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RESEARCH ARTICLE

A superfast muscle in the complex sonic apparatus of *Ophidion rochei* (Ophidiiformes): histological and physiological approaches

Loïc Kéver[1,*], Kelly S. Boyle[2], Branko Dragičević[3], Jakov Dulčić[3] and Eric Parmentier[1]

ABSTRACT

In teleosts, superfast muscles are generally associated with the swimbladder wall, whose vibrations result in sound production. In *Ophidion rochei*, three pairs of muscles were named 'sonic' because their contractions affect swimbladder position: the dorsal sonic muscle (DSM), the intermediate sonic muscle (ISM), and the ventral sonic muscle (VSM). These muscles were investigated thanks to electron microscopy and electromyography in order to determine their function in sound production. Fibers of the VSM and DSM were much thinner than the fibers of the ISM and epaxial musculature. However, only VSM fibers had the typical ultrastructure of superfast muscles: low proportion of myofibrils, and high proportions of sarcoplasmic reticulum and mitochondria. In females, each sound onset was preceded by the onset of electrical activity in the VSM and the DSM (ISM was not tested). The electromyograms of the VSM were very similar to the waveforms of the sounds: means for the pulse period were 3.6±0.5 and 3.6±0.7 ms, respectively. This shows that the fast VSM (ca. 280 Hz) is responsible for the pulse period and fundamental frequency of female sounds. DSM electromyograms were generally characterized by one or two main peaks followed by periods of lower electrical activity, which suggests a sustained contraction over the course of the sound. The fiber morphology of the ISM and its antagonistic position relative to the DSM are not indicative of a muscle capable of superfast contractions. Overall, this study experimentally shows the complexity of the sound production mechanism in the nocturnal fish *O. rochei*.

KEY WORDS: Fast muscle, Fish, Sound

INTRODUCTION

Sounds for communication purposes are known in many vertebrates and arthropods (Bradbury and Vehrencamp, 1998). In each case, sound production involves the vibration of body structures (Bradbury and Vehrencamp, 1998), always involving muscle activity. Superfast muscles evolved independently in several vertebrate and arthropod taxa (Rome et al., 1996; Josephson et al., 2000; Elemans et al., 2011). In vertebrates, these muscles are always associated with sound production and are known from some species of snakes (Rome et al., 1996), birds (Elemans et al., 2008), bats (Elemans et al., 2011), and fishes (Tavolga, 1964; Millot et al., 2011). The contraction rate of superfast muscles determines the fundamental frequency of the sound in fish (Skoglund, 1961; Fine et al., 2001; Millot et al., 2011), allows rapid modulations of sound characteristics in birds (Elemans et al., 2008), and sets the call rate in echolocating bats (Elemans et al., 2011). Rome et al. (Rome et al., 1996) also considered that rattlesnake tail shaker muscles are used 'to produce sounds at the frequency at which the muscle contracts'. Though these muscles are used to move the rattle, the last statement is questionable notably because other authors showed that the dimension of the proximal segment of the rattle determines sound frequencies (Young and Brown, 1995).

All vertebrate skeletal (locomotor and sonic) muscles are 'synchronous': each twitch is preceded by an activation potential (Josephson and Young, 1985; Josephson et al., 2000; Syme and Josephson, 2002) and Ca^{2+} must be released and re-sequestered by the sarcoplasmic reticulum to perform a contraction cycle (Rome et al., 1996). However, locomotor and fast sonic muscles differ in their design because the latter muscles manipulate lower masses at higher frequencies (Josephson et al., 2000; Rome, 2006; Elemans et al., 2008). To increase Ca^{2+} transient, superfast synchronous muscles generally have smaller muscle fibers that contain more sarcoplasmic reticulum and smaller myofibrils (Revel, 1962; Tavolga, 1964; Eichelberg, 1977; Fine et al., 1990; Fine et al., 1993; Josephson et al., 2000). Sonic muscles of *Opsanus tau* (toadfish), which are the most extensively studied, are also characterized by faster off-rates of Ca^{2+} from troponin, faster cross-bridge detachment rates, more Ca^{2+} pumps, more ATPases, and more parvalbumin (Appelt et al., 1991; Rome, 2006). Because locomotion generally requires more force than sound production, locomotor muscles have larger fibers with less sarcoplasmic reticulum and a larger proportion of myofibrils (Fine et al., 1990; Rome and Lindstedt, 1998). Consequently, force and speed are mutually exclusive in synchronous muscles: no vertebrate muscle can deliver a lot of force at very high frequency (Rome and Lindstedt, 1998). Results for sonic muscles of cicadas *Okanagana vanduzeei* (Josephson and Young, 1985), suggest that similar conclusions can be drawn for the synchronous muscles of insects. However, asynchronous (action potentials/twitches <1) muscles described in wing muscles of some insects have overcome this limitation: their fibers contain large proportions of myofibrils because they achieve high frequency twitches without high rates of Ca^{2+} cycling (Josephson and Young, 1985; Josephson et al., 2000; Syme and Josephson, 2002).

Physiology and histology of sonic muscles have been investigated in relatively few fish species (Fawcett and Revel, 1961; Tavolga, 1964; Gainer et al., 1965; Eichelberg, 1977; Fine et al., 1990; Fine et al., 2001; Connaughton, 2004; Parmentier and Diogo, 2006; Parmentier et al., 2006b). However, many studies have examined the functional morphology of sonic muscle fibers in *Opsanus tau* (Skoglund, 1961; Fine et al., 1990; Appelt et al., 1991; Fine et al., 1993; Rome et al., 1996; Loesser et al., 1997; Feher et al., 1998; Fine et al., 2001; Rome, 2006; Mitchell et al., 2008). This muscle is composed of thin fibers (ca. 20 µm in diameter) that are not completely tetanized at 500 Hz (Fine et al., 1990; Fine et al., 2001). Fast contracting sonic muscles were also described in *Pygocentrus nattereri* (Millot et al., 2011) and several holocentrid species (Gainer

[1]Laboratoire de Morphologie Fonctionnelle et Evolutive, AFFISH-RC, Institut de chimie, Bât. B6c, Université de Liège, B-4000 Liège, Belgium. [2]Département d'Ecologie et de Gestion de la Biodiversité, Muséum National d'Histoire Naturelle, 57 rue Cuvier, Case postale 55, 75231, Paris Cedex 5, France. [3]Institute of Oceanography and Fisheries, POB 500, 21000 Split, Croatia.

*Author for correspondence (loic.kever@ulg.ac.be)

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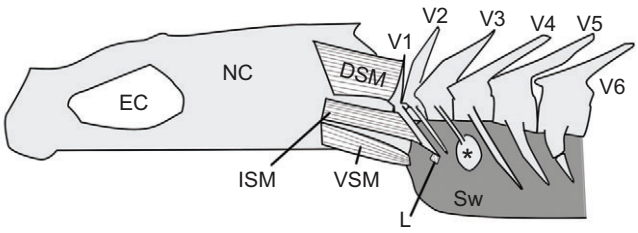

Fig. 1. Schematic representation of the sonic apparatus of female *Ophidion rochei*. *Swimbladder plate. DSM, dorsal sonic muscle; EC, eye cavity; ISM, intermediate sonic muscle; L, ligament; NC, neurocranium; Sw, swimbladder; V1–V6, vertebra 1–6; VSM, ventral sonic muscle.

et al., 1965; Parmentier et al., 2011). Again, superfast activity appears to be paralleled by the typical fast fiber morphology (Gainer et al., 1965; Eichelberg, 1977; Parmentier et al., 2011). In *O. tau*, *P. nattereri* and *Holocentrus rufus*, the fundamental frequency of the sound corresponds to the contraction rate of the sonic muscle (Fine et al., 2001; Millot et al., 2011). In *Carapus acus* (Carapidae), Parmentier et al. (Parmentier et al., 2006a) demonstrated that sonic muscles inserting on the swimbladder can also produce sounds at very low contraction rates (sonic muscle tetanized between 10 and 20 Hz). This example, however, involves important specializations of the sonic muscle and swimbladder: the sonic muscle has a hook that is attached to a small tubercle of the swimbladder wall at rest (Parmentier et al., 2006a), and muscle fibers and myofibrils have a unique helical disposition (Parmentier et al., 2003). Here, sound frequency is not determined by the contraction rate of the sonic muscle (Parmentier et al., 2006a).

Sonic muscles are present in many ophidiid species (Rose, 1961; Courtenay, 1971; Bowne, 1982; Carter and Musick, 1985; Parmentier et al., 2006b; Fine et al., 2007; Kéver et al., 2012). However, their physiology and fiber morphology is poorly documented: fiber diameters were measured in sonic muscles of *Ophidion barbatum* (Parmentier et al., 2006b) and a seasonal hypertrophy of a pair of sonic muscles was observed in *Lepophidium profondurum* (Nguyen et al., 2008). The present paper focuses on *Ophidion rochei* Müller 1845. Juvenile and adult *O. rochei* are characterized by three bilaterally paired sonic muscles (Kéver et al., 2012): the ventral sonic muscle (VSM), intermediate sonic muscle (ISM), and dorsal sonic muscle (DSM). The VSM originates on the neurocranium in all *O. rochei* but inserts on a mineralized structure (rocker bone) at the front of the swimbladder in males while in females (Fig. 1), it inserts directly on the swimbladder wall (Kéver et al., 2012). In both sexes (Fig. 1), the DSM and ISM originate on the neurocranium and insert on the modified first neural arch (neural rocker) and on the modified first epineural (wing-like process), respectively. A DSM contraction induces a dorsal anterior rotation of the neural rocker, which pulls the distal tip of the wing-like process backwards, the latter structure being connected to the swimbladder wall (or rocker bone in males) by ligaments (Parmentier et al., 2010b; Kéver et al., 2012). The ISM is considered to be an antagonist of the DSM.

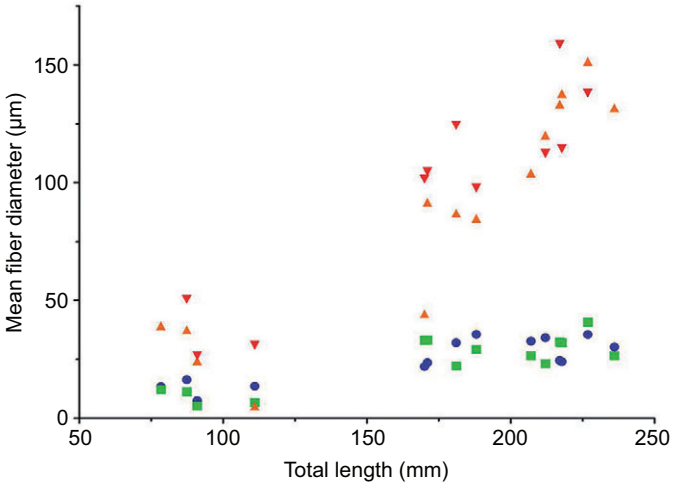

Fig. 2. Plot of the sonic and epaxial mean fiber diameters measured in 14 *O. rochei*. Fiber diameter (µm) of epaxial muscle (EM) and dorsal (DSM), ventral (VSM) and intermediate (ISM) sonic muscles plotted against total length (mm). EM: red inverted triangles; DSM: green squares; VSM: blue circles; ISM: orange triangles.

The multiple-pulsed call of males generally lasts several seconds and their pulse periods are ca. 120 ms (Kéver et al., 2012). Based on morphological data and male sounds, Parmentier et al. (Parmentier et al., 2010b) developed two different hypotheses to determine the action of the sound producing mechanism in *O. rochei*. The 'pulley' hypothesis proposed that the alternate contractions of the DSM and VSM are responsible for the two parts present in the waveform of each pulse (a low amplitude cycle followed by several high amplitude cycles). The 'bow' hypothesis suggests that a sustained contraction of the DSM during the whole call increases tension in the sonic apparatus while each contraction/relaxation cycle of the VSM produces each pulse. Both mechanisms do not require the use of fast sonic muscles. In contrast to male calls, female sounds are much shorter and tonal-like with a pulse period of ca. 4 ms (Kéver et al., 2012). This suggests that at least one sonic muscle should be able to contract very fast in females.

The aim of this paper is to give further insight on the sound production mechanism of *O. rochei* with an investigation of sonic muscle fiber morphology and activation patterns. This is the first study to experimentally demonstrate sound production based on more than one pair of swimbladder muscles in a group of fishes, meaning complex sound producing mechanisms are not restricted to higher vertebrates.

RESULTS

Histology

Muscle fiber diameter

Fiber diameters of the DSM, VSM, ISM and epaxial musculature (EM) were compared in juveniles, males and females. In each group, EM and ISM fibers were larger than VSM and DSM fibers (Table 1).

Table 1. Fiber diameter in different muscle types of *Ophidion rochei*

	VSM (µm)			DSM (µm)			ISM (µm)			EM (µm)		
	N	Mean	s.d.	*N*	Mean	s.d.	*N*	Mean	s.d.	*N*	Mean	s.d.
Juveniles	4	13	4	4	9	3	4	26	16	3	36	13
Males	2	30	11	2	32	2	2	67	31	2	102	1
Females	8	29	6	8	30	6	8	120	22	3	125	22

VSM, ventral sonic muscle; DSM, dorsal sonic muscle; ISM, intermediate sonic muscle; EM, epaxial musculature; *N*, number of fish sampled.

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The overall fiber diameter (regardless of muscle type) differed significantly between adults and juveniles [general linear model with repeated measures (rmGLM), F=36.2, d.f.=1, P<0.001]. Moreover, *post hoc* tests showed that fiber diameter of the four muscle types in juveniles was significantly smaller (P<0.001) than the fiber diameter in adult ISM and EM. In adults, the ISM and EM differed significantly (P<0.001) from the VSM and DSM. In juveniles, despite the apparent differences between these pairs of muscle types (Table 1), the *post hoc* tests found no differences (P>0.05) between the muscle types.

Linear regression for log-transformed fiber diameters against log-transformed total lengths (TL) (juveniles and adults) gave the following slopes: 1.01 for the VSM, 1.39 for the DSM, 1.79 for the ISM, and 1.57 for the EM. Although positive (>1) allometries were found for each muscle type, allometric growth was more pronounced in the EM and ISM. All together, these results showed that the four muscle types have relatively similar mean fiber diameters in juveniles while the mean fiber diameter in adults showed a pronounced dichotomy between the EM and ISM on one hand, and the VSM and DSM on the other (Fig. 2).

Muscle fiber ultrastructure

The ultrastructure of the ventral sonic muscle differed greatly from the three other muscle types in juveniles, males and females (Fig. 3). In the VSM, the most conspicuous characters concern the thickness of the band of sarcoplasm on the cell periphery that is not filled with myofibril packs (Fig. 3). This peripheral band is mainly occupied by mitochondria and small vesicles. In some cases, whorl bodies consisting of flattened or circular stacks of membranes that appear to be continuous with the sarcoplasmic reticulum were observed

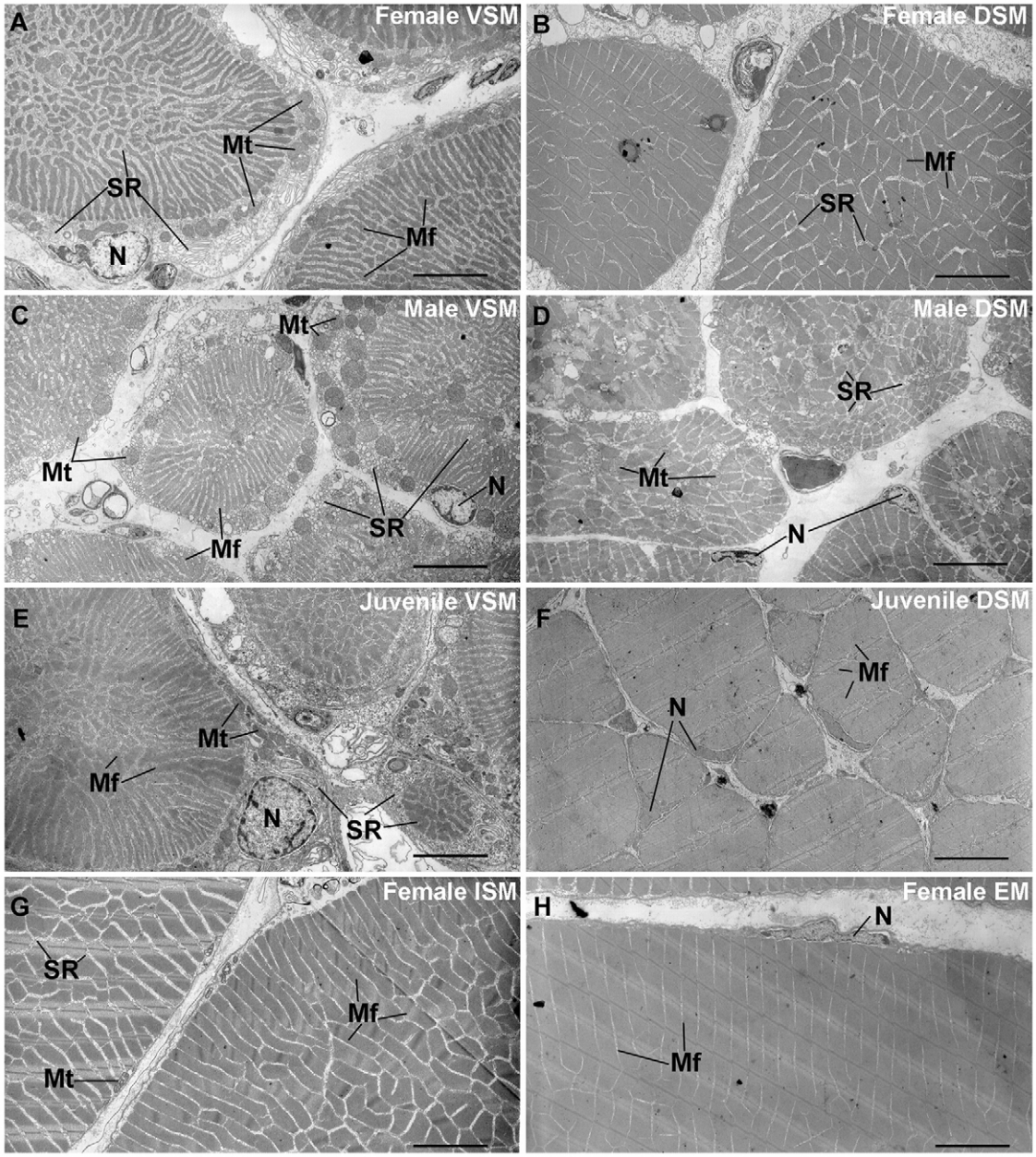

Fig. 3. Fiber ultrastructure of four types of muscles from *O. rochei*. (A) Ventral sonic muscle (VSM) of a female, (B) dorsal sonic muscle (DSM) of a female, (C) VSM of a male, (D) DSM of a male, (E) VSM of a juvenile, (F) DSM of a juvenile, (G) intermediate sonic muscle (ISM) of a female and (H) epaxial musculature (EM) of a female. Mf, myofibrils; Mt, mitochondria; N, nucleus; SR, sarcoplasmic reticulum. Magnification: ×2500. Scale bars: 5 µm.

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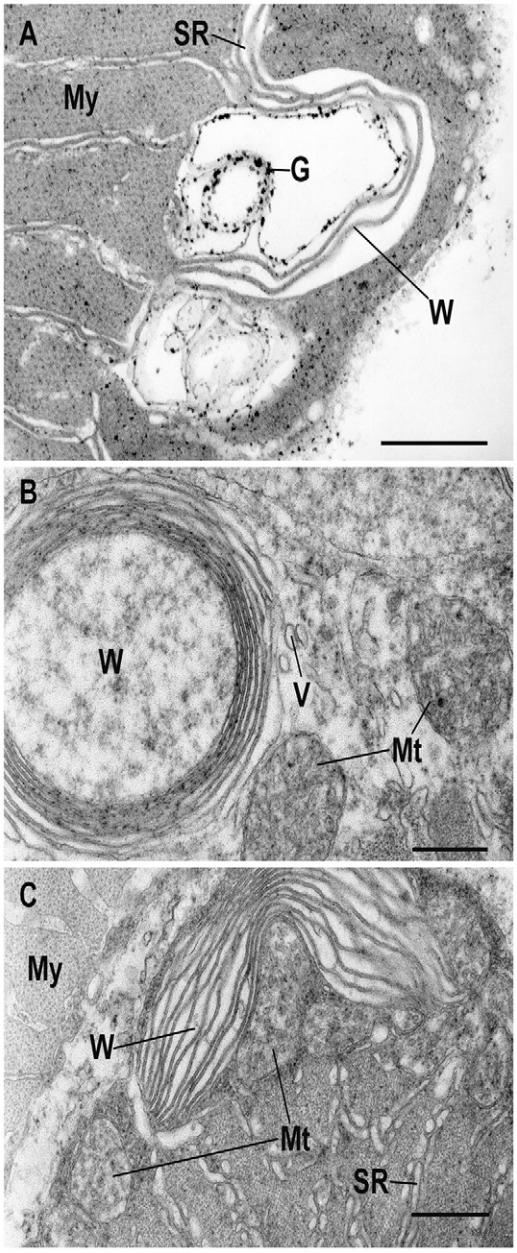

Fig. 4. Whorl bodies observed in ventral sonic muscles of *O. rochei*. (A) A whorl body and its connection with the sarcoplasmic reticulum (magnification: ×30,000). (B) A circular whorl body (magnification: ×25,000). (C) An elongated whorl body (magnification: ×25,000). G, glycogen granules; Mf, myofibrils; Mt, mitochondria; SR, sarcoplasmic reticulum; V, vesicles; W, whorls. Scale bars: 500 nm.

(Figs 3, 4). These membranes often contained densely stained granules and some of the whorl bodies had a central core of sarcoplasm (Fig. 4). These whorls were very rarely observed in other muscle types investigated.

Some other differences were observed between muscle types: (1) mitochondria were less dense and bigger in the DSM, ISM and EM than in the VSM, (2) the nuclei of the VSM and DSM were rounder than they generally are in other muscles (Fig. 3), (3) there was more space saved for sarcoplasmic reticulum between the myofibrils in the VSM (some DSM and ISM fibers also show more empty space than in the EM) than in other muscle types, and (4) some DSM fibers had many mitochondria between the myofibrils (Fig. 3D).

Electromyography

Trains of electromyograms (EMGs) were recorded from the VSM and DSM (Fig. 5). Action potential onsets in the VSM and DSM were always observed before the onset of sounds (mean latency was 6.5±3.1 and 11±3.9 ms, respectively). The latency between action potential and sound onsets was significantly (Wilcoxon test, $P<0.001$) shorter for the VSM than DSM. This implies that the DSM is activated before the VSM (Fig. 6).

The pattern of VSM EMGs of the four tested fish (Fig. 7) clearly showed that the activity of the VSM correlates with the occurrence of each pulse within the call (Table 2). We did not find significant differences (Table 3) between (1) the number of compound action potential peaks (7.6±2.5) and sound pulses (7.6±2.6), (2) the peak (3.6±0.5 ms) and pulse (3.6±0.7 ms) periods, and (3) the EMG (26.2±8.1 ms) and sound (25.9±7.8 ms) duration. Some differences, however, were observed between the fish: latency, for example, was almost two times longer in Fish 5 than in Fish 2.

The pattern of DSM EMGs differed significantly (Table 3) from sounds in many respects (Table 4, Figs 5, 7): (1) the number of compound action potential peaks (1.5±0.7) was lower than the number of sound pulses (6.6±2.4), (2) the peak period (12.5±6 ms) was longer than the pulse period (3.6±07 ms) and (3) mean EMG duration (26.3±18.5 ms) was longer than mean sound duration (22.4±8 ms). In addition, the DSM EMG pattern was more variable compared with the VSM EMG (Figs 5, 7): it was characterized by one or few pronounced peaks (always less than the number of pulses in the associated sound), followed by less intense electrical activity (oscillations just greater than electrical background noise). Peak period from compound action potentials was only measured for some DSM EMGs because a single peak was observed in 62% of the EMGs. In Fish 5, all the DSM EMGs recorded showed a single peak of high intensity. The number of peaks in DSM EMGs was significantly ($P<0.05$) but very weakly ($r=0.30$) correlated to the duration of the associated sound. The mean latency varied between fish: compared with Fish 3, latency is more than 1.5 times longer in Fish 1. Briefly, the DSM is activated before the sound production onset and its contraction appears to be sustained during the call. It is, however, important to note that the DSM was activated prior to each sound, indicating that these muscles are required to obtain calls.

DISCUSSION

This study provides the first experimental confirmation of a swimbladder sound production mechanism involving more than one pair of muscles in sound production in fishes, meaning that complex mechanisms are not restricted to higher vertebrates. Histological data clearly indicate that different kinds of muscles are found in the sound-producing apparatus of *O. rochei*. Fibers of the VSM and DSM were always thinner than in the EM and ISM, but this difference is more pronounced in adults. Similar differences were described in other teleosts (Fine et al., 1990; Millot and Parmentier, 2014). Thus, the functions of the VSM and the DSM in sound production probably necessitate conserving thin fibers in adults. However, differences at the ultrastructural level suggest that these two muscles differ in their functions.

Parmentier et al. (Parmentier et al., 2010b) formulated two hypotheses to explain the male sound characteristics. The ‘pulley’ hypothesis would require an alternate contraction of the VSM and DSM to form each pulse. The ‘bow’ hypothesis involves the sustained contraction of the DSM during the entire call to place the rocker bone under tension and a suite of rapid contraction/relaxation cycles of the VSM to create the successive sound pulses.

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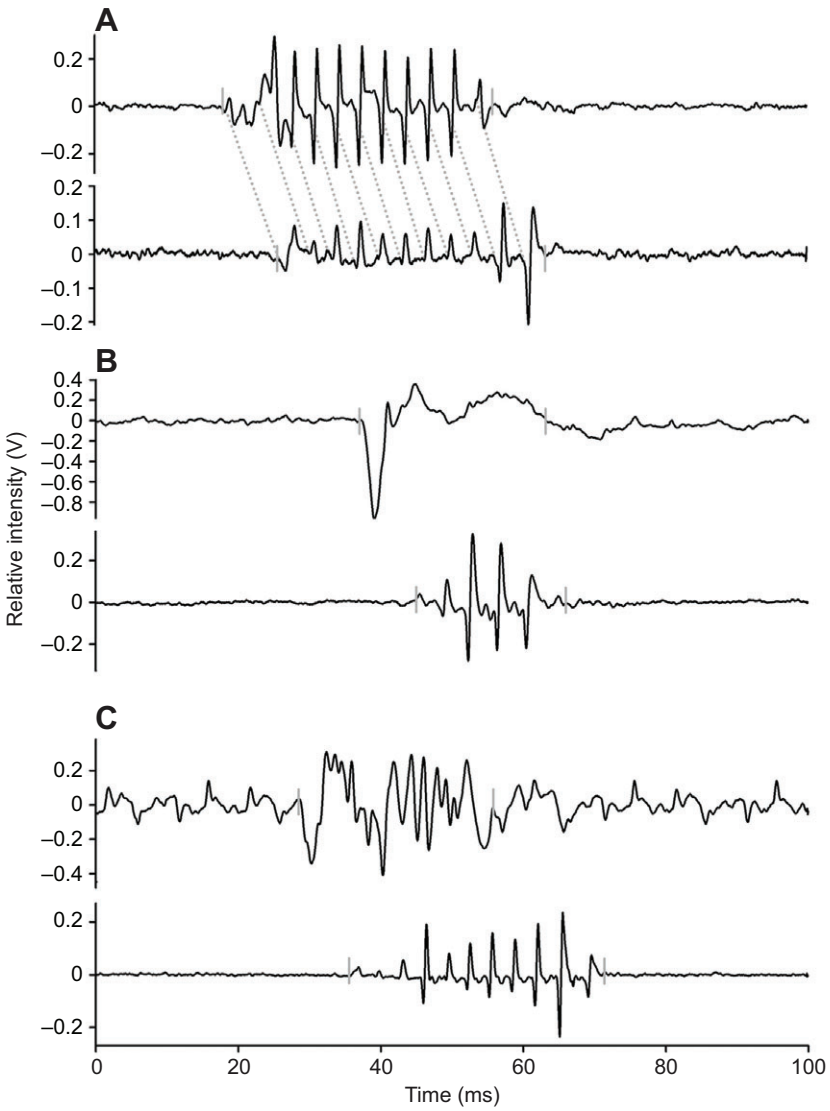

Fig. 5. Ventral and dorsal sonic muscle electromyograms (EMGs) and associated sounds. (A) A VSM EMG (top) and the associated sound (bottom). (B,C) Two DSM EMGs (top) and their associated sounds (bottom). The DSM EMGs illustrate the different patterns observed for this muscle. The gray lines show the onset and offset of each signal. The dotted grey lines show the corresponding pattern between the EMG and the sound.

In female *O. rochei,* EMGs of both the VSM and DSM support the bow hypothesis (Figs 5, 7). Each peak of the VSM electromyogram probably corresponds to muscle activation for separate contractions that produce each sound pulse. Because vertebrate muscles are synchronous (1 activation pattern:1 twitch), the short period in VSM EMGs indicates rapid muscle twitches. Based on mean action potential rate (period^{-1}), the VSM contracts at ~280 Hz during sound production at 23.5°C, placing these muscles among the fastest vertebrate muscles (Gainer et al., 1965; Fine et al., 2001; Elemans et al., 2008; Elemans et al., 2011). The cell ultrastructure of the VSM is consistent with this finding because fast-contracting muscles have a small fiber diameter (Tavolga, 1964; Fine et al., 1990; Parmentier and Diogo, 2006), a well-developed sarcoplasmic reticulum (Fawcett and Revel, 1961; Revel, 1962; Josephson and Young, 1985; Appelt et al., 1991; Schaeffer et al., 1996; Rome and Lindstedt, 1998; Syme and Josephson, 2002), a high proportion of space in the sarcoplasm (Millot and Parmentier, 2014), and numerous mitochondria (Rome et al., 1996; Schaeffer et al., 1996). The whorl bodies are generally continuous with sarcoplasmic reticulum and were more common in the VSM. These structures were also reported notably in fast sonic muscles of other fish species but their function is still unknown (Brantley et al., 1993; Loesser et al., 1997).

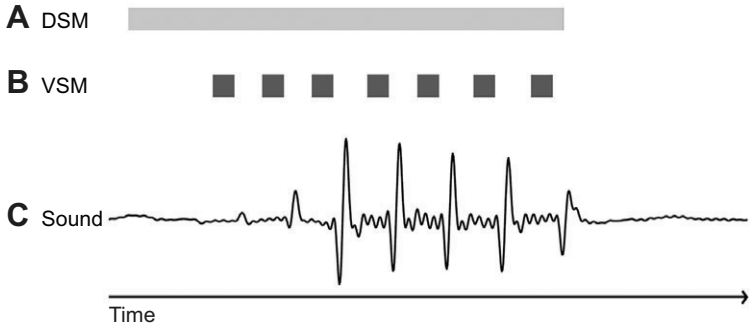

Fig. 6. Schematic representation of the hypothetical sound-producing mechanism of female *O. rochei*. (A) Schematic representation of the period of electrical activity in the dorsal sonic muscle (DSM) and (B) ventral sonic muscle (VSM). (C) The waveform of the associated female sound.

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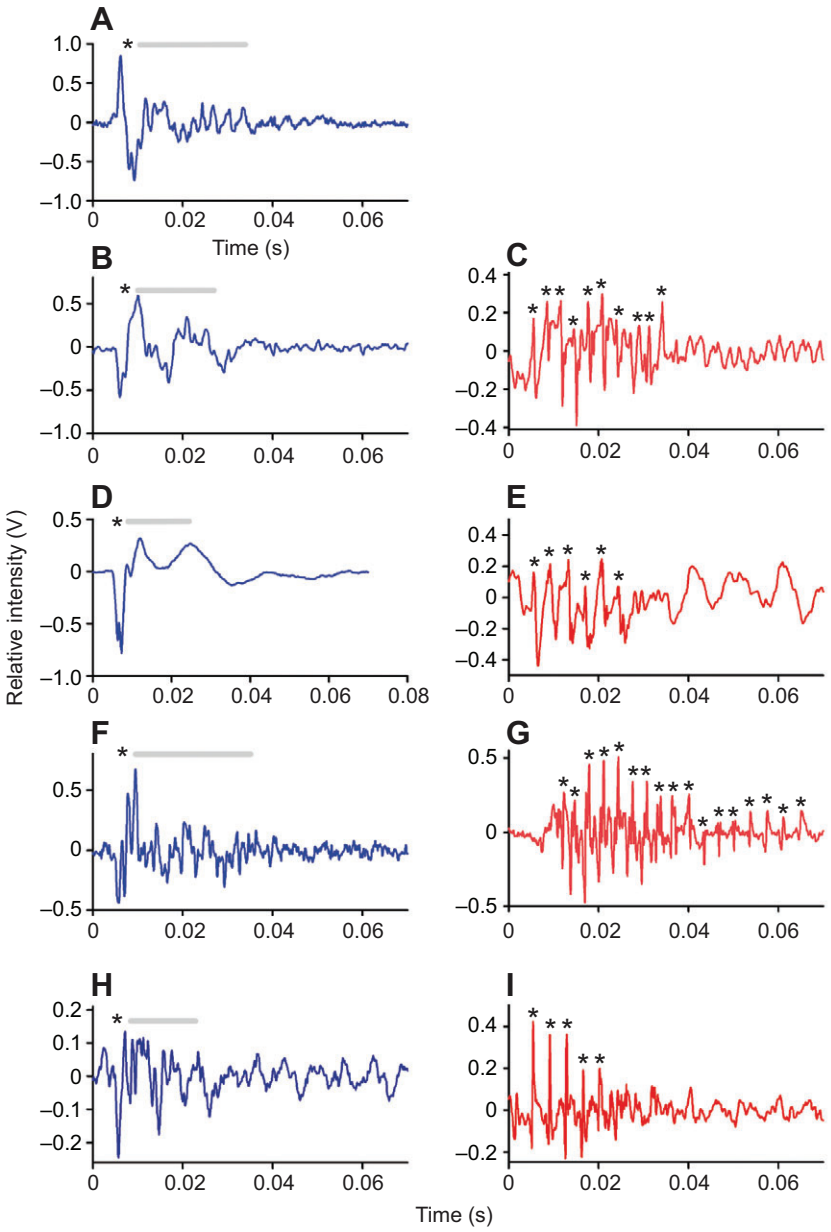

Fig. 7. Means of the ventral and dorsal sonic muscle electromyograms for each fish. EMGs were recorded in Fish 1 (A), Fish 2 (B,C), Fish 3 (D,E), Fish 4 (F,G) and Fish 5 (H,I). Mean traces are shown for the DSM (left) and VSM (right). EMGs were down-sampled at 22,050 kHz and band-passed at 3 kHz. *EMG peaks. The gray lines denote the period of activity that followed the first peak of DSM EMGs (a second peak was often observed during this period).

Electromyograms from the DSM were always characterized by a pronounced and short duration peak before the onset of sound production. Typically a second obvious peak was observed after the first one. The signal generally stayed slightly above the electrical background noise after each peak, which explains the relatively long duration obtained for DSM EMGs. Though the DSM EMG pattern differed from the superfast muscle pattern observed from the VSM, the pulse period (12.5±6 ms; see Table 4) of the pronounced peaks suggests a relatively fast contraction rate (ca. 80 Hz). However, the second activation potential may also happen before complete muscle relaxation, inducing partial tetany. The link between DSM activity and sounds is difficult to draw from EMG data because the number of EMG peaks differed from pulse number and the correlation between the DSM EMG peak number and sound duration was significant but very low. However, the DSM is clearly not responsible for the pulse rate of sounds. The DSM contraction is antagonistic to the VSM: it pulls the anterior part of the swimbladder caudally. The prior contraction of the DSM can have at least two effects. It may increase the tension at the VSM insertion on the swimbladder wall and consequently help its (VSM) relaxation. Moreover, stretching a muscle can increase the tension it delivers during its contraction (Brown, 1971). The role of the DSM would be to rapidly restore the position of the swimbladder after the VSM contraction.

The bow hypothesis was first developed to explain the male sound production mechanism. The question arises: is the sound production mechanism of males and females based on the same principle? The answer could be positive for parsimonious and comparative reasons. At juvenile stages, males and females have the same sound production mechanisms (Kéver et al., 2012). Sounds produced by juveniles are very similar to those of adult females (Kéver et al., 2012). The insertions and ultrastructures of the DSM and VSM are quite comparable in both sexes, indicating that these muscles should have similar roles. The differences are at the level of the swimbladder and epineurals (Kéver et al., 2012) with the VSM inserting on the rocker bone in males. This heavy mineralized structure derived from the anterior part of the swimbladder partially explains the substantial differences between male and female sound production. Sounds with

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Table 2. Relationship between sound features and ventral sonic muscle activity in four *O. rochei*

	N	Mean	s.d.
EMG peak number	55	7.6	2.5
EMG period (ms)	364	3.6	0.5
EMG duration (ms)	55	26.2	8.1
Latency (EMG onset−sound onset) (ms)	55	6.5	3.1
Sound pulse number	55	7.6	2.6
Sound period (ms)	358	3.6	0.7
Sound duration (ms)	55	25.9	7.8

EMG, electromyogram.

a high pulse rate were never recorded from mature males (Parmentier et al., 2010a; Kéver et al., 2012; Kéver et al., 2014). The lower pulse rate could be related to the rocker bone inertia or to differences in the rate of activation (an adaption to produce longer calls and favor source location?). According to Rome and Lindstedt (Rome and Lindstedt, 1998), force and speed are mutually exclusive in synchronous muscles: no vertebrate muscle can deliver a lot of force at very high frequency. The ultrastructure of male VSM suggests that the VSM is a fast muscle. In order to move the heavy rocker bone, males probably increase the VSM strength by adding fibers. This prediction is consistent with the present results and the large VSM observed in males (Kéver et al., 2012).

ISM action potentials were not recorded because fiber ultrastructure suggested no specialization. Their insertion on the first epineural suggests that their action is antagonistic to the DSM. The ISM could be active after a sound in order to return the swimbladder to its resting position. This muscle was considered as part of the sonic apparatus because it inserts on the first epineural, which is connected to the swimbladder in *O. rochei*. However, the ISM may not be involved in sound production because muscles that originate on the neurocranium and insert on the first ribs for locomotion are common in fish.

Conclusions

Histological and physiological data show that the VSM is probably the fastest of the three sonic muscles in *O. rochei*. In females, the fast VSM (ca. 280 Hz) is responsible for the pulse period and fundamental frequency of sounds. DSM fibers are activated prior to sound emission and muscle activity seems sustained over the course of the call, indicating that this muscle is required in sound production, at least by increasing the tension in the swimbladder.

In most teleost fishes that produce swimbladder sounds, two symmetric muscles are used to contract at a given rate, making stereotyped calls. This study experimentally shows that sonic mechanisms can be more complex in some fish species, suggesting the important role of sound production in communication. We highlight that the complexity occurs not only in structural organization (see Parmentier et al., 2010b; Kéver et al., 2012) but also involves the associated physiology. In this species, sounds are produced by the co-ordination of muscles that have differences in ultrastructure, contraction ability and neuronal motor patterns. Moreover, the comparison between males and females shows that the activation pattern (but not the ultrastructure) of VSM is sexually dimorphic. In males, the muscle does not make continuous fast contractions, but is active over long calls at a lower rate (though probably fast twitches).

The overall evidence suggests that the acoustic communication in Ophidiiformes that live mainly in deep and dark environments is complex. A good comprehension of the relationships between morphology, physiology and sound characteristics in shallow water Ophidiiformes will be crucial for future studies on less accessible species.

Table 3. Comparisons between EMG and sound characteristics

	DSM EMG sound			VSM EMG sound		
	N	*Z*	*P*-value	*N*	*Z*	*P*-value
Peak number	85	8.01	**<0.001**	55	0.66	0.507
Period	37	5.3	**<0.001**	343	1.66	0.098
Duration	85	2.2	**0.028**	55	0.27	0.789

Wilcoxon non-parametric tests. Significant *P*-values are bold (sequential Bonferroni correction). Peak number, number of peaks in the EMGs and number of pulses in their associated sounds; period, peak period in the EMGs and pulse period in their associated sounds; duration, duration of the EMGs and duration of their associated sounds; *Z*, critical values of the Wilcoxon tests for large sample sizes.

Table 4. Relationship between sound features and dorsal sonic muscle activity in five *O. rochei*

	N	Mean	s.d.
EMG peak number	85	1.5	0.7
EMG period (ms)	37	12.5	6.0
EMG duration (ms)	85	26.3	18.5
Latency (EMG onset–sound onset) (ms)	85	11.0	3.9
Sound pulse number	85	6.6	2.4
Sound period (ms)	473	3.6	0.7
Sound duration (ms)	85	22.4	8.0

MATERIALS AND METHODS

Histology

Samples from the VSM, DSM, ISM and EM of 14 *O. rochei* (four juveniles: 78−111 mm TL; eight females: 171−236 mm TL; two males: 170−188 mm TL) were fixed with glutaraldehyde (1%). These fish were sampled at different periods of the year (e.g. three females were sampled in May and five in September) but no details are given in the present paper because no clear effects on fiber diameter or ultrastructure were observed (this could be related to the small number of individuals sampled). After fixation, these samples were dehydrated in a series of ethanol-propylene oxide and embedded in epoxy resin (SPI-Pon 812).

First, semi-thin sections (0.5 µm) of muscles for the four juveniles (three for EM), the eight females (six for EM), and the two males were colored with toluidine blue (0.5% in a 1% borax solution), and photographed under a binocular microscope. For the 53 photographs, the mean diameters (d) of 25 randomly selected fibers (three homemade grids with 25 dots placed at the intersection of the grid and used randomly) were calculated using fiber areas [$d=2\sqrt{(A/\Pi)}$] measured in Adobe Photoshop CS4 (Adobe, San Jose, CA, USA).

Second, ultrathin sections (60–80 nm) were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (JEOL JEM 100SX) under an 80 kV accelerating voltage. This allowed for a qualitative description of fiber ultrastructure.

Electromyography

Five female *O. rochei* (no live males were available) were tested in order to describe the activity of their DSM and VSM: FISH 1 (133 mm TL), FISH 2 (143 mm TL), FISH 3 (150 mm TL), FISH 4 (166 mm TL) and FISH 5 (242 mm TL). These fish were held in a 280-liter tank fed with seawater at 23.5°C (15 h:9 h light:dark cycle).

Each fish was anesthetized with MS 222 (200 mg l^{-1}). Bipolar electrodes were placed with 27.5-gauge hypodermic needles in the DSM and VSM on one side of the fish (both sides were tested and no lateralized behavior was observed). Electrode wires were secured to the dorsal fin with a suture and cyanoacrylate glue. Then the fish was ventilated with oxygenated seawater and placed in a small net in the middle of the holding tank.

Bipolar electrodes were prepared as described by Parmentier et al. (Parmentier et al., 2013). The signal obtained from these electrodes was amplified 10,000 times, bandpassed (100−10,000 Hz), and notched filtered (50 Hz) with a differential amplifier (AM Systems model 1700, Sequim, MA, USA). It was then digitized with a USB sound card (Creative model SB0270, Creative Labs, Singapore) and recorded at a sampling rate of 44,000 Hz in Adobe Audition 2.0 software.

Simultaneously, sounds were recorded with an Orca hydrophone (sensitivity −186 dB re. 1 V μPa^{-1}) connected to a Tascam HD-P2 stereo audio recorder (Wiesbaden, Germany). Line output from the audio recorder was connected to one channel of the USB sound card instead of one of the two electrodes after each sound recorded to allow manual synchronization in Adobe Audition 2.0. In some cases one electrode came out of the fish, which explains the difference in the number of EMGs recorded for the DSM and the VSM. In such situations, the output line of the audio recorder was continuously placed into one of the channels of the USB sound card. Thus sounds and EMGs were automatically synchronized.

EMG and sound recordings were both downsampled at 22,000 Hz and manually investigated in Avisoft-SAS Lab Pro version 4.33 software (Avisoft Bioacoustics, Glienicke, Germany). For each signal, peak period (called pulse period for sounds), number of peaks (called pulse number for sounds), and signal duration (called EMG duration for EMGs and sound duration for sounds) were investigated. In addition, the latency between the EMG onset and sound onset was measured. Note that the background noise was observed long before and after the signal.

After the EMGs were performed, one fish was radiographed in ventral and dorsal views and two specimens were dissected with caution to confirm electrode location.

Statistical analyses

Fiber diameter

The normality of variables was investigated with Kolmogorov–Smirnov tests. A general linear model with repeated measures was performed to compare mean fiber diameter (dependent variable) obtained for the different muscle types (repeated measures). The variable ‘groups’ was selected as a fixed factor. In this variable ‘groups’, adults and juveniles were represented by two different codes. Tukey’s honest significant difference *post hoc* tests allowed for comparisons between the two groups and between the four muscle types.

Mean fiber diameter obtained for each muscle type in fish was log-transformed and plotted against log-transformed total length. Slopes obtained from the linear regression were used to investigate allometries in fiber growth.

Electromyogram and sound data

The normality of the variables was tested using Kolomogorov–Smirnov tests. The non-parametric Wilcoxon test was used to compare periods, durations and number of peaks measured on EMGs and the sounds. Alpha levels were adjusted with a sequential Bonferroni correction (Rice, 1989). The Wilcoxon test was also use to compare the VSM EMG sound latency to the DSM EMG sound latency. In the latter case, only sounds for which the VSM and DSM were simultaneously recorded were tested. All statistical tests were performed in STATISTICA 10 (StatSoft Inc., Tulsa, OK, USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.K., K.S.B. and E.P. conceived and designed the experiments. B.D. and J.D. collected the fish. L.K. carried out the experiments, analysed the data and wrote the manuscript. K.S.B., E.P., B.D. and J.D. revised the manuscript. E.P. gave final approval for submission.

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