P. notatus* hums had a maximum amplitude intermediate between those of the two other groups (Fig. 7B).

Spectrographic cross-correlation and PCo analysis of different batrachoidid hoots and the hoot-like portion of midshipman growls (included because of structural similarity to *Opsanus* boatwhistles)
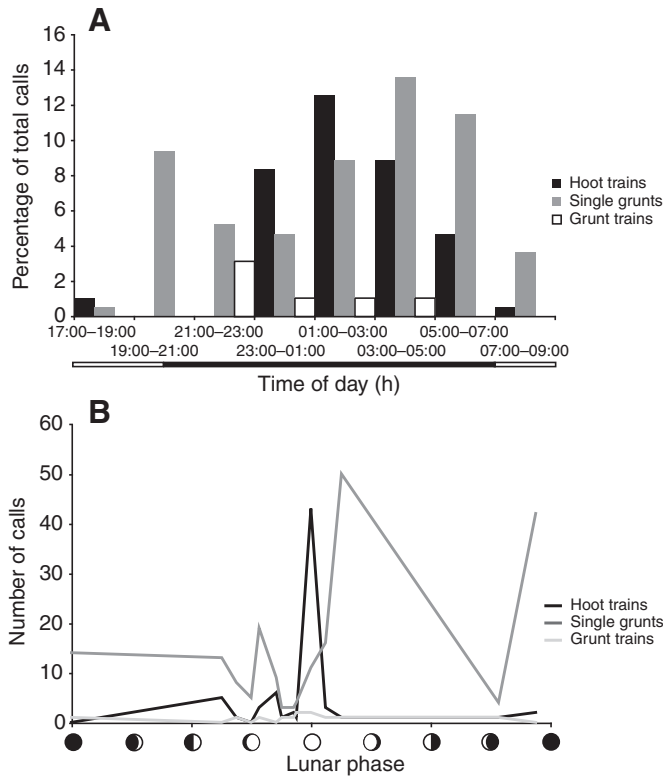
Fig. 6. (A) Diel patterns of calling frequency of different call types in *B. trispinosus.* Horizontal bar beneath *x*-axis labels indicates light cycle: open bar represents the period of ambient lights on, filled bar represents the period of ambient lights off (with only moonlight on). (B) Number of different call types over the lunar cycle in *B. trispinosus.*

showed that the first three principal coordinates accounted for 27.96% of the total variation (PCo1: 13.83%, PCo2: 7.47%, PCo3: 6.66%). Cross-correlation analysis of the waveform produced similar results to spectrographic cross-correlation. Vocalizations from within species were clustered closely together in the PCo (Fig. 8). *Batrachomoeus trispinosus* formed a distinct cluster apart from all the other species with a positive distribution along PCo1, and some dispersion along PCo2 (0.1 to –0.1). *Opsanus tau* and *O. phobetron* formed clusters extending to the lower left of the plot (with negative PCo1 and PCo2 values). Though there were fewer samples of *O. phobetron*, the variance along PCo1 was approximately the same as that of *O. tau*. *Opsanus beta* had a similar distribution along PCo1 to the other *Opsanus* species, but had positive PCo2 values. *Halobatrachus didactylus* calls and *P. notatus* growls overlapped with the calls of *O. beta* and *O. phobetron*, while *Porichthys* hums were tightly clustered near the center of the plot. When maximum amplitude position was regressed against PCo1, the result was a strong and highly significant relationship between these variables (R^2=0.60, d.f.=165, F=231.13, P<0.0001), suggesting that the position of maximum amplitude within the call (Fig. 7) was influencing PCo1.

Swimbladder morphology

The swimbladder of *B. trispinosus*, unlike that of any other toadfish studied so far, was found to be laterally divided into two asymmetrical, physically separate swimbladders (Fig. 9). Within the peritoneal cavity, the swimbladder extended anteriorly to the pelvic girdle and posteriorly to the caudal third of the cavity. The intrinsic

swimbladder muscles spanned the entire lateral wall of each swimbladder and were whitish in coloration in unfixed material. The vocal nerve inserted on the dorsomedial edge of each bladder muscle. Each swimbladder had its own rete mirabele, located on the posterior dorsal end of bladder, rather than a single rete as in other batrachoidids [described by Greene (Greene, 1924)]. Each swimbladder also had its own latitudinal septum; a pore in the center of the septum [described by Fänge and Wittenberg, and by Tower (Fänge and Wittenberg, 1958; Tower, 1908)] was visible in four out of seven specimens examined.

A single muscle was attached to the lateral wall of each swimbladder. When viewed in cross-section, the lateral wall was concave in smaller fish (7.1 cm standard length; Fig. 10A), becoming more convex in larger body size fish (15.1 cm standard length; Fig. 10B). Muscle fibers were arranged vertically, perpendicular to the long axis of each bladder (Fig. 10A,B). The bladder muscle was thicker in the smaller fish (standard length of 7.1 cm), while it was larger in the dorsoventral axis in larger fish (standard length of 15.1 cm; Fig. 10A,B). A thin membrane, appearing to be part of the external swimbladder wall, covered the swimbladder muscle (Fig. 10C).

The left and right swimbladders were obviously asymmetrical in terms of length (Fig. 9), with the anterior poles of the two adjacent; however, one side was not consistently longer. Of the specimens measured (*N*=25), the left swimbladder was longer than the right in 15 fish (nine males and six females), the right was longer in nine (seven males and two females), and only one (a female) had equal-sized swimbladders. Differences in the width of the left and right bladders did not necessarily correspond to differences in length. Of the 15 with the longer left bladder, seven also had a wider left bladder; of the nine with the longer right bladder, six also had a wider right bladder.

Swimbladder length and width increased with standard length (Fig. 11; see Table 1 for statistics) and were sexually dimorphic (Fig. 11; see Table 2 for results from statistical tests); however, while males had wider swimbladders, females had longer ones. The degree of asymmetry of either length or width (i.e. the difference for each measurement between the two bladders) showed no strong relationship with body size (R^2=0.04).

There was no consistent difference in the proportional muscle mass (i.e. corrected for body mass) between the left and right bladders (two-tailed paired *t*-test: d.f.=5, t=–0.00000025, P=0.99); there was also no significant sex difference in this parameter for either the left or right bladder (Table 2). We attempted to get accurate volumetric measurements for each swimbladder, but were unable to successfully separate the vocal muscle from each bladder and keep the entire bladder intact.

Evolutionary relationships of the toadfishes

The maximum likelihood analysis resulted in a topology with a negative log likelihood score of 17838.52 (Fig. 12). Likelihood bootstrapping analysis showed high support (>75) in 9 of 11 nodes. *Halobatrachus* was the most basal out of the toadfishes included in the analysis, followed by *Batrachomoeus trispinosus+Allenbatrachus grunniens*. *Opsanus* spp. and *Porichthys* spp. were sister clades and the most derived, and a monophyletic Thalassophryninae (*Daector+Thalassophryne*) (Collette, 1966) was their sister group.

DISCUSSION

Like other toadfishes, *B. trispinosus* produces an array of different vocalizations. However, in contrast to other studied members of the
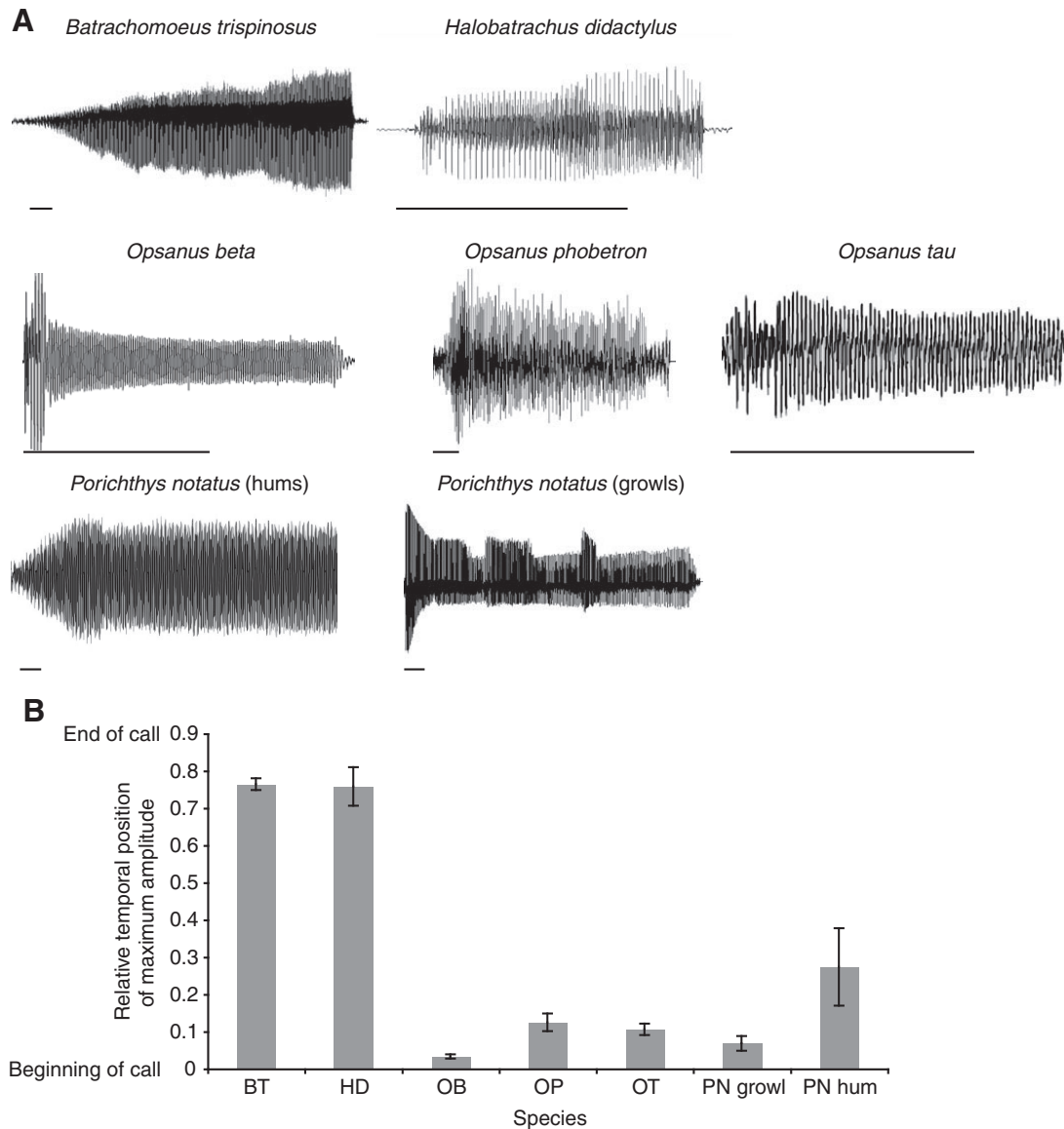
Fig. 7. (A) Representative waveforms of different toadfish vocalizations. Horizontal scale bars represent 250 ms; amplitude of the different calls is not to scale. (B) Relative temporal position of the occurrence of the maximum amplitude in toadfish harmonic calls, for *B. trispinosus* (BT), *Halobatrachus didactylus* (HD), *Opsanus beta* (OB), *O. phobetron* (OP), *O. tau* (OT), and the growl and hum of *Porichthys notatus* (PN).

family, *B. trispinosus* has evolved a novel swimbladder morphology – laterally divided into two separate bladders – and is capable of producing sounds with beats: both unique features among vocal fish. As discussed below, these unique morphological and behavioral adaptations of *B. trispinosus* may represent one of the most highly derived vocal systems in fishes, convergent with tetrapod taxa in their ability to produce acoustic beats.

Swimbladder development and functional morphology

Despite the morphological diversity of actinopterygian swimbladders, the completely divided swimbladder of *B. trispinosus* is unique among those species so far studied. Species of *Opsanus* and *Porichthys* have a single, heart-shaped swimbladder (Bass and Baker, 1991; Fänge and Wittenberg, 1958; Tower, 1908). The swimbladder of *Batrachomoeus* most closely resembles that of *H. didactylus* (dos Santos et al., 2000) and descriptions of that of *Allenbatrachus grunniens* (=*Opsanus grunniens*) (Rauther, 1945),

in which the two halves of the swimbladder are connected by a short hollow tube in the posterior third of the bladders, and perpendicular to their long axis. The available phylogenetic evidence suggests that the swimbladder of *Batrachomoeus* represents a divergent condition from that of the semi-connected swimbladders of *Halobatrachus* and *Allenbatrachus* (Fig. 12).

Interestingly, the highly derived swimbladder of *B. trispinosus* appears to be relatively basal for the batrachoidids. A combination of more complete taxon sampling in creating a phylogeny of the Batrachoididae as well as determining the, as yet, unclear sister taxon to the family (Greenfield et al., 2008; Miya et al., 2005; Patterson and Rosen, 1989; Regan, 1912; Smith and Wheeler, 2006) will help clarify patterns and processes in toadfish swimbladder evolution. The systematic relationships of toadfish inferred from our molecular phylogeny are largely congruent with the visual consensus tree from a recent, more thorough morphological phylogenetic study of the Batrachoididae (Greenfield et al., 2008). The largest discrepancy
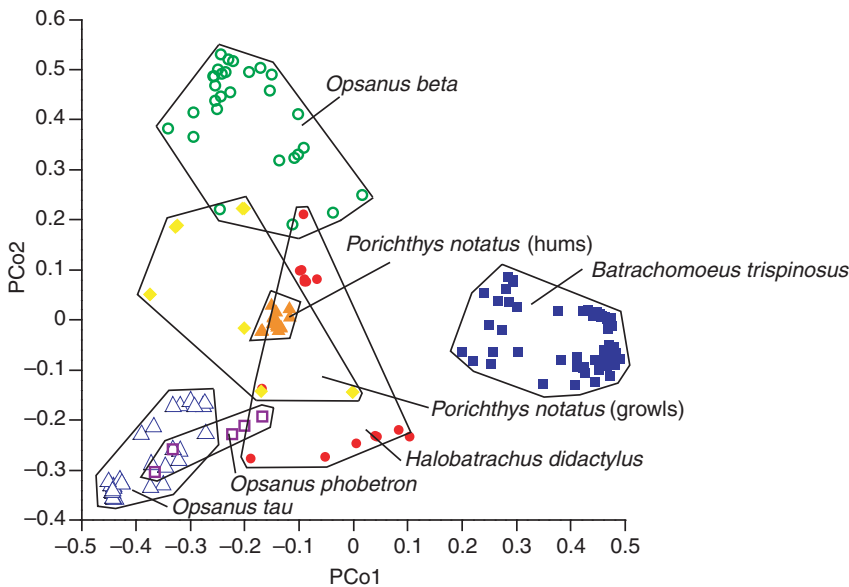
Fig. 8. Results from a principal coordinates analysis (PCo2 *versus* PCo1) of spectrographic cross-correlation (SPCC–PCo) (Cortopassi and Bradbury, 2000) of 166 vocalizations from six species of toadfishes. Sounds analyzed included harmonic hoots from the boatwhistle vocalizations of *B. trispinosus* (filled blue squares), *H. didactylus* (filled red circles), *O. beta* (open green circles), *O. phobetron* (open purple squares), *O. tau* (open blue triangles) and the hum of *P. notatus* (filled orange triangles). Due to their temporal similarity to toadfish boatwhistles, 'growls' of *P. notatus* (filled yellow diamonds) were also analyzed.

between our phylogeny and those from Greenfield et al. (Greenfield et al., 2008) is that the clade of *Batrachomoeus+Allenbatrachus* is basal to *Halobatrachus*. However, even with the phylogenetic topology of Greenfield and colleagues (Greenfield et al., 2008), the swimbladder condition of *B. trispinosus* is uniquely derived (autapomorphic) within the family, given the retained close relationship between *B. trispinosus* and *A. grunniens* (with an undivided swimbladder).

The *Batrachomoeus* swimbladder phenotype raises intriguing questions about mechanisms of its development: whether it originates as a single structure and laterally divides during ontogeny or initially develops as two separate bladders. During the development of *Opsanus* and *Porichthys*, the swimbladder originates as a single outpocketing of the esophagus, remaining attached by a duct to the esophagus through a physostomous phase, after which the duct atrophies and the swimbladder becomes completely separate or physoclistous (Lindholm and Bass, 1993; Tracy, 1911). The swimbladders of *O. tau* and *P. notatus* possess a longitudinal septum on the anterior portion of the bladder (Bass and Baker, 1991; Tracy, 1911). It is possible that the extended growth of the septum ultimately divides the swimbladder into two lateralized structures, different progressions of which give rise to either a partially or a completely separated swimbladder across species. The diversity of swimbladder morphologies in this family may be a fruitful area for investigation of the evolution and consequences of variation in bilateral patterning during development, inclusive of genetic control.

The collagenous membrane covering the swimbladder muscle (Fig. 10C) appears to be another unique feature of *B. trispinosus* compared with *O. beta*, *O. tau* and *P. notatus*. While the exact role of this membrane is unclear, it may serve to increase intramuscular pressure during vocal motor activity, which may in turn increase tensile forces along the swimbladder wall (*sensu* Wainwright et al., 1978; Westneat et al., 1998), resulting in higher resonant frequencies.

Sexually dimorphic bladders have been reported in *H. didactylus, O. tau, O. beta* and *P. notatus* (Brantley and Bass, 1994; Brantley et al., 1993; Fine, 1975; Fine et al., 1990; Modesto and Canário, 2003; Walsh et al., 1987; Walsh et al., 1989). The sexual dimorphism in the swimbladders of *B. trispinosus* is complex; males had wider swimbladders, while females had longer ones. The potential functional significance of differences in either of these dimensions remains unclear. More detailed biomechanical studies of each

swimbladder (e.g. Fine et al., 2001) will be required to better understand the role of dimorphisms in sound production.

Vocal repertoire

Vocalizations produced by *B. trispinosus* resemble the major acoustically and behaviorally defined classes of vocalizations described in other batrachoidids, namely agonistic grunts that are brief in duration, and comparatively longer duration, multiharmonic advertisement boatwhistles and hums (e.g. Brantley and Bass, 1994; dos Santos et al., 2000; Gray and Winn, 1961; Ibara et al., 1983; Tavolga, 1958; Thorson and Fine, 2002b). Because of the similarities of the acoustical properties of *B. trispinosus* calls to those of other toadfishes, we suggest that the different classes of calls have a homologous social context: the long duration, multiharmonic hoot serves as an advertisement or courtship call, while the short duration grunts and grunt trains function in aggression and territorial defense. A number of dissected females from the tanks were found to have ripe eggs in the ovaries (*N*=4), which is suggestive of courtship activity and reproductive behavior, as observed in other toadfishes (e.g. Brantley and Bass, 1994; Gray and Winn, 1961). The presence of reproductively mature females within the population further supports the hypothesis that the hoots serve a role in courtship behavior.

With the exception of *P. notatus* hums, *B. trispinosus* hoots are substantially longer than those in other toadfishes: individual *B. trispinosus* hoots last up to 6s (compared with <1s for *Halobatrachus*, *Opsanus* and *Sanopus*) (Amorim and Vasconcelos, 2008; Amorim et al., 2008; Mann et al., 2002); the hoot-like hums of *P. notatus* last upwards of an hour (Bass et al., 1999; Brantley and Bass, 1994; Ibara et al., 1983). The rich harmonic structure of *B. trispinosus* grunt trains also appears to be distinct from that of most other toadfish (see above references). Like other toadfishes, *B. trispinosus* showed increased levels of calling at night (e.g. Brantley and Bass, 1994; Breder, 1968; Ibara et al., 1983; Thorson and Fine, 2002b). However, it appears that other toadfish species do not display the same degree of lunar synchrony in calling patterns as *B. trispinosus* (Breder, 1968).

To our knowledge, the beats in the individual vocalizations of *B. trispinosus* are the first to be reported in fishes [though groups of chorusing *P. notatus* can collectively produce beats (McKibben and Bass, 2001)]. This demonstrates that fishes have also
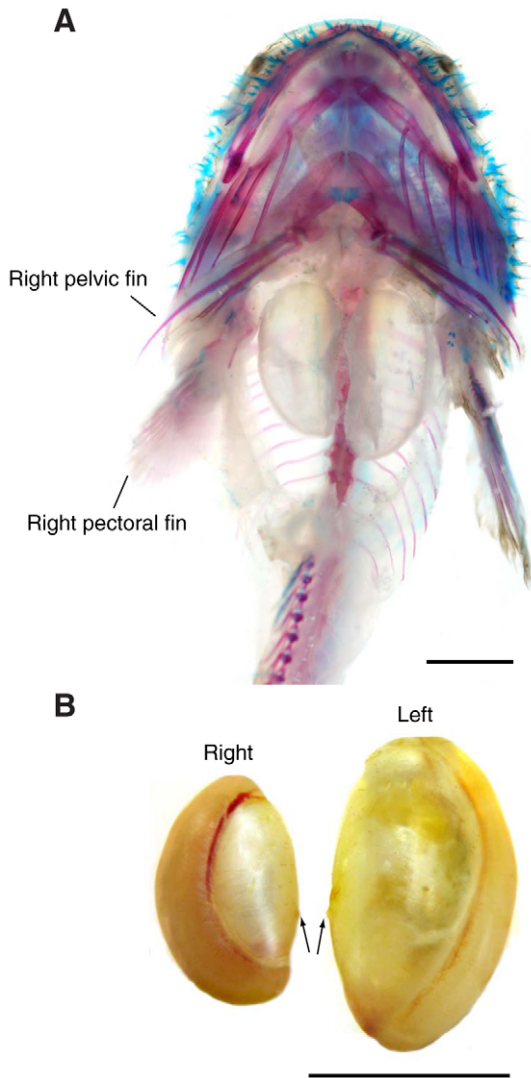
Fig. 9. (A) Ventral view of a cleared and stained *B. trispinosus* specimen (6.6 cm standard length) showing the bilateral swimbladder. The skin and viscera of the abdominal cavity were removed and the swimbladder was left in place. Specimen was cleared and stained for cartilage (with Alcian Blue) and bone (with Alizarin Red) following the protocol of Song and Parenti (Song and Parenti, 1995). Scale bar represents 1 cm. The right pelvic and pectoral fins are labeled for reference orientation. (B) Ventral view of dissected swimbladders from a 9.9 cm standard length female, showing asymmetry between left and right bladders. Arrows indicate points of attachment, where the bladders are connected to each other by connective tissue. Scale bar represents 1 cm.

Fig. 10. Cross-section (70 μm thick sections) of swimbladder wall and intrinsic swimbladder muscle of *B. trispinosus,* stained with Methylene Blue from 7.1 cm standard length male (A) and 15.1 cm standard length male (B). The medial side of the swimbladder is to the left. Scale bar represents 1 mm for A and B. (C) High magnification view of junction of swimbladder wall and muscle from the area represented by the box in A. Medial side of the swimbladder is to the left. Scale bar represents 1 mm.

independently evolved harmonically complex acoustical signals similar to those of other tetrapods, such as birds (Nowicki and Capranica, 1986) and frogs (Suthers et al., 2006). We propose that the ability of *B. trispinosus* to produce beats is dependent upon their bilaterally divided swimbladder, reminiscent of the two halves of the avian syrinx (Suthers, 1990; Suthers, 2001). Given the variability of the properties (i.e. dominant frequency, beat duration) of sounds with beats, it seems likely that the physiological generation of beats is under active control by the central vocal motor system (see Bass and McKibben, 2003). Alternatively, beats may be generated passively due to the asymmetry of the swimbladder, similar to the asymmetric (type III) avian syrinx, as in oilbirds and penguins
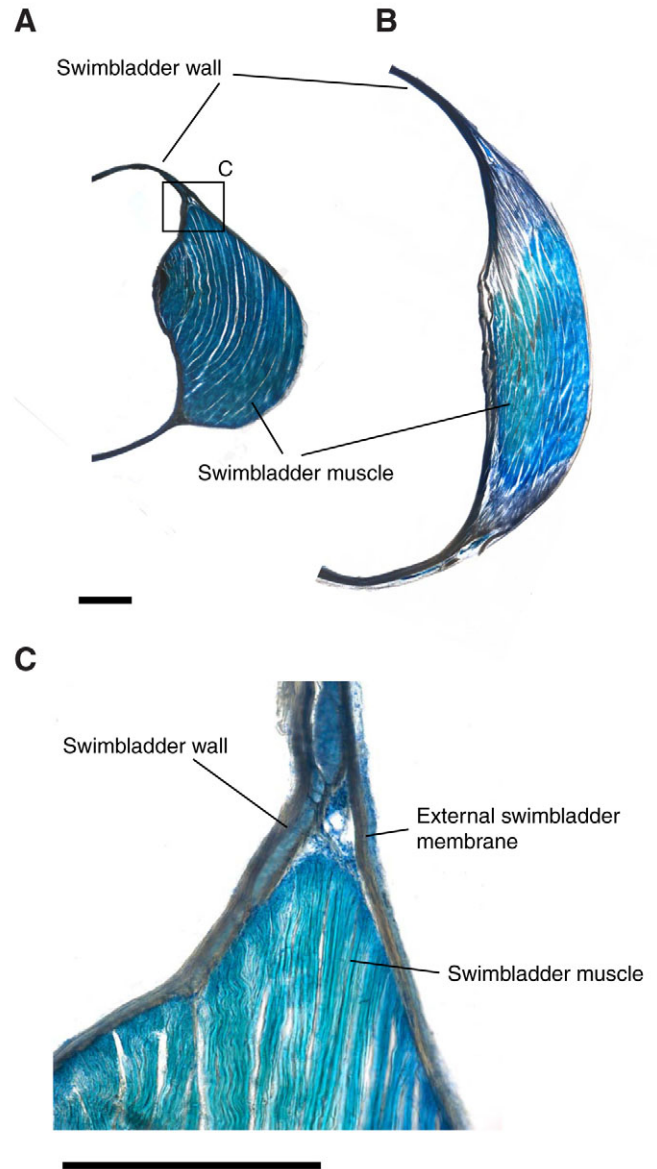
(Aubin et al., 2000; Bradbury and Vehrencamp, 1998; Suthers and Hector, 1985). However, passive anatomical generation of beats seems unlikely, as the majority of *B. trispinosus* specimens examined had some degree of asymmetry between the left and right bladders, but the majority of sounds did not exhibit beats.

The spectral properties of the acoustic beats observed here are reminiscent of the spectra for the two-voice/biphonation calls in mockingbirds (Zollinger et al., 2008). In *Batrachomoeus* calls, the difference in frequency between the two major high frequency peaks, rather than between each of the three peaks, approximates the call's modulation rate; hence our interpretation of the signals as beats rather than as amplitude-modulated signals. Interestingly, there is also a
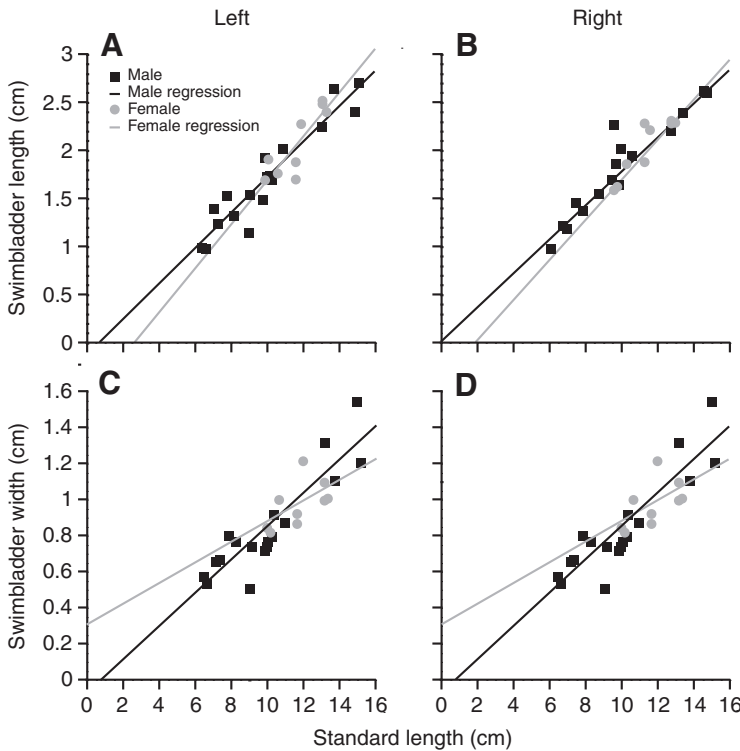
Fig. 11. Ontogenetic changes in swimbladder morphology of *B. trispinosus* males and females. (A) Length of left swimbladder *versus* standard length; (B) length of right swimbladder *versus* standard length; (C) left swimbladder width *versus* standard length; (D) right swimbladder width *versus* standard length. Solid lines represent regression lines of males (black) and females (gray). For all parameters measured, males were significantly different from females (ANCOVA, *P*<0.0001); females had longer swimbladders and wider swimbladder muscles than males, while males had wider swimbladders than females.

significant amount of energy near 200 Hz, close to the fundamental frequency of non-beat grunts. Whether these non-linear phenomena (see Fitch et al., 2002; Zollinger et al., 2008) arise from the activity of one or both swimbladders remains to be investigated.

The high frequency dominant portion of grunts with beats (2000–2500 Hz) raises the question of whether *B. trispinosus* can, in fact, detect this component of the signal. The available studies show that this dominant frequency is probably outside of the auditory sensitivity of other studied batrachoidids, namely *H. didactylus*, *O. tau* and *P. notatus* (e.g. Fay and Edds-Walton, 1997; Fish and Offutt, 1972; Sisneros et al., 2004; Vasconcelos and Ladich, 2008). However, increased hearing sensitivity to higher frequencies (>1000 Hz) has independently and repeatedly evolved in fishes (Braun and Grande, 2008). The detection of these sounds is often facilitated by mechanical transduction mechanisms between the ear and anatomical structures with a different density from water; one of the most common auditory specializations for sensitivity to higher sound frequencies in fishes involves modifications of the swimbladder (e.g. Braun and Grande, 2008; Popper et al., 2003). From the dissections of *B. trispinosus*, the anterior end of the swimbladder is angled dorsally, pointing towards the neurocranium, and terminates 1.25 mm behind the sacculus (measured in one specimen). The swimbladder of *B. trispinosus* is much more rostral, elongate and closer to the ear than that of *O. tau*, in which the swimbladder has been suggested to play no role in audition (Yan

et al., 2000). Thus, given the unique morphology and position of the swimbladder in *B. trispinosus* for signal production, it may also be involved in signal reception.

Underwater playback studies in midshipman investigated the discrimination of two-tone beats with modulation rates up to 10 Hz and showed that both beat frequency and the depth of modulation contribute to acoustic recognition (McKibben and Bass, 1998; McKibben and Bass, 2001). Comparable experiments with the distantly related goldfish (*Carassius auratus*) show similar discrimination for two-tone beat stimuli, in this case with beat frequencies ranging up to 200 Hz (Fay, 1998), close to the modulation rate of the *Batrachomoeus* calls observed here. Single neuron recording studies of the auditory system of batrachoidids also show the temporal encoding of two-tone beat stimuli. For midshipman, sensitivity to stimuli with 1–10 Hz beat frequencies overlaps that of the naturally occurring beats generated by the concurrent humming of neighboring males during the breeding season (Bodnar and Bass, 1997). The auditory system of the toadfish *O. beta* also encodes beats, but mainly for beat frequencies >10 Hz (Bass et al., 2001). Future behavioral and sensory experiments will be needed to investigate the recognition and behavioral significance of acoustic beats.

The coral reef, nearshore and estuarine environments inhabited by *B. trispinosus* (Greenfield, 1999) are complex acoustic environments (Bass and Clark, 2003). In shallow (10–100 m) and

Table 1. Results of linear regressions from morphological measurements of the left and right *Batrachomoeus trispinosus* swimbladders

Swimbladder measurement	R^2 overall	d.f.	F	P	R^2 male	R^2 female
Length						
Left	0.88	26	185.54	*P*<0.0001	0.90	0.74
Right	0.89	24	179.77	*P*<0.0001	0.90	0.81
Width						
Left	0.77	26	81.53	*P*<0.0001	0.80	0.34
Right	0.83	24	109.77	*P*<0.0001	0.88	0.69

Table 2. Tests for sexual dimorphism of swimbladder and muscle morphology of *Batrachomoeus trispinosus*

Swimbladder measurement	d.f.	F	P
Length			
Left	26	59.61	*P*<0.0001
Right	24	56.96	*P*<0.0001
Width			
Left	26	26.72	*P*<0.0001
Right	24	37.41	*P*<0.0001
Muscle mass			
Left	10	0.7422	*P*=0.4113
Right	10	0.4563	*P*=0.5163

Results from analyses of covariance of swimbladders of males and females, with the fish's standard length as the covariate. Differences in muscle mass were tested with an analysis of variance using the residuals of muscle mass regressed against body size.

very shallow (<5 m) water systems (see Bass and Clark, 2003), lower frequency components of an acoustic signal have a higher level of attenuation, while the higher frequency components propagate many times farther (Bass and Clark, 2003; Fine and Lenhardt, 1983; Mann and Lobel, 1997). Fine and Lenhardt demonstrated that the frequencies around the fundamental frequency of the boatwhistle of *O. tau* (around 200 Hz) had an attenuation of –29.5 dB over 7 m, while upper harmonics around 800 Hz only had an attenuation of –13 dB over the same distance (Fine and Lenhardt, 1983). Thus, for *B. trispinosus*, the high-frequency harmonic components of many of the hoots, grunts and particularly the grunts with beats may be an adaptation to increase the propagation distance of the call in shallow water habitats. However, playback studies (*sensu* Fish, 1972;

McKibben and Bass, 2001; Remage-Healey and Bass, 2005) are needed to confirm any behavioral significance of the higher frequency components of *B. trispinosus* calls, and whether increased propagation distance is biologically meaningful, advantageous or simply an artifact of swimbladder mechanics during sound production.

Diversity of toadfish vocalizations

The quantitative comparison of toadfish vocalizations offers an interesting insight into the diversity and perhaps the evolution of toadfish acoustic communication. Taxonomically, the between-genus diversity of toadfish sounds is primarily distributed along PCo1, whereas within-genus diversity (*Opsanus*) is along PCo2. The PCo analysis also showed that the two most closely related species, *O. phobetron* and *O. tau* (Fig. 12) have the most similar sounds (Fig. 8). Unfortunately, while SPCC allows for the objective discrimination of sounds based on complex spectrographic and temporal components, its principal shortcoming is that comparisons between spectrograms result in a univariate similarity score and the PCo analysis is then conducted on the similarity matrix (Clark et al., 1987; Cortopassi and Bradbury, 2000). As such, it is impossible to determine which specific components of the sound (e.g. fundamental frequency, call duration, etc.) are specifically influencing their distribution in the PCo analysis. However, the correlation between amplitude modulation pattern and PCo1 suggests that amplitude envelope shape may be influencing the statistical discrimination of the different sounds (Fig. 7B). The hoots of *O. beta* and *O. tau* and the growls of *P. notatus* have decreasing amplitude throughout the call; *P. notatus* hums, *O. phobetron* and *H. didactylus* have little amplitude modulation in their calls, whereas most *B. trispinosus* calls continually increase in amplitude
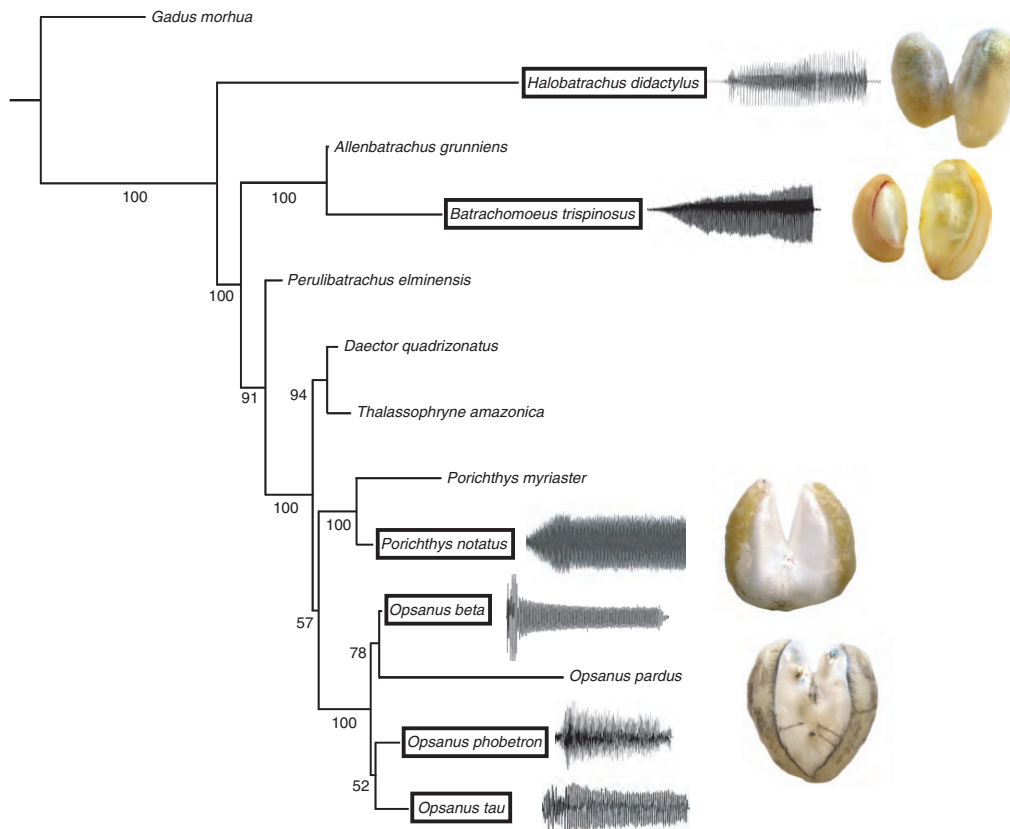

Fig. 12. Phylogenetic relationships of toadfishes generated from a maximum likelihood analysis of genes from available sequences (*16S*; *28S*; cytochrome oxidase subunit I, *COI*; cytochrome b, *CytB*) in GenBank. Branch lengths are drawn proportional to the amount of character change. Bootstrap values are shown next to nodes. The gadid, *Gadus morhua*, was used as an outgroup for the Batrachoididae. Boxes around species names indicate taxa used in the comparative sound analysis, and a representative waveform of the species' call and swimbladder is shown for each taxon analyzed.

throughout the duration of the call (Fig. 7B). Interestingly, the hoots of the closely related *B. trispinosus* and *H. didactylus* shared the latest position of maximum amplitude in their calls among the species studied. The behavioral importance of amplitude modulation is also shown by playback studies in midshipman fish (Bodnar and Bass, 2001; McKibben and Bass, 1998; McKibben and Bass, 2001).

The diversity of intraspecific vocalizations in toadfishes parallels the diversification of acoustic signals in other fish families (e.g. Amorim et al., 2004; Gerald, 1971; Lobel, 2001; Malavasi et al., 2008; Rice and Lobel, 2003). While acoustic call diversity and evolution are frequently discussed in tetrapods (e.g. Price and Lanyon, 2002; Ryan, 1986), similar evolutionary questions have not been extensively tested in fishes (but see Malavasi et al., 2008). Due to the relative simplicity of many central and peripheral vocal mechanisms (compared with tetrapods), fish typically lack the ability to produce complex and dynamic, frequency-modulated calls (Bass, 1997; Bass and McKibben, 2003; Demski et al., 1973; Rice and Lobel, 2003). Consequently, it is often variation in either temporal patterning or frequency that is primarily responsible for vocal differences among fish populations and species (Kihslinger and Klimley, 2002; Malavasi et al., 2008; Mann and Lobel, 1998; Parmentier et al., 2005). Again, underwater playbacks in midshipman fish show that individual fish can discriminate sounds based on fine temporal structure, i.e. fundamental frequency (McKibben and Bass, 1998; McKibben and Bass, 2001). The diversity of amplitude, frequency and beat-modulated vocalizations of *Batrachomoeus* may present the most complex pattern of call structure so far shown for any toadfish species or, for that matter, any fish. How such variation shapes social communication at both behavioral and neural levels of organization remains to be explored.

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