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Characterization of the primary sonic muscles in *Carapus acus* (Carapidae): a multidisciplinary approach

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Sound production in carapid fishes results from the action of extrinsic muscles that insert into the swim bladder. Biochemical, histochemical and morphological techniques were used to examine the sonic muscles and compare them with epaxial muscles in *Carapus acus*. Sonic fibres are thicker than red and thinner than white epaxial fibres, and sonic fibres and myofibrils exhibit an unusual helicoidal organization: the myofibrils of the centre are in a straight line whereas they are more and more twisted towards the periphery. Sonic muscles have both features of red (numerous mitochondria, high glycogen content) and white (alkali-stable ATPase) fibres. They differ also in the isoforms of the light chain (LC3) and heavy chain (HC), in having T tubules at both the Z-line and the A–I junction and in a unique parvalbumin isoform (PAI) that may aid relaxation. All these features lead to the expression of two assumptions about sound generation: the sonic muscle should be able to perform fast and powerful contractions that provoke the forward movement of the forepart of the swim bladder and the stretching and 'flapping' of the swim bladder fenestra; the helicoidal organization allows progressive drawing of the swim bladder fenestra which emits a sound when rapidly released in a spring-like manner.

Keywords: sonic muscle; Carapidae; helix; parvalbumin; myofibrils; ATPase activity

1. INTRODUCTION

Many fish species have developed mechanisms allowing them to emit species-specific sounds (Hawkins 1993; Carlson & Bass 2000; Fine *et al.* 2001). One of these sound-producing mechanisms is the result of swim bladder vibration due to the action of specialized muscles. These muscles are extrinsic when they attach to the swim bladder and an external element pertaining to the bladder in various Ophidiiformes (Howes 1992), Holocentridae (Carlson & Bass 2000) or Sciaenidae (Ono & Poss 1982; Connaughton *et al.* 1997; Sprague 2000). The action of these muscles induces a production of sounds with a fundamental frequency ranging from 100 to 300 Hz. This value corresponds to the muscular contraction speed, placing them among the fastest muscles present in vertebrates (Loesser *et al.* 1997; Fine *et al.* 2001). This characteristic, coupled with their ability to support activity over long periods (Fine *et al.* 1990), results from numerous morphological and biochemical adaptations such as the specialization of protein isoforms (Hamoir & Focant 1981; Huriaux *et al.* 1983) and the high concentration of intracellular components (Pennypacker *et al.* 1985; Rome *et al.* 1999).

The fibres and myofibrils of sonic muscles are thinner (Evans 1973; Fine *et al.* 1993), and possess a more developed sarcoplasmic reticulum (Hamoir *et al.* 1980; Hamoir & Focant 1981; Feher *et al.* 1998) than the fast white fibres (Eichelberg 1976). This set of characteristics could facilitate rapid flows of metabolites and calcium (Eichelberg 1976; Fine *et al.* 1990; Feher *et al.* 1998). Moreover, a sufficient energetic inflow is supplied by abundant mitochondria and a high glycogen content (Ono & Poss 1982; Fine *et al.* 1993; Connaughton *et al.* 1997). Fast contractions of sonic muscles could also be associated with the parvalbumins acting as the releasing factor that binds calcium ions before sarcoplasmic reticulum re-accumulation (Gillis 1985). The sonic muscles of *Opsanus tau* contain the highest parvalbumin concentration ever measured (Hamoir *et al.* 1980; Feher *et al.* 1998).

The interest generated in pearlfish (Carapidae) results from the ability of certain species to live inside different invertebrate hosts (Parmentier *et al.* 2000). The species belonging to this family possess extrinsic muscles: primary and secondary sound-producing muscles. The first group originate on the orbital roof and insert into the anterior wall of the swim bladder. The second group originate on the epiotic and insert into the distal portion of the first and second epipleural ribs (Parmentier *et al.* 2002). In *Carapus boraborensis*, *Carapus homei* and *Encheliophis gracilis*, the contraction of the first muscle creates the production of a species-specific sound (Parmentier *et al.* 2003).

This study is intended to discover, by a multidisciplinary approach, the peculiarities of the primary sound-producing muscle in *Carapus acus* Brünnich 1768. Three different approaches are used. (i) The biochemical approach comprises the electrophoretic study of the myofibrillar proteins and parvalbumins (PAs). (ii) The histochemical approach aims at determining the glycogen content and the ATPase activity. (iii) Finally, the techniques of optical and electronic microscopy were used for the morphological approach. In each of the three disciplines, the epaxial muscles serve as a comparison for the characterization of the sonic muscles.

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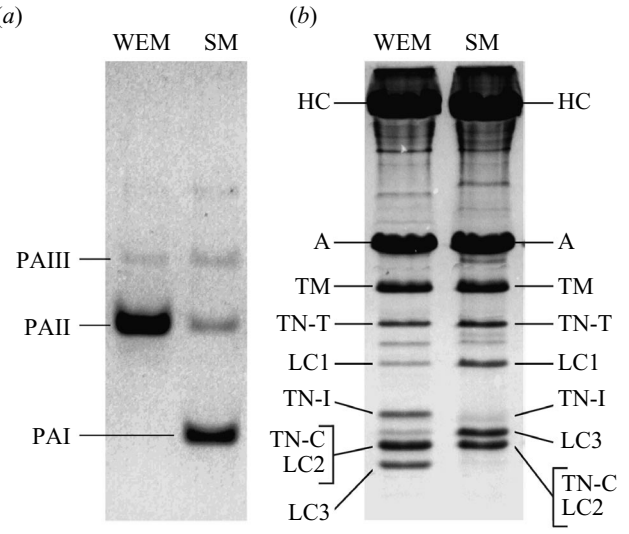

Figure 1. Electrophoretogram of (*a*) non-denaturing PAGE (glycerol 10%, pH 8.6) of parvalbumin isoforms and (*b*) SDS–PAGE (pH 8.4) of myofibrillar proteins in white epaxial muscle (WEM) and sonic muscle (SM) in *Carapus acus*.

Table 1. Physico-chemical properties and distribution of the parvalbumins in white epaxial and sonic muscle.
(M_r, relative molecular mass; pI, isoelectric point.)

isoforms	quantity (%)	M_r	pI
white	$n = 10$	$n = 6$	$n = 7$
PAII	94.24 ± 1.72	11320 ± 130	4.57 ± 0.11
PAIII	5.76 ± 1.72	12170 ± 260	
sonic			
PAI	68.58 ± 16.34	11630 ± 130	4.62 ± 0.11
PAII	22.03 ± 12.58	11310 ± 260	4.56 ($n = 1$)
PAIII	9.87 ± 4.37	12170 ± 160	4.87 ($n = 1$)

2. MATERIAL AND METHODS

(a) *Biological material*

Twenty-four *C. acus* (total length of 8–15 cm) were collected during the dissection of 182 specimens of *Holothuria tubulosa* obtained in front of the STA.RE.SO. station (Calvi Bay, Corsica). Ten specimens were frozen (−20 °C) for the PA and myofibril studies. Four were fixed in Bouin for the production of serial histological sections. Small samples of the primary sonic muscle (1 cm^3) were taken from four specimens and fixed in glutaraldehyde 2.5% for electronic microscopy (TEM). Six fishes were used for research on myofibrillar ATPase activity: blocks of myotomal and sonic muscles were immersed in isopentane, cooled to near its freezing point by liquid nitrogen, directly after death.

(b) *Biochemical methods*

White epaxial muscle (20–70 mg) and the two primary sound-producing muscles (53–227 mg) were dissected out in the 10 frozen specimens. Samples were weighed and suspended in 10 vol. of a preservative solution (Tris 10 mM, KCl 50 mM, DTT 10 mM, NaN_3 0.005% and glycerol 50%), kept at 4 °C for 24 h and stored at −18 °C.

(i) *Preparation and extraction of parvalbumins*

Sarcoplasmic extracts obtained by centrifuging (17 500*g* for 20 min at 4 °C) thawed muscle homogenates in preservative glycerol solution were heated at 100 °C for 4 min and centrifuged at 17 500*g* for 10 min. The supernatant containing parvalbumins was incubated in 1 vol. of an incubation solution for polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions (Focant *et al.* 1992). A portion of the glycerol solution was diluted in 1 vol. of sodium dodecyl sulphate (SDS) incubation solution and heated at 100 °C for 2 min for use in electrophoresis in the presence of SDS (Laemmli 1970). A portion of the glycerol solution was also diluted in 2 vol. of 8 M urea, ampholine pH 4–6 (BIO-RAD) 4%, ampholine pH 3–10 (BIO-RAD), β-mercaptoethanol 0.1%, bromophenol blue for use in an isoelectrofocusing (IEF) system.

Analytical PAGE separations of PA isoforms were performed in a BIO-RAD Mini-PROTEAN II cell (vertical plate 0.075 cm × 8.3 cm × 6 cm) under three sets of conditions (Focant *et al.* 1999): in a non-denaturing system, in the presence of SDS and in an IEF system. Conditions for electrophoresis, electrofocusing, staining, densitometry and estimation of isoelectric points (pI) are those determined by the authors. Isoforms were identified by comparing them with isolated *Cyprinus carpio* PAs and apparent relative molecular masses (M_r) were determined by comparison with purified PA of *Heterobranchus longifilis* (Focant *et al.* 1999).

(ii) *Preparation and extraction of myofibrillar proteins*

The preparation was done according to Huriaux *et al.* (1999). Myofibrils were washed in a sucrose solution. Myofibril incubation, electrophoretical analysis and pI determinations were also done according to Huriaux *et al.* (1999) with slight modifications. Myosin heavy chain (HC) separation was performed in the presence of 50% glycerol at pH 8.4. The apparent relative molecular mass of myofibrillar proteins was estimated by using standard kits covering the range 14.4–97.4 kDa (Huriaux *et al.* 1999).

(c) *Histochemical methods*

The ATPase activity was demonstrated according to the method of Meyer-Rochow *et al.* (1994). Cross-sections were pre-incubated at room temperature at 14 different pH values (from 4.35 to 11) for various lengths of time (0–60 min). Cross-sections of 6–7 μm were stained for glycogen using Schiff's periodic acid method (PAS) (Hotchkiss 1948).

(d) *Morphological methods*

The general morphology of sonic and epaxial muscles was observed on 6–7 μm sections stained using Masson's trichrome method (Ganter & Jollès 1970). The cellular ultrastructure was examined on ultrathin sections (60–80 nm) stained with uranyl acetate and lead citrate. The sections were viewed with a JEOL JEM 100SX electron microscope.

(e) *Measurements*

Measurements of fibres and myofibrils were accomplished with the DesignCaD software. Fibre diameters were estimated from sections stained by ATPase at ×200 magnification. Myofibrillar diameters were measured from electronic sections at ×20 000 magnification.

Table 2. Physico-chemical properties and distribution of the myofibrillar proteins in white epaxial and sonic muscle. (M_r, relative molecular mass; pI, isoelectric point. Figure in brackets is the number of samples.)

	white muscle		sonic muscle	
	M_r	pI	M_r	pI
actin	44 780 ± 500 (5)		44 680 ± 380 (9)	
tropomyosin	37 710 ± 580 (6)	4.99 ± 0.03 (4)	37 890 ± 480 (9)	5.00 ± 0.04 (2)
LC1	27 590 ± 470 (6)	5.09 ± 0.01 (4)	27 500 ± 410 (6)	5.10 ± 0.04 (2)
LC2	19 220 ± 360 (11)	5.05 ± 0.02 (4)	19 460 ± 280 (11)	5.06 ± 0.03 (2)
LC3	16 810 ± 1020 (6)	4.52 ± 0.02 (4)	20 670 ± 300 (10)	4.68 ± 0.03 (3)
troponin-T	32 450 ± 290 (6)		32 300 ± 460 (10)	
troponin-I	22 440 ± 290 (6)		21 930 ± 240 (6)	
troponin-C	19 560 ± 450 (2)		20 220 ± 490 (2)	3.91

3. RESULTS

(a) *Biochemical approach*

(i) *Parvalbumin identification*

PAs from epaxial and sonic muscles were separated and identified by non-denaturing PAGE (figure 1), SDS–PAGE and IEF–PAGE (not shown). The white muscle of *C. acus* contains two PA isoforms: PAII and PAIII; in the sonic muscle PAI, PAII and PAIII are found. PAII is predominant in the white muscle whereas PAI is the most abundant form in the sonic muscle.

The apparent relative molecular mass and the isoelectric points of the various PAs are listed in table 1. The PAII and PAIII isoforms are identical in the white and sonic muscles.

(ii) *Myofibrillar proteins*

Comparison of the different electrophoretograms shows that the myofibrils of the white and sonic fibres differ from one another in three of their constituents.

(i) In the thin filaments, the troponin I (TN-I) band of the sonic muscles migrates slowly and is less intense (figure 1) in SDS–PAGE (pH 8.4). By contrast, actin (A), tropomyosin (TM), troponin C (TN-C) and troponin T (TN-T) in both muscles have the same molecular mass (table 2). TM and TN-C also show the same isoelectric points. TN-T and TN-I are high-alkali proteins and cannot be detected under the classical conditions of IEF–PAGE (Huriaux *et al.* 1999). The separation of TN-C and TN-I was done on urea 8 M gel in the absence of Ca^{2+} ions. This property allows us to confidently identify the TN-C, to isolate it and to establish its molecular mass and pI.

(ii) White and sonic fibres have differences in the light chains (LC3) and heavy chains (HC) of myosin. The white LC3 muscle possesses a higher electric charge, a more acidic pI and a lighter M_r compared with the sonic muscle (table 2). The LC1 and LC2 possess the same M_r and pI in both muscles (table 2).

(iii) The myosin HC of the sonic muscle presents a minor M_r.

(b) *Histochemical approach*

(i) *ATPase activity*

The highlighting of the myofibrillar ATPase activity is mainly due to two variables: pH and the preincubation time (PT). In the trunk musculature, a thin layer of red fibres located under the dermis surrounds the white fibre layer. The white fibres become stained when preincubated at pH 5, whereas their optimum coloration is at pH 10.4. The red fibres are optimally stained at pH 4.35. They are lightly stained when the pH ranges from 4.5 to 10.6 and only if the PT does not exceed 5 min. Sonic muscle fibres are stained at a pH range between 6 and 10.6. Their maximal activity is at pH 10.4 with a PT of 0 or 1 min. However, the depth of colour appears lighter than in the white fibres.

At pH 10.4 and PT between 15 and 60 min, all the muscular ATPases are inactive. However, a greyish staining is present on the periphery of red muscle fibres, concentrated at some points of the circumference in sonic fibres and absent in white fibres. These staining patterns correspond to the mitochondrial ATPases (Johnston *et al.* 1975).

(ii) *Glycogen*

The red fibres are highly stained. The white fibres have a light staining, confined to the cellular periphery. The colour depth is intermediate in the sonic muscle fibres.

(c) *Morphological approach*

(i) *Gross morphology*

In *C. acus*, the orientation of the epaxial musculature fibres and of their myofibrils are classical—they are parallel and in a straight line.

The situation appears to be different in the primary sonic muscle. The myofibrils that limit the circumference of the fibre have an oblique disposition whereas the myofibrils of the middle of the cell are in a straight line (figure 2). In electronic microscopy cross-sections, the central myofibrils show irregular sections with the typical organization of the contractile filaments: one myosin filament surrounded by six actin filaments (figure 2). The sections become more and more oblique towards the periphery and the filament shows the progressive striation usually observed in longitudinal sections. In the longitudinal sections, the central myofibrils are the only ones to be longitudinally cut, the myofibril section area diminishes towards the cellular periphery because the myofibrils are more and more twisted and thus more and more oblique with regard to the plan section.

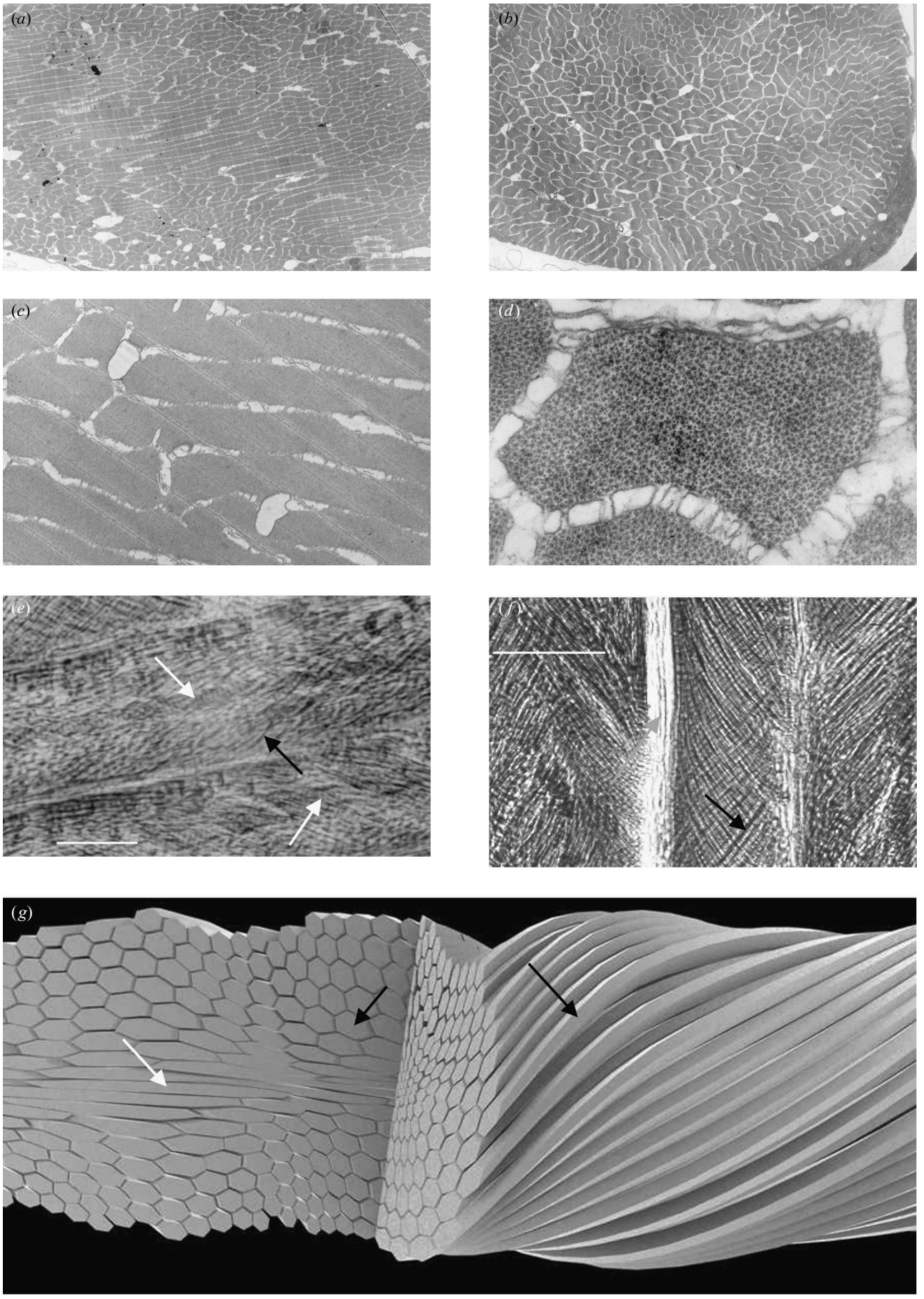

Figure 2. (*Caption overleaf.*)

Figure 2. Sonic fibre in *Carapus acus*. (*a*) Longitudinal (×1500) and (*b*) transverse (×2000) section of the sonic muscles section. (*c*) Transverse section in the periphery of the fibres, the myofibrils are obliquely cut (×3000). (*d*) Transverse section in a myofibril in the centre of the fibre (×40 000). Longitudinal section showing (*e*) the straight myofibrils (white arrows) in the centre of the fibres with the surrounding external twisted myofibrils (black arrows) and (*f*) the twisted myofibril in the periphery; grey arrow: edge of individual fibres (scale bar, 20 μm). (*g*) Diagram of the helicoidal disposition formulated based on the previous figures. A lower number of myofibril bundles are used to simplify the figures.

(ii) *Cellular content*

The myofibrils of the sonic fibres have a more developed membrane system than that of the white fibres (figure 3). In the white muscle, the T system/sarcoplasmic reticulum (SR) (1 T tubule + 2 terminal system of the sarcoplasmic reticulum) only surround the sarcomere at the Z-line level. In the sonic muscle, the T system/SR are found at the Z-line level and at the A/I junctions. Their sarcoplasmic reticulae are also wider (figure 3).

The mitochondria are more numerous in the sonic fibres than in the white fibres. In the sonic muscle, they are concentrated in packs under the sarcolem (figure 3), close to the blood capillary. This situation corresponds to the detection of mitochondria by ATPase activity (figure 3). In the white muscle, they are less numerous and more isolated.

The sonic fibre diameter (37 ± 10 μm, $n = 160$) is 1.3 (ANOVA, $p < 0.05$) times larger than the red fibres (28 ± 10 μm, $n = 45$) but it is 2.5 times thinner (ANOVA, $p < 0.05$) than the white fibres (88 ± 19 μm, $n = 57$). The diameter of the bundle of myofibrils in the sonic fibres is 0.634 μm (± 0.13, $n = 60$) or 1.7 (Student *t*-test, $p < 0.05$) times thinner than the white fibres (1.08 ± 0.19 μm, $n = 60$).

4. DISCUSSION

In the Carapidae, the sound production results from the action of extrinsic muscles inserted into the anterior part of the swim bladder. The fast forward movement of the swim bladder should result in vibration of its thinner zone (Parmentier *et al.* 2003). The muscle is a machine in which the contraction/relaxation properties depend on its structural components and on the efficiency of its energetic metabolism.

(a) *Biochemical approach*

This study corresponds with the pattern of understanding of sound production in teleosts, as the only species in which the muscle PAs have been biochemically characterized is *Opsanus tau* (Hamoir *et al.* 1980; Hamoir & Focant 1981; Appelt *et al.* 1991).

(i) *Parvalbumins*

In *C. acus*, the differences in PAs between the white and the sonic muscle are principally qualitative. PAI is present in only the sonic muscle, where it is present in a greater amount than PAII and PAIII, both found in the white muscles. These differences could be the origin of different contractile properties between both muscles because the expression of peculiar isoforms in a type of muscle would correspond to its functional requirements (Gerday 1982; Huriaux *et al.* 1997; Focant *et al.* 2000). The sonic muscles of *O. tau* contain the same three isoforms of PA as the white muscles, but the total concentration in PAs is three times higher in the sonic muscle, which could be related to the high relaxation rate (Hamoir *et al.* 1980; Gillis 1985). The relatively high amount of PAI is also striking because this isoform is usually only present in high levels in the red muscle of adult teleosts (Gerday 1982) or in the white muscle of larvae (Huriaux *et al.* 1996; Focant *et al.* 1999).

(ii) *Myofibrillar proteins*

The actin, tropomyosin, LC1, LC2, TN-T and TN-C are identical in both muscles. By contrast, differences are noticed at the level of the LC3, the TN-I and in the HCs of myosin. These results differ from those of *O. tau* in which LC2 is different in the two types of muscle (Hamoir *et al.* 1980). In *C. acus*, the biochemical differences at the level of the LC3 are particularly interesting because these LCs are involved in the ATPase activity of the myosin (Pette & Staron 1990), the latter being correlated with the muscular contraction speed (e.g. Johnston *et al.* 1975). The presence of a special LC3 in the sonic muscle could thus represent an adaptation to swift contractions. The difference in molecular mass in the HC corresponds to its composition in amino acids that could also affect the enzymatic activity of the myosin and therefore the contraction speed (Pette & Staron 1990). The TN-I isoform of the sonic muscle might also affect the muscle contraction requirements (Huriaux *et al.* 1999).

(b) *Histochemical approach*

The white fibres in *C. acus* possess a high ATPase activity, are alkali-stable and have a low glycogen and mitochondria content. The red fibres have a lower ATPase activity, are acid-stable and have a higher glycogen and mitochondria content (Meyer-Rochow *et al.* 1994; Chen & Huang 2000; Devincenti *et al.* 2000). These data correspond to the metabolisms assigned to these fibres: fast-twitch contraction with an anaerobic metabolism for the white fibres and slow-twitch contraction with an aerobic metabolism for the red fibres (te Kronnie *et al.* 1983; Chen *et al.* 1998). The histochemical features of the sonic muscles in *C. acus* should be related to the unusual contractile properties; they have characteristics of both red and whites fibres. As for the white fibres, sonic muscle ATPase is alkali-stable. However, the staining of the sonic muscle ATPase is less marked than in the white fibres in each of the experimental assays, which could be related to the presence of the LC3 isoform or of the HC. On the other hand, the fibres of the sonic muscle come closer to the red fibres with the higher amount of glycogen and the presence of more numerous mitochondria than in the white muscle. These features should allow a better resistance to fatigue in the sonic muscles than in the white fibres (Akster 1981). This histochemical approach allows us to reasonably suppose that the sonic muscles in *C. acus* are able to contract in a fast and sustained manner. Whether the muscles are intrinsic as in *O. tau*, or extrinsic such as in *Terapon jarbua* or *Cynoscion*

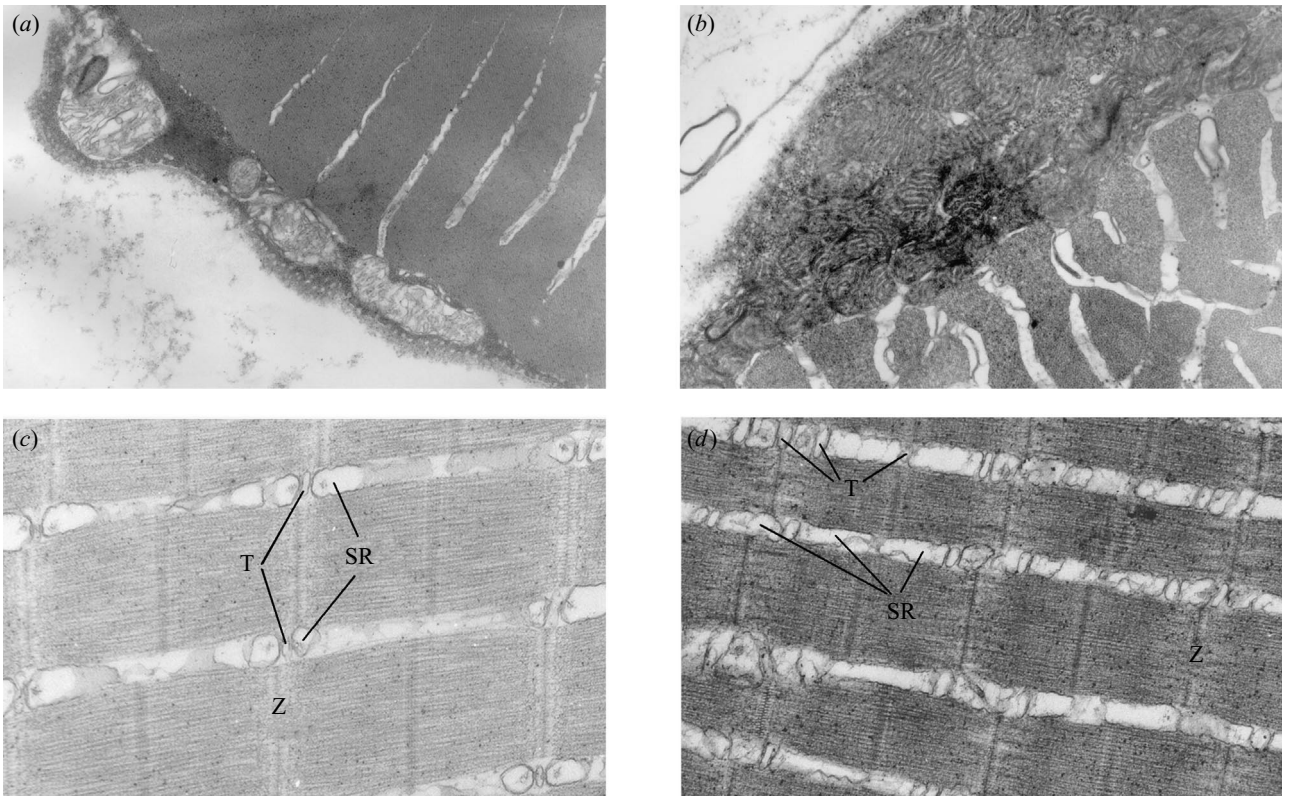

Figure 3. Transverse section in the periphery of the epaxial fibre (*a*) and in the sonic fibres (*b*) showing the mitochondria (×10 000). Longitudinal section in the epaxial fibre (*c*) and in the sonic fibres (*d*) (×20 000). Abbreviations: T, tubule; SR, sarcoplasmic reticulum; Z, Z-line.

regalis, the fibres of the sonic muscle are alkali-stable and present a higher amount of glycogen and mitochondria than in their respective white muscles (Ono & Poss 1982; Fine *et al.* 1990, 1993; Chen *et al.* 1998). Moreover, the disposition of the numerous mitochondria looks more like that of the sonic muscle in *Porichthys notatus* males, a fish that is able to generate sound for long periods of time (Bass & Marchaterre 1989).

(c) *Morphological approach*

In teleosts, the sonic muscle seems to be constructed to increase the exchange surfaces and to reduce the diffusion distances between the different parts intervening in muscular contraction.

As in *C. acus*, the fibres and the myofibrils of fast-twitch sonic muscles of teleosts are characterized by a smaller diameter compared with the white muscles (Evans 1973; Ono & Poss 1982; Fine *et al.* 1990, 1993; Connaughton *et al.* 1997; Loesser *et al.* 1997). This feature gives them a higher surface : volume ratio that would facilitate fast flows of metabolites (Fine *et al.* 1990).

The position, the number and the size of the T tubules and the sarcoplasmic cisternae would also be different factors limiting the diffusion distance. The T system/SR of the white fibres are typically at the Z-line level (Akster 1981). In the sonic muscles of *O. tau* and *T. jarbua*, they are at the A/I junction's level, which allows a reduction of the diffusion time of calcium (Eichelberg 1976; Loesser *et al.* 1997). In the sonic muscles of *C. acus* these properties are particularly well developed because the T system/SR are at the level of both the Z-lines and A/I junctions. To the best of our knowledge, this adaptation is unique in vertebrate muscle and should accelerate the diffusion time of calcium. Moreover, this sonic muscle also differs from other typical sonic muscles in not having a central sarcoplasm core and a radial arrangement in cross-sections (Ono & Poss 1982; Fine *et al.* 1993). It could be linked with the helicoidal organization process.

(i) *Helicoidal organization*

The helix organization of the myofibrils in the sonic muscle fibres in *C. acus* is striking. Many hypotheses may be formulated for its functional significance. The helix allows a higher serial sarcomere disposition for a given length. It should result in a greater number of actin–myosin links and thus a greater force involved (Walker & Liem 1994). However, no muscle force can be generated at extreme shortening (Herrel *et al.* 2002). The helicoidal organization could allow the sarcomeres to contract at different moments and progressively stretch the swim bladder fenestra, which begins to vibrate. The swim bladder fenestra is a thinner zone in front of the swim bladder, just behind the insertion of the sonic muscle. It is covered by an osseous plate that could be an amplification system of the sound (Parmentier *et al.* 2003). In other teleosts, the sonic muscles act in such a way that they deform all the tissues of the swim bladder (Demski *et al.* 1973). In *C. acus*, all the work of the sonic muscle must be applied to a single zone.

A second hypothesis for the generation of sounds is that the rapid relaxation of the muscle could vibrate the stretched swim bladder fenestra like a guitar string. The myofibrillar helix should constitute an advantageous system during relaxation because it may provide the muscle

with spring-like mechanical properties. The sonic muscle does not have an antagonist muscle. The force allowing it to return to the original length arises from the elasticity of the swim bladder and from the gas pressure existing inside the swim bladder. When contracting, the myofibrils shorten and the helix step is reduced. The helix could then increase the efficiency of the relaxation by uncoiling, like a spring.

5. CONCLUSIONS

The helicoidal organization of myofibrils of the sonic muscles allows a relative increase of the total force and of the shortening velocity of the whole muscle. Moreover, the small diameter of the fibres and myofibrils linked with an increase of the exchange surface, due to a more developed sarcoplasmic reticulum and T system, should ensure a faster diffusion of the metabolites involved in the contraction and the relaxation.

The presence of more mitochondria and a more important glycogen concentration than in the white fibres indicates, in the sonic muscle, the ability to maintain sustained work.

Although this multidisciplinary approach provides numerous elements about the characterization of the sonic muscles in *C. acus*, it should be complemented by other studies to describe, in greater detail, the aerobic or anaerobic functioning of the muscle. The recording of *C. acus* sounds and electromyographic studies will also prove valuable in the investigation of the contraction speed of the sonic muscles in *C. acus* and to test the two hypotheses of sound generation.

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