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RESEARCH ARTICLE

Vocal production complexity correlates with neural instructions in the oyster toadfish (*Opsanus tau*)

Coen P. H. Elemans[1,2,*], Allen F. Mensinger[3,4] and Lawrence C. Rome[3,5]

ABSTRACT

Sound communication is fundamental to many social interactions and essential to courtship and agonistic behaviours in many vertebrates. The swimbladder and associated muscles in batrachoidid fishes (midshipman and toadfish) is a unique vertebrate sound production system, wherein fundamental frequencies are determined directly by the firing rate of a vocal-acoustic neural network that drives the contraction frequency of superfast swimbladder muscles. The oyster toadfish boatwhistle call starts with an irregular sound waveform that could be an emergent property of the peripheral nonlinear sound-producing system or reflect complex encoding in the central nervous system. Here, we demonstrate that the start of the boatwhistle is indicative of a chaotic strange attractor, and tested whether its origin lies in the peripheral sound-producing system or in the vocal motor network. We recorded sound and swimbladder muscle activity in awake, freely behaving toadfish during motor nerve stimulation, and recorded sound, motor nerve and muscle activity during spontaneous grunts. The results show that rhythmic motor volleys do not cause complex sound signals. However, arrhythmic recruitment of swimbladder muscle during spontaneous grunts correlates with complex sounds. This supports the hypothesis that the irregular start of the boatwhistle is encoded in the vocal pre-motor neural network, and not caused by peripheral interactions with the sound-producing system. We suggest that sound production system demands across vocal tetrapods have selected for muscles and motorneurons adapted for speed, which can execute complex neural instructions into equivalently complex vocalisations.

KEY WORDS: Acoustic communication, Deterministic chaos, Sound production, Swim bladder, Toadfish, Superfast muscle

INTRODUCTION

Vocalisations are controlled by distinct neural circuits highly conserved across fish and all major lineages of vocal tetrapods (Bass et al., 2008). The activity of these neural circuits drives an often highly nonlinear vocal system, capable of generating acoustic signals ranging from whistles to complex, irregular vocalisations that exhibit low-dimensional chaos (Elemans et al., 2010; Fitch et al., 2002). Such complex sounds have been reported in fish (Rice et al., 2011), frogs (Suthers et al., 2006), birds (Fletcher, 2000; Zollinger et al., 2008) and various mammals (Fitch et al., 2002). The biomechanics of a peripheral system can impose constraints on its

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neural control, but also provide opportunities for the emergence of complexity in expressed behaviours (Chiel and Beer, 1997). Exploiting the nonlinear physical properties of sound-producing organs could enhance the acoustic complexity of vocalisations, causing, for example, sudden frequency jumps or chaotic oscillations, without equivalently complex neural control (Fee et al., 1998), and function as an individual signature (Fitch et al., 2002).

An excellent model system to study the interactions between neural control and peripheral mechanics in sound production is the batrachoidid fishes (midshipman and toadfish), which produce sound by swimbladder wall movements (Fine et al., 2009; Fine et al., 2001; Skoglund, 1961) actuated by pure-fibre superfast muscles that are among the fastest vertebrate skeletal muscles (Rome, 2006; Rome et al., 1996). Each swimbladder muscle (SBM) is innervated by vocal motor neurons specialised for superfast muscles (Chagnaud et al., 2012) and receives input from a vocal network consisting of distinct hindbrain nuclei (Chagnaud et al., 2011). In midshipman (*Porichthys notatus*), pre-pacemaker neurons in the ventral medullary nucleus control call duration and project on pacemaker neurons (Bass and Baker, 1990; Bass et al., 1994), which set the firing rate and project on vocal motor neurons that control swimbladder contractions. The firing rate of this vocal-acoustic neural network directly determines SBM contraction frequency, which in turn results in swimbladder compression and sound.

The oyster toadfish [*Opsanus tau* (Linnaeus 1766)] is an animal model system widely used in biomedical studies (Walsh et al., 2007) and its superfast swimbladder muscles are a leading model for understanding superfast muscle physiology (Rome, 2006). This highly specialised muscle type, because of its speed and highly ordered structure, has become an important model for studying regular skeletal muscle function and design (Rome, 2006) and calcium movements during contraction (Harwood et al., 2011; Nelson et al., 2014).

Oyster toadfish produce at least two kinds of sounds: the grunt, an agonistic call (peak frequency ~90 Hz) produced by both sexes (Maruska and Mensinger, 2009), and the 'boatwhistle', a 200–700 ms long tonal call (fundamental frequency 80–220 Hz) that males can produce up to six to 15 times per minute from their nest to attract females and establish territories (Edds-Walton et al., 2002; Fine, 1978; Mensinger, 2014; Tavolga, 1958). The boatwhistle consists of two parts (Fig. 1A). Part 1 (P1) consists of a 50–100 ms long irregular waveform, while part 2 (P2) consists of a regular repetitive harmonic waveform (Edds-Walton et al., 2002).

If the irregular waveform of P1 is a complex waveform that has an underlying chaotic attractor (Elemans et al., 2010; Fitch et al., 2002; Wilden et al., 1998), this raises the question of whether this irregular sound waveform is caused by peripheral nonlinearities of the sound production system or is centrally encoded. If the origin lies in peripheral nonlinearities, such as interaction between swimbladder and superfast muscle mechanics, this would enhance the animals' acoustic complexity without equivalently complex

List of abbreviations	
EMG	electromyography
P1	part 1 of the boatwhistle call, irregular waveform
P2	part 2 of the boatwhistle call, harmonic waveform
SBM	swimbladder muscle
VN	vocal motor nerve
VPN	vocal pacemaker nucleus

neural control (Fee et al., 1998). If this is not the case, the acoustic complexity might be driven by neural instruction.

We investigated whether the start of the boatwhistle is indicative of a chaotic strange attractor and tested the competing hypotheses that the irregular waveform in P1 of the toadfish boatwhistle is an emergent property of the peripheral sound-producing system, or that it originates in the central nervous system due to irregular motor volleys of the vocal motor nerve (VN). Sound production and SBM activity were recorded in awake, freely behaving fish during VN stimulation, while VN and SBM activity were recorded during spontaneous grunts.

RESULTS

We recorded boatwhistle advertisement calls of male oyster toadfish in saltwater ponds. As previously described, these natural vocalisations consisted of an irregular P1 sound waveform and a harmonic P2 waveform (Fig. 1) (Edds-Walton et al., 2002). In chaotic

nonlinear signals, the phase-space plot has a distinctive three-dimensional, aperiodic structure, whereas linear signals are periodic and often two-dimensional (Elemans et al., 2010; Fitch et al., 2002; Rice et al., 2011). In phase-space, 10 overlaid calls of one male show a distinctive three-dimensional aperiodic attractor (BW1 in Fig. 1C). Therefore, although the waveform is irregular it is consistent between calls and indicative of a low-dimensional chaotic signal. In contrast, P2 consists of a harmonic waveform in the time domain and a stable two-dimensional limit cycle in phase-space (BW2).

We hypothesise that the complex waveform in P1 of the toadfish boatwhistle is either (1) an emergent property of the peripheral interaction between superfast muscle contraction and swimbladder biomechanics, or (2) finds its origin in the activation pattern of the VNs. To test the first hypothesis, vocalisations were evoked by rhythmical stimulation of the bilateral VNs in implanted fish. Previous VN recordings during oyster toadfish grunts (Skoglund, 1961) and midshipman calls (Bass and Baker, 1990) show that the two bilateral VNs fire in phase. Therefore, we applied a stimulation pattern to the VNs that was bilaterally in phase. Rhythmic bilateral VN stimulation resulted in sound that consisted entirely of harmonic waveforms in all individuals (N=5). Fig. 1B shows the recordings of three representative individuals. Phase-space representation of the sound waveform shows a two-dimensional limit cycle that gradually grows in amplitude (i.e. spirals outwards) to the final maximal amplitude oscillation (ST3–5 in Fig. 1C). The prolonged presence of sound after stimulation is due to dissipating acoustic energy in the
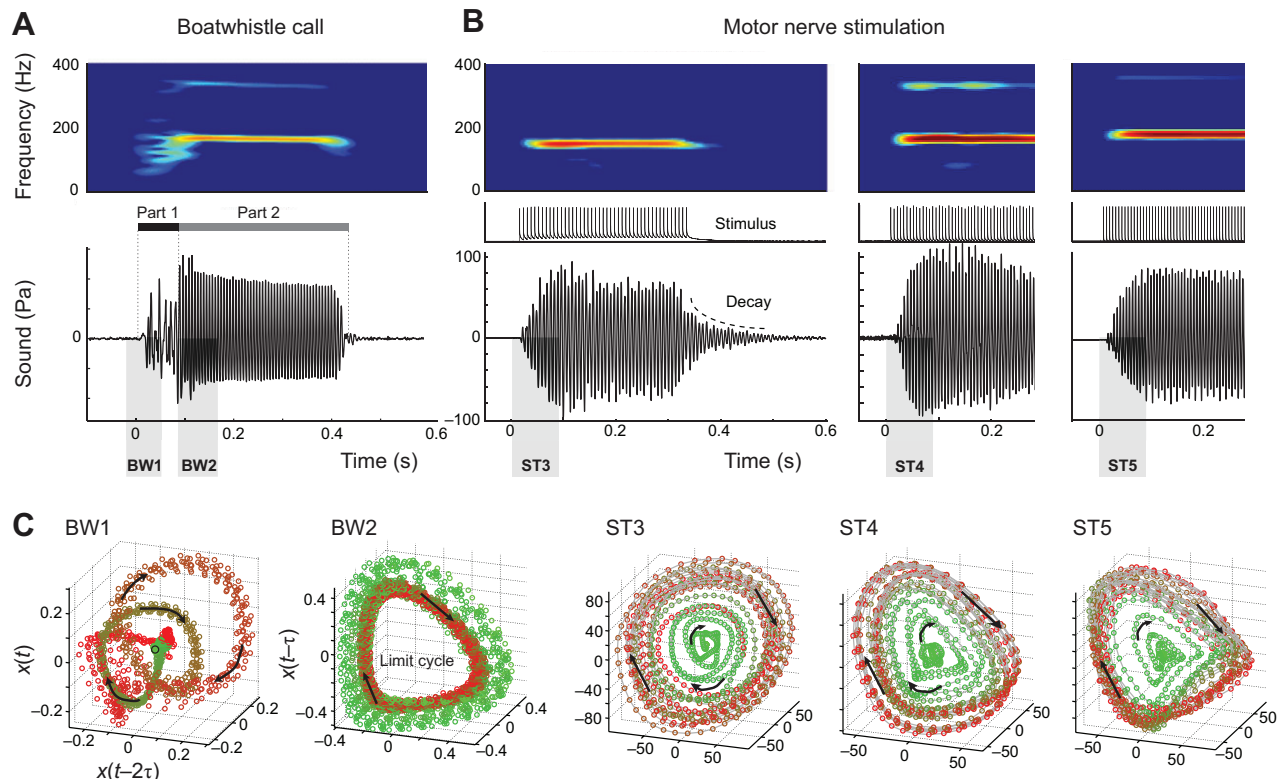
Fig. 1. The male oyster toadfish (*Opsanus tau*) advertisement call, or boatwhistle, contains irregular and harmonic components. (A) Representative spectrogram (*nfft*=1024, d.f.=3.0 Hz, dt=0.5 ms) and oscillogram of a spontaneous boatwhistle call recorded in saltwater ponds (20°C). The sound waveform of the boatwhistle consists of a 50–100 ms irregular waveform (part 1, P1) followed by a harmonic waveform (part 2, P2). (B) Representative spectrograms (*nfft*=1024, d.f.=5.9 Hz, dt=0.33 ms, dynamic range 20 dB) and oscillograms of sounds generated by rhythmic, synchronous stimulation of the bilateral motor nerves in three individuals at 150, 165 and 175 Hz, respectively (23.0°C). The sound waveforms do not contain an irregular P1. (C) Phase-space plots (τ=1 ms) of five segments as indicated by light grey areas in A and B. BW1: although the oscillogram of P1 seems irregular, the phase-space plot reveals a robust underlying attractor (only 10 calls overlaid for clarity). BW2: the phase-space plot of P2's harmonic waveform reveals a limit cycle oscillation. ST3–5: the phase-space plots of sound during stimulation show that the sound amplitude increases regularly (spiral) and does not go through a differently shaped attractor as the spontaneous boatwhistle. Marker colour indicates progressing time (start, green; end, red).
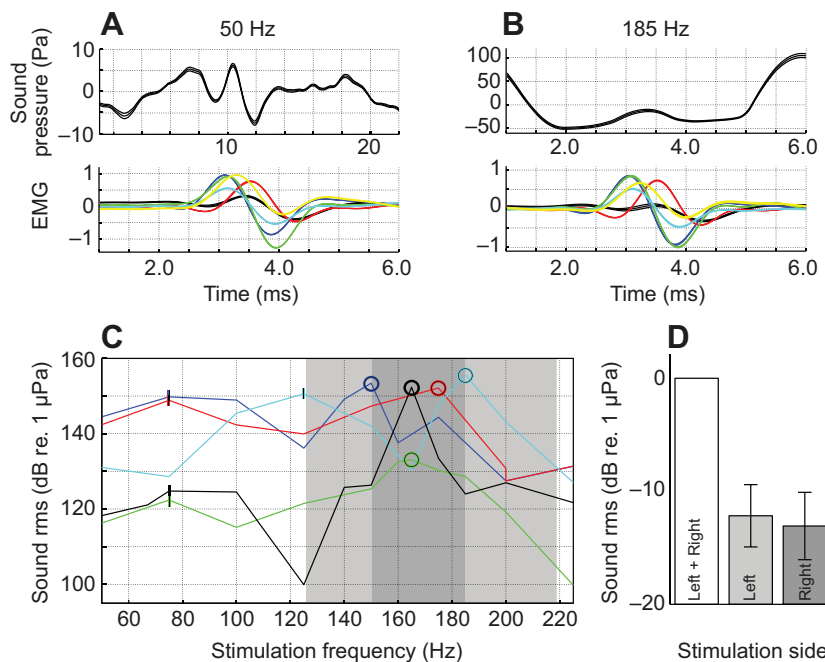
Fig. 2. Synchronous bilateral vocal motor nerve stimulation leads to consistent muscle fibre depolarisation and sound waveforms. Sound pressure (top) and normalised electromyography (EMG) traces (bottom, colour-coded for electrode position) during rhythmic bilateral stimulation of motor nerves at (A) 50 Hz and (B) 185 Hz from one oyster toadfish. Signals (means ± s.e.m.) are aligned to the first EMG maximum (blue trace). (C) Each individual fish has an optimal stimulation frequency where the radiated sound is maximal (N=5). The peak frequency range observed (dark grey area) falls well within the observed range of fundamental frequencies of boatwhistles recorded in the field (light grey area). (D) Radiated sound pressure decreases significantly (P<0.01, N=5) when stimulated unilaterally versus bilaterally. Sound pressure amplitude normalised to bilateral stimulation value.

tank ('Decay' in Fig. 1B). Rhythmic bilateral stimulation did not produce irregular P1 waveforms in any of the individuals tested (N=5).

Rhythmic bilateral VN stimulation resulted in depolarisations of the swimbladder muscle fibres at the six electromyography (EMG) electrodes over a wide range of frequencies. Fig. 2 shows two examples of the resulting sound pressure and muscle EMG for one individual stimulated for 300 ms at 50 Hz (Fig. 2A) and 185 Hz (Fig. 2B) at 23°C. At both frequencies, VN stimulation resulted in repetitive sound pressure and EMG patterns. Muscle depolarisations faithfully responded to each stimulation cycle up to 250 Hz, which is well within the range of natural vocalisations, while higher frequency stimulation (500 Hz) of the VNs resulted in skipped depolarisations.

SBM excitation resulted in harmonic sound pressure waveforms of which the amplitude varied with VN stimulation frequency and was highest between 150 and 185 Hz (Fig. 2C) at the temperature studied (23°C). Each individual fish demonstrated a slightly different optimal frequency where sound pressure was highest. The sound pressure produced ranged from 152 to 156 dB SPL re. 1 μPa except for one individual fish that produced its loudest call at 134 dB. A second, lower sound amplitude peak was observed around half of the optimal frequency, at 80–100 Hz for four out of

five fish and for one individual at 130 Hz. This lower amplitude peak was separated from the higher amplitude peak by a trough ranging from 8 to 25 dB. Unilateral VN stimulation at the individual's optimal frequency resulted in sound production, but caused the sound pressure amplitude to drop significantly (P<0.01, N=5) by 12.2±2.8 dB (left-sided) and 13.1±3.0 dB (right-sided) (Fig. 2D).

Subsequently, we explored whether the origin of P1 complexity was derived from asynchronous activation of the bilateral VNs, i.e. firing bilaterally out of phase. We measured sound pressure waveforms as a function of phase-lag between left–right stimulation (Fig. 3), and only observed regular waveforms and two-dimensional orbits in 3D phase-space at the onset of the sound waveforms (N=5). Together, these results show that rhythmic VN stimulation does not lead to complex P1 waveforms.

To determine whether complex P1 waveforms are caused by arrhythmic activity of the VN, the goal was to measure VN activity during boatwhistle production. However, the implanted fish did not produce spontaneous boatwhistles during the course of the experiments, but bilateral VMN activity was recorded during 105 grunts in two individuals. The acoustic energy generated by the grunts reverberates for up to 400 ms in the experimental tank. The left and right VNs recruit the SBMs in
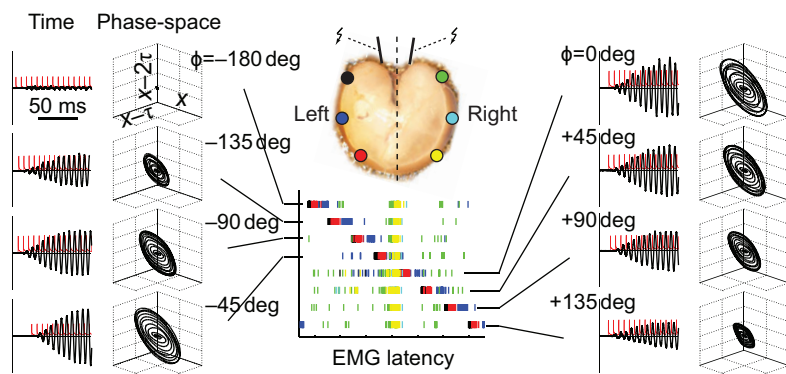
Fig. 3. Asynchronous bilateral vocal motor nerve stimulation does not evoke irregular sound waveforms. Shifting phase (φ) between bilateral VN stimulus trains leads to concurrent EMG latency shifts (central raster plot, with electrode positions colour-coded) but does not evoke irregular sound waveforms. Sound (black) and left VN stimulus (red) plotted in time and 3D phase-space (τ=5 ms). Note that at phase shift φ=180 deg, destructive interference cancels out the sound waveform.
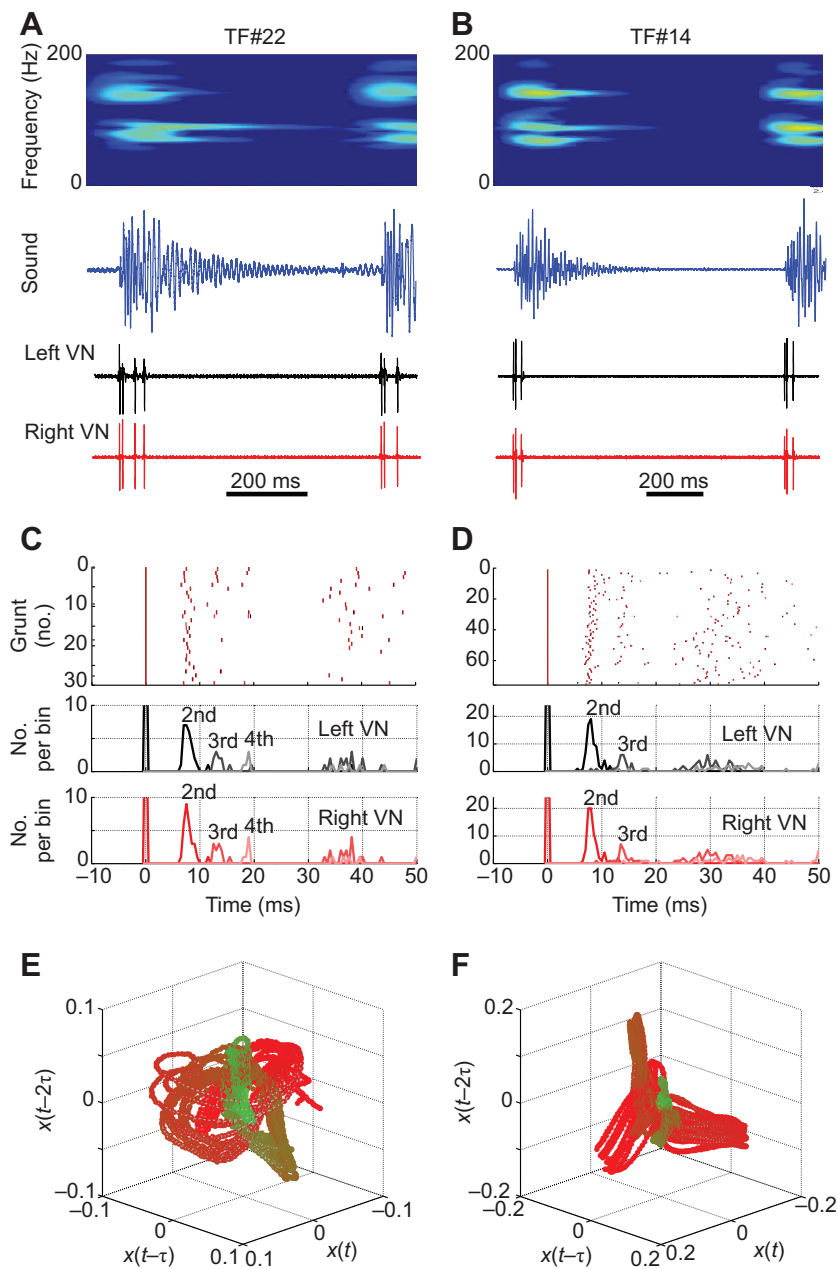
Fig. 4. Evoked grunts show in-phase left–right arrhythmic firing of the vocal nerves.
(A,B) Representative spectrograms, raw traces of sound and associated left and right vocal motor nerve (VN) activity in two oyster toadfish. (C,D) Rasterplot and histograms of VN activity aligned to the first spike. The left and right VN fire synchronously in all grunts (N=30 and N=75 for the two individuals, respectively). The spikes were summed in the histograms according to number in the sequence as indicated by the colour intensity of the lines of the first, second, third and fourth spikes. (E,F) Three-dimensional phase-space plots (τ=5 ms) of the sound reveal underlying attractors (only 10 calls overlaid for clarity) that are different for each individual. Marker colour indicates progressing time (start, green; end, red).

phase in both individuals (Fig. 4A,B). Fig. 4C,D shows VN spike timing histograms aligned to the first action potential. The peaks of these timing distributions indicate that the temporal spacing between the first and subsequent spikes for both left and right is 7.3, 13.0 and 19.0 ms for one individual (TF#22; Fig. 4C), and 8.0 and 14.0 ms for the other individual (TF#14; Fig. 4D). These timing distributions have considerable tails, especially for the third and fourth spikes, and therefore mean values are higher than the peak locations in the histograms. For TF#22, the mean ± s.d. (N=30) values of temporal spacing were 7.9±0.9, 28.1±13.0 and 29.1±13.8 for the second, third and fourth spikes, respectively. For TF#14, the mean ± s.d. (N=75) values of temporal spacing were 8.9±4.1 and 25.2±8.5 for the second and third spikes, respectively. The temporal spacing of the spike timing peaks is not at regular intervals and therefore reflects an arrhythmic recruitment of the superfast vocal muscles. The spectrograms show similarities in spectral structure compared with the boatwhistle P1 (Fig. 1A).

However, although the sound waveform is irregular, overlaid phase-space plots of the sounds reveal a consistently shaped three-dimensional attractor for each individual (Fig. 4) indicative of a low-dimensional chaotic signal.

DISCUSSION
There are three primary conclusions that can be drawn from the results of our study. First, spontaneous boatwhistle vocalisations of oyster toadfish start with a complex sound signal. Second, rhythmic stimulation of vocal motor nerves (VNs) does not elicit the emergence of such complex signals in underwater vocalisations (Fig. 1), but results in highly rhythmic sound waveforms (Fig. 1B,C, Fig. 2A, Fig. 3). Third, arrhythmic VN volleys, and therefore arrhythmic SBM recruitment, is correlated with complex sound waveforms in spontaneous grunts (Fig. 4). Thus the data support the hypothesis that complex signals in the P1 of the boatwhistle are not due to emergent nonlinearities of the peripheral sound-producing

system, and most likely find their origin in arrhythmic motor volleys generated in the central nervous system.

Toadfish tolerate electrode implantation well and as the anaesthesia effects dissipate within 1 h, they often resume normal behaviour within 12–24 h of surgery (Palmer and Mensinger, 2004). Although 'tethered' by the electrode wires, the preparation allowed investigation of an intact system in a freely swimming animal. Rhythmic VN stimulation volleys up to 300 Hz showed no irregular P1 waveforms (Figs 1, 2), which contrasts with previous a report on toadfish removed from the water where the first few cycles of contraction were partially fused before the muscle exhibited regular contractions at frequencies over 200 Hz (Fine et al., 2001). At the onset of nerve stimulation bursts, we observed a gradual increase of sound amplitude in all fish during the first six to 10 stimuli (Fig. 1B,C). This gradual increase could be explained by tank resonances during constant intensity sound production, or by increasing intensity of sound production. We deliberately chose small tank dimensions to avoid acoustic resonances as much as possible, but they cannot be entirely eliminated due to the complications of small tank acoustic fields. The constant EMG amplitude during stimulation suggests that the muscle recruitment and thus sound production is constant. Therefore, additional work is necessary to resolve this point. However, the above phenomena emphasise the importance of studying the intact system underwater, especially when looking at irregular waves, because aerial exposure decreases acoustic loading and the bladder's damping ratio.

The toadfish swimbladder produces sound only when driven by muscle contraction. Each contraction causes bladder displacement (Fine et al., 2001; Skoglund, 1961), radiating a pressure sound waveform that is repetitive during rhythmic stimuli (Fig. 2A). Because superfast muscles trade off force for speed (Rome et al., 1999) and energetic cost (Rome, 2006), the design for high-frequency contractions results in reduced muscle power output (Young and Rome, 2001) and lower acoustic power radiated. The acoustical power radiated as a function of stimulation frequency (Fig. 2C) appears to have two maxima for each fish. However, because we have a limited number of data points for each individual, we cannot test statistically whether the distribution is bimodal. The first peak corresponds to the peak frequency of grunts, which is around 90 Hz (Maruska and Mensinger, 2009). The second peak has also been observed during stimulation in air (Fine et al., 2001) and ranges from 150 to 185 Hz (Fig. 2C), which lies within the natural range of boatwhistles (80–219 Hz) (Edds-Walton et al., 2002; Fine, 1978). We suggest that these peaks do not represent resonance properties of the swimbladder, but are likely due to the interaction of power generation by superfast SBMs and mechanics of the bladder. To parse out the origin of these sound production optima, we need to conduct work-loop experiments of isolated SBMs at the full range of fundamental frequencies found in boatwhistles.

An irregular P1 is also observed in other batrachoidids, such as the Lusitanian toadfish (*Halobatrachus didactylus*) (Amorim and Vasconcelos, 2008) and the gulf toadfish (*Opsanus beta*) (Thorson and Fine, 2002). Midshipman fish, however, do not exhibit a P1 in their calls (Rice et al., 2011). Of all batrachoidids, the motor control networks in midshipman fish are the best known and considered conserved amongst the batrachoidids as well as vocal tetrapods (Bass et al., 2008). In midshipman fish, a vocal pacemaker nucleus (VPN) functions as the frequency pacemaker network driving the vocal motor nucleus and VN activity, while call duration is set by a vocal pre-pacemaker network that gates and/or modulates VPN activity for the duration of a call (Chagnaud et al., 2011). In toadfish, mid- and hindbrain stimulation evoked a wide range of

vocalisations, of which some examples seem to contain a P1 (Fine, 1979), but no simultaneous VN recordings were presented, which would be required to make firm conclusions about the origin of those irregularities.

Although we cannot ensure in toadfish that motor volleys during grunts are similar to those during P1 of the boatwhistle, we predict that the VPN generates a motor pattern during the boatwhistle that is (1) bilaterally in phase and (2) at first arrhythmic (during P1), as during spontaneous grunt recordings, and subsequently changes into a rhythmic motor volley (during P2). It is unknown what mechanism underlies the generation of the arrhythmic vocal network activity and what causes the transition from arrhythmic (P1) to rhythmic (P2) network activity during the oyster toadfish boatwhistle. We speculate that the origin resides in the VPN network, behaving as a nonlinear oscillator that bifurcates from an arrhythmic to rhythmic oscillation, driving the vocal motor nucleus and subsequently the motor nerves. Local temperature manipulation (Long and Fee, 2008) of the VPN and pre-pacemaker nucleus could separate their respective roles in setting the temporal dynamics of the network.

Exploiting peripheral dynamics of vocal organs has been suggested to add complexity in tetrapod vocalisations that could function as a unique signature of individuality to the voice of its producer (Fitch et al., 2002). Although the behavioural significance of P1 is not known, acoustic parameters of the P2 of the boatwhistle, such as pulse rate and duration, have been suggested to signal the physiological condition of the animal (Remage-Healey and Bass, 2005; Vasconcelos et al., 2012) as well as male fitness (Sisneros et al., 2009). Because the P1 of the boatwhistle seems to be neurally encoded, we speculate that the irregular, but repeatable, P1 may also encode a robust and thus consistently recognisable individual signature. We speculate that P1 and P2 could hence have separate functions behaviourally, akin to the two-part call of coqui frogs (Narins and Capranica, 1978).

The biomechanics of motor systems dictates strong constraints on neural control limits. The performance demands on motor systems associated with sound production and modulation have selected for the evolution of extreme superfast muscles (Elemans et al., 2011; Elemans et al., 2008; Elemans et al., 2004; Rome, 2006; Rome et al., 1996; Young and Rome, 2001) and highly synchronised and specialised motor neurons (Chagnaud et al., 2012). Our results show that complex signals, even in a simple sound-producing organ, can emerge from reliable neural inputs. It is therefore possible that complex vocal signals across vertebrates are driven by neural rather than biomechanical complexity. If complex signals indeed convey behaviourally relevant information, then neurally encoded acoustic complexity may be a common feature in vertebrate vocal systems.

MATERIALS AND METHODS
Subjects
Adult male oyster toadfish (*Opsanus tau*) were obtained from the Marine Biological Laboratory (MBL; Woods Hole, MA, USA) and maintained in large, flow-through seawater tanks at 20°C. All experimental procedures were conducted at MBL and conformed to institutional animal care protocols.

Outdoor recordings
Toadfish were placed in a 20×20 m saltwater pond at Woods Hole Oceanographic Institution and provided with artificial habitat. Sound was recorded with a Brüel & Kjær hydrophone (type 8103) and digitised at 10 kHz (ADInstruments). Waveforms were 50–500 Hz band-pass filtered with a second-order Butterworth filter.

The theory of nonlinear dynamics established that the behaviour of a dynamical system depends on the geometry of the underlying attractor

(Strogatz, 1994). The most common low-dimensional attractors found in voiced sound production by vertebrates are fixed point (a constant value), limit cycle (periodic oscillations, such as pure tones and harmonic stacks), torus (e.g. amplitude modulated sounds) and chaotic attractors (nonperiodic oscillations such as 'noisy' sounds) (Fitch et al., 2002; Wilden et al., 1998). These attractors leave characteristic signatures when embedded in phase-space representation of the sound amplitude time series that allow for unambiguous identification (Elemans et al., 2010). Three-dimensional phase-space plots of the sound waveforms were reconstructed from the time series $x(t)$ by time-delayed embedding it in a three-dimensional phase-space: $[x(t), x(t-\tau), x(t-2\tau)]$, with τ typically in the order of one-tenth of the period [for a detailed explanation, see Elemans et al. (Elemans et al., 2010) and Fitch et al. (Fitch et al., 2002)].

Surgical procedure and electrode design

Fish were anaesthetised by immersion in 0.001% MS222 (ethyl 3-aminobenzoate methanesulfonate; Sigma Chemical Co., USA) in seawater and placed ventral side up in an acrylate stereotactic tank with the anaesthetic solution continuously recirculated through the buccal cavity (Palmer and Mensinger, 2004). The swimbladder was exposed, and stimulation electrodes were sutured bilaterally into the vocal motor nerves ~15 mm from their entry into the SBM. Two bare platinum wire (75 μm, A-M Systems, WA, USA) hook electrodes spaced 1 mm apart terminating onto lead-out, multi-strand wire served as stimulation electrode. Three EMG electrodes were inserted 10–15 mm apart into the lateral side of each SBM, and were secured with sutures and cyanoacrylate glue to the muscle fascia. EMG electrodes consisted of double twisted-wire Teflon-coated stainless steel wire (A-M Systems) 75 cm long and 50 μm in diameter, with 1 mm of coating removed at the tip. A chlorided silver ground electrode was sutured onto the rostral side of the abdominal wall.

Electrodes were inserted to stimulate and record from the bilateral motor nerves and swimbladder muscles in 11 individuals [29.9±1.2 cm standard length (mean ± s.e.m.); 530±69 g wet mass]. We attempted to record EMG and VN activity during spontaneous or evoked grunts from four animals. We followed procedures as above, but only one pair of EMG electrodes was inserted on each side of each SBM tested.

Recording setup

Following surgery, fish were transferred to a 51×25×30 cm glass tank containing seawater (23±0.5°C). An opaque 20×25 cm (diameter × height) PVC cylinder was placed in the rear of the tank to provide habitat and ensure the fish were in the same position and orientation during experiments. The small size of the tank reduces the amount of interference of sound pressure waves (Okumura et al., 2002). The tank was placed on a vibration isolation table surrounded by a 1×1×1 m Faraday cage padded with 2 inch (5.08 cm) thick Sonofoam sound absorption material and four LENRD bass traps (Auralex Acoustics, Indianapolis, IN, USA).

Sound pressure was recorded with two Brüel & Kjær hydrophones (model 8103) and one HTI hydrophone (model 643002, Hydroacoustic Technology Inc., WA, USA). The Brüel & Kjær hydrophones were fixed parallel, 20 mm apart, in an acrylic frame and positioned 9 cm from the bottom, and 7 cm from the front in the tank. Temperature was logged at 10 s intervals with an S-TMB-M002 temperature probe (accuracy <±0.2°C) and Hobo data logger U14-002 (Onset Technologies, MA, USA).

Signal acquisition and conditioning

EMG electrodes were connected to a pre-amplifier (Grass P511-J). EMG signals were band-pass filtered (1 Hz to 3 kHz) and amplified 100–500 times. Hydrophone signals were band-pass filtered (10 Hz to 3 kHz) and amplified 1000 times (A-M Systems model 1700). All signals were digitised at 20 kHz (NI-6259 USB, National Instruments). Nerve stimulation was performed with a two-channel stimulator (Grass S88) and two stimulation-isolation units (Grass SIU5). All control, recording and analysis software was written in MATLAB (The MathWorks) and LabView (National Instruments).

Stimulation protocol and data analysis

Stimulation amplitudes were optimised independently for the left and right sides of the VN for maximal SBM EMG amplitude. All further stimulation experiments were performed with these settings. To investigate the effects of stimulation frequency, we used bilateral in-phase 300 ms duration stimulation trains at 50 through 250 Hz (25 Hz increments). To study the effects of unilateral stimulation, we applied 300 ms duration stimulation trains to the left and right VNs separately and simultaneously at the frequency with highest sound pressure output from the previous experiment. We applied 200 ms duration stimulation trains that were phase shifted by −180 deg through 180 deg (45 deg increments) to investigate the effects of out-of-phase bilateral stimulation. The sound pressure and EMG signals were low-pass filtered (1 kHz, sixth-order Butterworth filter) and segmented per stimulus in the stimulus train. Latency was defined as the time from stimulus onset to maximum EMG amplitude.

Evoked grunts and data analysis

Implanted fish did not produce spontaneous boatwhistles during the time frame of the experiments, but could be stimulated to produce grunts by holding them underwater in nets close to the hydrophones. The calls, associated EMG activity and VN compound action potentials were extracted. Hydrophone signals were high-pass filtered (20 Hz, sixth-order Butterworth filter) and power spectral density was estimated from 100 ms segments starting from the compound action potential of each call using the multi-taper method (Percival and Walden, 1993) with four data-tapers 8192 point fast Fourier transform (nfft).

Statistics

ANOVA tests were performed to test for difference of means. All data presented are means ± s.e.m. unless indicated otherwise.

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