

# Ultrastructure of the sarcoplasmic reticulum in cardiac myocytes from Pacific bluefin tuna

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**Abstract** Pacific bluefin tuna are active teleost fish with a large capacity for heat conservation and endothermy. They have a high metabolism, and hence the myocardium must be capable of sustaining elevated levels of cardiac output over a wide range of temperatures. To examine the way that the myocardial cells of bluefin tuna respond to their unique cardiac physiology, we have studied the ultrastructure of the internal membrane system and mitochondria of atrial and ventricular myocytes by light and electron microscopy. Our results reveal that cardiomyocytes of juvenile bluefin tuna possess a relatively high content of sarcoplasmic reticulum (SR), together with a large volume of mitochondria within the two (compact and spongy) ventricular compartments and in the atrial myocardium. The mitochondrial structure and distribution in bluefin tuna myocardium follow specific metabolic zonation resulting in a higher volume and lower cristae density in the compact ventricular layer than in atrium and spongy layer. The presence of junctional SR

profiles and an extensive network of free SR within cells may ensure a rapid delivery of  $\text{Ca}^{2+}$  to the myofibrils. This, in conjunction with transsarcolemmal  $\text{Ca}^{2+}$  entry, might contribute to a faster excitation-contraction-relaxation cycle and thus enhance cardiac performance, cardiac output, and the maintenance of excitability at low temperatures. We propose that the mitochondrial configuration together with the developed SR ultrastructure of bluefin tunas myocardium are important evolutionary steps for the maintenance of high heart rates and endothermy in this teleost fish.

**Keywords** Sarcoplasmic reticulum · Mitochondria · Heat conservation · Endothermy · Myocardium · Ventricle · Atrium · Pacific bluefin tuna · *Thunnus orientalis* (Teleostei)

## Introduction

The ultrastructure and morphology of the teleost heart has been studied for 50 years in a variety of species (Kisch 1954; Bloom 1962; Kisch and Philpott 1963, 1966; Kilarsky 1967; Santer and Cobb 1972; Santer 1974; Basile et al. 1976; Tota 1978; Leknes 1980, 1981; Midttun 1980; Breisch et al. 1983; Santer et al. 1983; Johnston and Harrison 1987). The teleost heart is composed of the sinus venosus, the bulbus arteriosus, a single atrium, and a ventricle. The myocardium in fish, as in many other vertebrates, is constituted of two distinct regions: the compact and spongy layers (Tota 1978; Midttun 1980; Santer and Walker 1980; Breisch et al. 1983; Santer et al. 1983; Sanchez-Quintana and Hurlé 1987; Agnisola and Tota 1994; Simoes et al. 2002a, 2002b; Icardo et al. 2005). In general, the atrium tends to be exclusively spongy, whereas the ventricle has a peripheral compact component and a luminal spongy layer, which constitutes a larger proportion

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of the ventricular myocardium in several fishes (Tota 1978; Midttun 1980; Santer and Walker 1980; Breisch et al. 1983; Santer et al. 1983; Sanchez-Quintana and Hurle 1987; Agnisola and Tota 1994; Simoes et al. 2002a, 2002b; Icardo et al. 2005). In addition, bony fishes have a unique anatomy of the bulbus arteriosus, which is composed of smooth muscle and cartilaginous tissue distributed within an elastin framework (Watson and Cobb 1979; Leknes 1982; Icardo et al. 1999, 2000; Braun et al. 2003).

The spongy tissue in ventricle consists of a complex trabeculated network of fibers formed by long interdigitating branching cells in close proximity to the luminal fluids (Leknes 1980, 1981; Midttun 1980). On the other hand, the compact layer is formed by well-oriented fibers packed in orderly arranged bundles covering the inner ventricular layer (Breisch et al. 1983; Agnisola and Tota 1994; Simoes et al. 2002a, 2002b; Icardo et al. 2005). The whole atrium has a general structure similar to the spongy tissue of the ventricle, but with a higher proportion of open spaces between the bundles of fibers and variability in their orientation (Santer and Cobb 1972; Leknes 1980, 1981; Midttun 1980).

A direct relationship exists between cardiac architecture, the lifestyle of the fish (i.e., its physiological ecology), and its circulatory supply (Tota 1978; Santer and Walker 1980; Tota et al. 1983; Feller et al. 1985; Farrell and Jones 1992). Active species with migratory behavior possess a large pyramidal-shaped ventricle, with a higher proportion of compact tissue (Santer and Walker 1980; Santer et al. 1983; Tota et al. 1983; Farrell and Jones 1992), whereas inactive fishes have a saccular or tubular ventricle, composed predominantly of trabeculated fibers (Santer et al. 1983; Santer 1985; Simoes et al. 2002a, 2002b). In fishes that are acclimated to cold temperature, the ventricular mass has been reported to enlarge, in parallel to an increased metabolic activity of mitochondria and changes in the force-frequency relationship (Seibert 1979; Goolish 1987; Driedzic et al. 1996; Rodnick and Sidell 1997; St-Pierre et al. 1998; Shiels et al. 2002a, 2002b; Tiitu and Vornanen 2002).

As regards the cytological architecture of cardiac myocytes in teleosts, the cells tend to have small diameters with peripheral myofibrils and centrally located mitochondria, glycogen, and lipid vesicles (Kisch 1954, 1966; Kisch and Philpott 1963; Kilarsky 1967; Yamamoto 1967). Transverse (T) tubules are absent, and sarcoplasmic reticulum (SR) is poorly or moderately developed (Santer and Cobb 1972; Midttun 1980; Breisch et al. 1983; Santer et al. 1983; Santer 1985; Farrell and Jones 1992). The latter is variable ranging from completely absent in some fishes (Kisch and Philpott 1963; Kilarsky 1964; Santer and Cobb 1972) to significantly present in active species (Leak 1969; Midttun 1980; Farrell and Jones 1992; Tibbits et al. 1992;

Tibbits and Kashihara 1992; Keen et al. 1994; Shiels et al. 2006).

Among the *Thunnus* genus, biochemical, morphological, and physiological studies of the cardiac system have been conducted in yellowfin, bluefin, and albacore tunas (Tota 1978; Breisch et al. 1983; Shiels et al. 1999, 2004; Block and Stevens 2001; Blank et al. 2002, 2004, 2007; Landeira-Fernandez et al. 2003; Castilho et al. 2007). Tunas are unique among teleosts because of the presence, in the circulatory system, of counter-current heat exchangers that allow for metabolic heat retention in slow-twitch muscles, viscera, brain, and eyes (Carey and Teal 1969; Carey et al. 1984). Differently from most fish, tuna retain metabolic heat in their tissues and elevate the temperature of their muscle, viscera brain, and eyes (e.g., bluefin) significantly above ambient water temperatures, resulting in gradients of up to 10–23°C between the internal tissues and the cardiac and gill tissues over a wide range of temperatures (Kitagawa et al. 2000; Marcinek et al. 2001; Block and Stevens 2001). In association with the conservation of metabolic heat and with the high cardiac output, tunas have a large ventricular mass relative to its body size and a higher ventral aortic pressure as compared with other fishes (Korsmeyer et al. 1997; Blank et al. 2004, 2007). The heart temperature, however, reflects changes in environmental temperature, regardless of body size and regional endothermy, because the anatomical position is outside the circulatory regions with counter-current heat exchangers, and the blood supply comes directly from the gills. The heart is located close to the ventral body wall where the heat loss via conduction is high.

To support the high metabolic rates necessary to maintain body temperature, tunas maintain high heart rates and cardiac outputs (Brill and Bushnell 2001; Blank et al. 2007; Korsmeyer et al. 1997). Bluefin tunas have a higher mass-specific metabolic rate than yellowfin tunas (Blank et al. 2007), and the heart of the bluefin tuna maintains its function over a wider range of ambient temperatures (Blank et al. 2004; Korsmeyer et al. 1997). Specifically, the bluefin tuna ventricle has an exceptional aerobic capacity (Blank et al. 2004) with high mitochondrial enzymatic activity and oxygen consumption (Basile et al. 1976), high myoglobin levels (Giovane et al. 1980), and a high affinity for oxygen (Marcinek et al. 2001). For the above reasons, which are also supported by anatomical studies, bluefin tunas have a large thick-walled and pyramidal heart, a high proportion of compact layer supplied by oxygenated coronary capillaries (Tota 1978; Farrell and Jones 1992), and a well-developed coronary circulation (Poupa et al. 1981; Farrell and Jones 1992).

Excitation-contraction (e-c) coupling in tuna myocardium depends on the influx of extracellular  $Ca^{2+}$  across the plasmalemma, via L-type  $Ca^{2+}$  channels (CaV1.2; Tibbits et

al. 1992; Vornanen 1997), and on release from the SR (Shiels et al. 1999, 2004; Keen et al. 1992; Tibbits et al. 1992; Landeira-Fernandez et al. 2003; Brill and Bushnell 2001). In teleost fishes, the L-type  $\text{Ca}^{2+}$  channels are responsible for the majority of  $\text{Ca}^{2+}$  entry (Vornanen 1997; Hove-Madsen and Tort 1998; Shiels et al. 2000); the  $\text{Na}^{2+}/\text{Ca}^{2+}$  exchanger also plays an important role (Vornanen 1999). Isolated SR vesicles from cold-temperate tunas (bluefin and albacore) have a high content of SR ATP-ase related to high pumping rates (Landeira-Fernandez et al. 2003), and this up regulation of SR proteins is similar to that seen in other cold-acclimated fishes (Landeira-Fernandez et al. 2003; Weng et al. 2005; Castilho et al. 2007).

In this study, we provide a qualitative and quantitative assessment of the organelles involved in calcium metabolism and energy production in the heart of the bluefin tuna. We demonstrate the extraordinary distribution and ultrastructure of the SR in relation to the e-c coupling mechanism.

## Materials and methods

### Animals

Pacific bluefin tuna (*Thunnus orientalis*) were captured by using 10–30 kg line and circle hooks off San Diego, Calif., USA. Individual fishes were pithed, and the heart was removed for fixation. In most cases, the atrium was still beating when the fixation protocols were initiated. Cellular myocardial preparations were prepared from Pacific bluefin tuna that had been transported to the Tuna Research and Conservation Center (Pacific Grove, Calif.) and held in captivity in tanks (Farwell 2001; Blank et al. 2004). The mean curved fork length (CFL) of animals used for experiments was  $90.5 \pm 6$  cm (mean  $\pm$  SD;  $n=9$ ; range: 70–95.5 cm). All procedures were in accordance with Stanford University protocols for the use of institutional animals.

### Electron microscopy

Small samples of tissue from the atrium and ventricle of wild tuna (either from the spongy cavity or from the compact layer) were dissected from the hearts after perfusion with a saline solution, pinned with Minutens pins in a dish (containing a layer of Sylgard), and fixed by immersion with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Myocytes were isolated from the atrium and the spongy layer of the ventricle of captive tuna, as described by Shiels et al. (2004). Cells were stored in fresh isolating solution for 1 h, fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and stored at 4°C. Small bundles of muscle teased either from the

atrium or the ventricle were postfixed in 2%  $\text{OsO}_4$  in 0.1 M sodium cacodylate for 1 h at room temperature, stained *en bloc* with saturated uranyl acetate in acetate buffer, and embedded in Epon. Some samples were also postfixed in 8%  $\text{K}_2\text{Fe}(\text{CN})_6$ , 2%  $\text{OsO}_4$  in 0.1 M sodium cacodylate for 2 h at room temperature and then stained as above. Most of the ultra-thin sections ( $\sim 50$  nm) were stained first with saturated uranyl acetate solution in 50% ethanol and then in Sato lead solution (Sato 1968). Other ultra-thin sections and thick sections ( $\sim 90$  nm) were stained with the lead solution only. Sections were observed by using a Philips 410 microscope (Philips Electron Optics, Mahwah, N.J.), and images were recorded either on film or by using a Hamamatsu C4742–95 digital imaging system (Advanced Microscopy Techniques, Chazy, N.Y.).

### Stereology

Estimates of the relative surface area and volume of total SR and mitochondria were obtained by point and intersection counting in digitally recorded images, excluding regions of the nuclei and adjacent Golgi apparatus, at a magnification of  $\times 61,000$  from cross sections of cardiac myocytes (Loud 1962; Mobley and Eisenberg 1975). The images were covered with an orthogonal array of dots at a spacing of 0.16  $\mu\text{m}$ . The ratio between the number of dots falling over an organelle to the total number of dots covering the image gave the volume fraction of the organelles (mitochondria and SR) relative to the total cell volume. The number of dots covering the cytoplasm was obtained by subtracting the number of dots over the organelles from the total number of dots covering the image. The ratio between dots overlapping with an organelle and/or area and the dots covering the entire image was equal to the ratio of that organelle or component volume and the fiber volume, excluding the nuclei and Golgi apparatus. The ratio of SR surface area to fiber volume (as above) was obtained from  $C/2dP_{\text{test}}$ , where  $C$  is the number of intersections,  $d$  is the spacing between the grid lines, and  $P_{\text{test}}$  is the number of grid intersections in the test area. We did not use a correction factor for SR anisotropy (Mobley and Eisenberg 1975), anisotropy not being a factor in a cross section of cardiac muscle.

### Isolated myocytes

Myocytes were isolated from myocardial tissue removed from the atrium of pithed captive Pacific bluefin tuna and the spongy layer of the ventricle based on the isolation protocols of Shiels et al. (2004). After isolation, cells were stored in fresh isolating solution for 1 h and then fixed. A sample of myocytes was processed for electron microscopy and other samples for light microscopy. The electron

micrographs of tuna myocytes used in this study were not considered for stereology and quantitation, primarily because the fish used for this preparation were held in captivity in tanks at the Tuna Research and Conservation Center.

### Immunolabeling

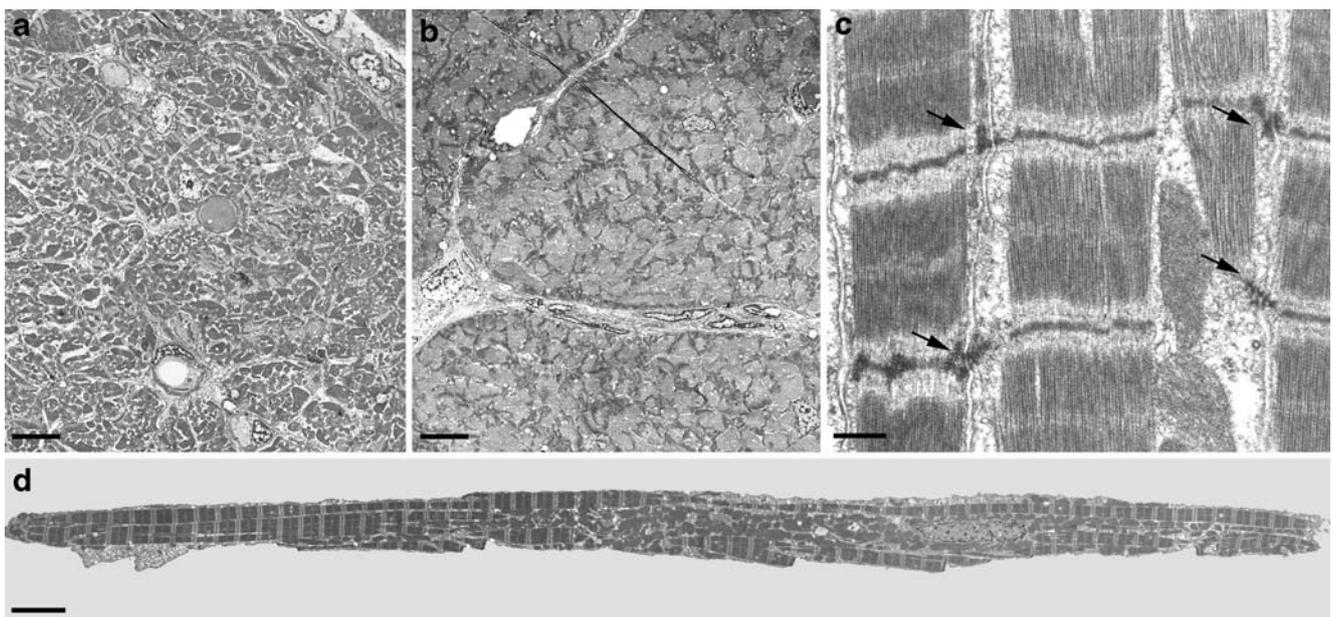
Small bundles of compact and spongy tissue were dissected from the hearts after perfusion with a saline solution, pinned in a Sylgard dish with Minutem pins, and fixed by immersion with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at 4°C. Single myocytes were fixed in the same fixative solution after isolation (see above). Samples of bundles and myocytes were blocked with PBS containing 1% bovine serum albumin (BSA) and 10% goat serum at 4°C for 1 h, incubated overnight with primary monoclonal antibody against ryanodine receptors (RyRs; kindly donated by Dr. T. Murayama), washed with PBS-BSA, incubated with secondary antibody (Texas Ted-Alexa 588 goat anti-rabbit IgG, kindly donated by Dr. C. Cardenas) for 1 h at room temperature, washed with PBS-BSA, and mounted in anti-bleaching solution (0.0025% para-phenyl-enediamine, 0.25% 1,4 diazobicyclo 2,2,2,octane, and 5% n-propylgaahte in glycerol). Specimens were examined with a Leica DM6000B confocal microscope.

## Results

### Ventricle ultrastructure: spongy and compact layers

The heart of the bluefin tuna has a pyramidal shape. The ventricle is composed of two layers: an inner spongy layer consisting of a trabeculated myocardial network of bundles in which the fibers are unevenly organized (Fig. 1a) and an external compact layer in which the fibers are packed into tightly arranged bundles (Fig. 1b). A distinctive feature between the spongy and compact layers is the different distribution of capillaries within the myocardium. The internal ventricular layer contains a slightly higher density of capillaries than the outer layer (0.19 and 0.18 N/100  $\mu\text{m}^2$ , respectively,  $P<0.05$ ) and higher intercapillary distances (Table 1). The outer membrane of the capillary wall in the spongy layer contains capillaries that are separated from one another by a space of  $22.8\pm 8.1 \mu\text{m}$  (mean $\pm$ SD,  $n=60$ ,  $P<0.05$ ), whereas in the compact layer, the same distance is  $19.2\pm 8.2 \mu\text{m}$  (mean $\pm$ SD,  $n=60$ ,  $P<0.05$ ; Table 1).

Ventricular myocytes are spindle-shaped (Fig. 1d) and have a slightly larger cross-sectional area (Table 1) than those from other teleosts (Santer and Cobb 1972; Shiels et al. 2004; Kilarsky 1967; Vornanen 1998). The cross-sectional areas of the spongy and compact layer cells were  $115.4\pm 36 \mu\text{m}^2$  and  $149\pm 78 \mu\text{m}^2$ , respectively (mean $\pm$ SD,



**Fig. 1** Cardiac muscle from the two different ventricular layers of the Pacific bluefin tuna: the compact (external) and the spongy (internal) layers. In the ventricle, the inner and outer layers can be distinguished mainly in areas where myofibrils are unevenly organized, as in the spongy layer (a), and in areas in which a compressed view of fibers are highlighted, as in the compact layer (b). In longitudinal sections of

the spongy layer (c) and in the compact layer, cell-to-cell junctions are visible as focal adhesions (arrows) at the level of the Z-line. (d) Single isolated myocyte from the inner ventricular wall of an acclimated Pacific bluefin tuna; note the sarcomeric pattern of the myofibrils and the internal organization of the organelles along the longitudinal axis of the cells. Bars 0.5  $\mu\text{m}$  (a-c), 5  $\mu\text{m}$  (d)

**Table 1** Measurements of the capillary density in atrial and ventricular myocardium of Pacific bluefin tuna. All values are expressed as mean±SD in experiments performed on eight animals

Measured parameter	Ventricle		Atrium
	Compact layer	Spongy layer	
Capillary density (N/100 $\mu\text{m}^2$ )	0.18±0.06	0.19±0.08	0.12±0.04
Intercapillary distance ( $\mu\text{m}$ )	19.2±8.1	22.8±7.9	25.94±13.5
Cross-sectional area ( $\mu\text{m}^2$ ); n=56 cells	149±78 <sup>#,*</sup>	115.4±36 <sup>#,*</sup>	147.4±58 <sup>*</sup>

<sup>#</sup> Student's *t*-test,  $P>0.05$

<sup>\*</sup> Student's *t*-test,  $P<0.05$

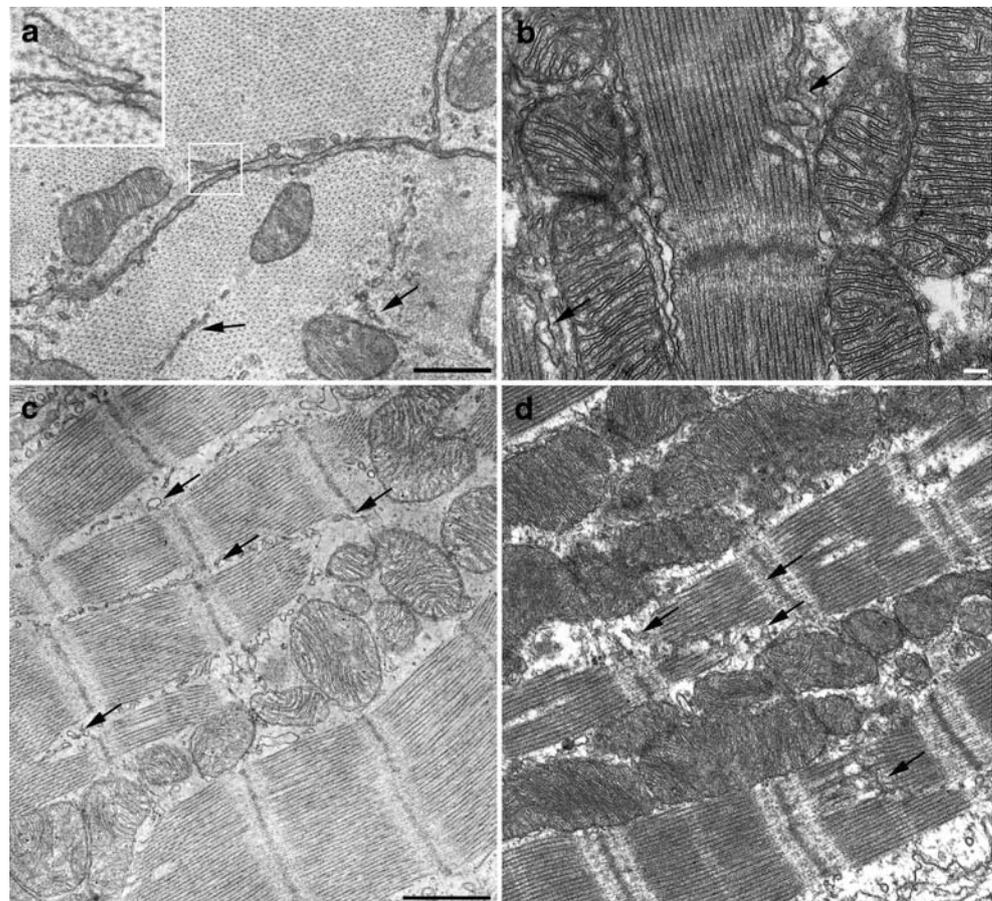
$n=51$  cells). The average sarcomere length of the two regions was  $1.6\pm 0.1$   $\mu\text{m}$  ( $n=20$  cells) and  $2.0\pm 0.1$   $\mu\text{m}$  (mean±SD,  $n=20$  cells), respectively. From this, we calculated approximate volumes for the cells of  $185\pm 54$   $\mu\text{m}^3$  and  $298\pm 156$   $\mu\text{m}^3$ ; these values were significantly different ( $P<4.2\times 10^{-6}$ ).

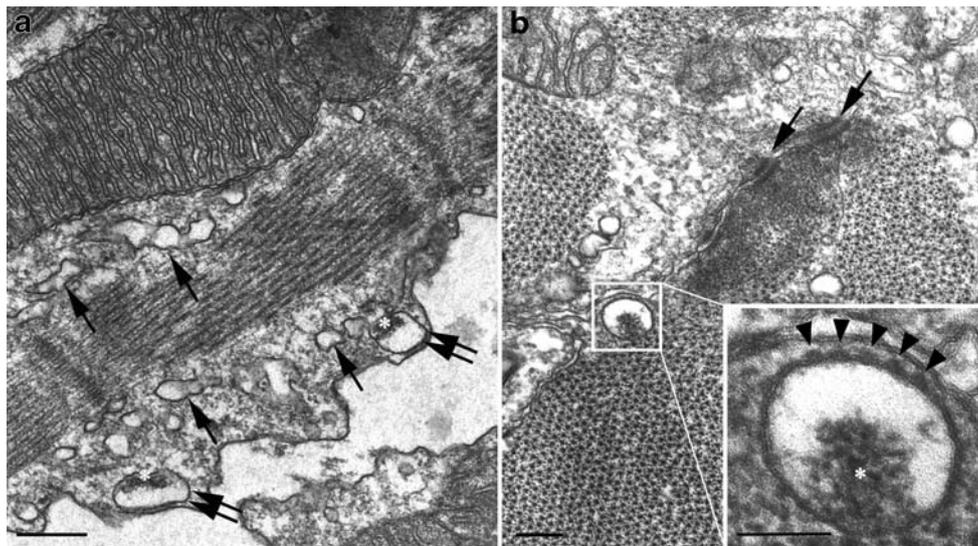
The main organelles (mitochondria and nuclei) within myocytes are centrally located, leaving the myofibrils at the periphery of the cell (Fig. 1d). At the Z-line level, junctional complexes (focal adhesions) are visible as interfibrillar material resembling, both in density and in

texture, the dense substance seen at the site of attachment of the myofilaments to the plasma membrane (Fig. 1c). This dense component seems to be continuous from the Z-lines at the lateral margin of one cell to another, presumably connecting two myocytes. At the attachment site of the myofilaments, desmosomes and intercalated disks can be frequently seen in both cardiac layers. No differences have been noted in the structure of the gap junctions or in the lateral connections between cells of different layers.

The SR is readily visible within the ventricular myocytes in both the compact (Fig. 2a,c) and the spongy (Fig. 2b,d)

**Fig. 2** Internal membrane system in the compact and spongy layers of the ventricle of the Pacific bluefin tuna. Note the usual configuration of a network (arrows) of free sarcoplasmic reticulum (fSR) distributed among the myofibrils. Various electron-microscopic techniques show several profiles with no structural differences among the two ventricular layers. **a** Transverse thick section of compact myocardium postfixed in  $\text{K}_2\text{Fe}(\text{CN})_6$  and  $\text{OsO}_2$ ; the fSR approaches the sarcolemma presumably forming peripheral coupling (**a**, inset). Several cisternae of fSR are also visible within the myofibrils. **b** Multiple disposition of the SR network among myofibrils and mitochondria of the luminal (spongy) compartment. **c**, **d** Thin sections stained with lead only and with uranyl acetate plus lead, respectively, showing the disposition of the fSR (arrows) between the myofibrils of the compact (**c**) and spongy layer (**d**). Bars 0.1  $\mu\text{m}$  (**a**, **b**), 0.5  $\mu\text{m}$  (**c**, **d**)





**Fig. 3** Distribution of the junctional SR (jSR) in peripheral couplings in the ventricle of the Pacific bluefin tuna. **a** Longitudinal thin section. The jSR appears as wide cisternae containing calsequestrin (white asterisk) and is located only at the periphery of the cells (double arrows). The extensive internal fSR network (arrows) is also visible throughout the cytoplasm. **b** Transverse section. The form and shape

of the jSR do not change. Note that, in peripheral couplings (**b**, inset) formed by the apposition of the jSR profile with the plasmalemma, rows of “feet” (ryanodine receptors; RyRs) are visible between the two membranes (**b**, arrowheads). Bars 0.3  $\mu\text{m}$  (**a**), 0.2  $\mu\text{m}$  (**b**), 0.1  $\mu\text{m}$  (inset)

cell layers. The two domains of the SR are the free  $\text{Ca}^{2+}$ -pumping SR forming a loose network around the myofibrils (Fig. 2 arrows) and the junctional SR (jSR; Fig. 3), which appears as wide cisternae in association with the sarcolemma, forming peripheral couplings (Figs. 2a, inset, 3a,b). In longitudinal sections, networks of free SR (fSR) tubules are prominent between the myofibrils, mostly opposite to the A band (Fig. 2b,d) and in the proximity of the sarcolemma. In cross sections, fSR varies in appearance from small cisternae, sometime well visible in rows, to large and wide profiles randomly located within the cytoplasm. The jSR has a rounded shape and is relatively large. On the surface associated with the plasmalemma, the cytoplasmic domain of the RyRs (feet) is visible (Fig. 3b, arrowheads in inset). Inside the junctional SR cisternae, the calsequestrin content is usually clustered on one side of the cisternae (Fig. 3, asterisks). In optical sections from confocal images of the compact ventricular layer labeled with antibodies against RyRs (Fig. 4a), the way that the foci are located only at the periphery of the cells resulting in rows longitudinally oriented is clearly seen. As in the compact layer, isolated myocytes from the spongy layer (Fig. 4b) exhibit RyR-positive foci located at  $\sim 2\text{-}\mu\text{m}$  intervals at, or in close proximity to, the Z-line.

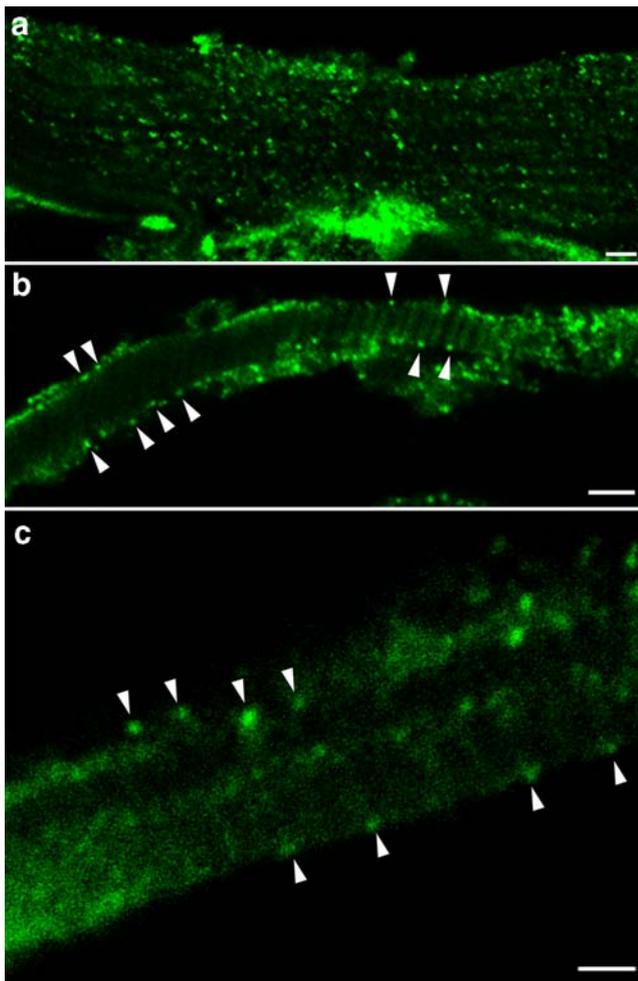
Bluefin tuna ventricular myocytes lack T-tubules, a common morphological condition in all fishes. The absence of the T-tubular system in the compact and spongy layers of tuna ventricular myocytes precludes, by definition, the formation of internal dyadic profiles (jSR cisternae in close

opposition to T-tubules). In addition, no corbular SR (jSR containing RyRs and calsequestrin but not opposed to T-tubules or the sarcolemma) have been found in any bluefin ventricular cell. At the periphery of the myocytes of both ventricular compartments, the only sarcolemmal processes noted have been identified as flask-shaped invaginations or caveolae (Fig. 2a, arrows). The distribution of caveolae within the ventricular myocytes is not as abundant as that noted in the atrium. No differences are apparent in the distribution of SR and mitochondria between myocytes of the two cardiac cell layers (cf. Fig. 2c,d; see also Table 2).

Within the spongy fibers, myofibrils and mitochondria are sparsely arranged, whereas the compact layer exhibits organelles and myofibrils densely packed within the fiber. In addition, the density of the mitochondrial cristae varies among the two layers. The difference results in the number of inner mitochondrial membrane folds being 1.3-times higher in the spongy layer than in the compact layer (Table 2).

#### Atrial ultrastructure

The bluefin tuna atrium is composed of numerous trabeculae of short lengths that form a unique complex network of bundles. The narrow and elongated atrial cells (Fig. 5a) have a sarcomere length and cross-sectional area ( $147.4 \pm 58 \mu\text{m}^2$ , mean  $\pm$  SD,  $n=56$ ; Table 1) similar to that of the compact ventricle layer. Other morphometric measurements (Table 1) show that the capillary density in atrial myocardium



**Fig. 4** Comparison of labeling with antibodies against RyR in the ventricle (**a, b**) and atrium (**c**) of Pacific bluefin tuna. Whole-mounts of compact ventricular myocardium (**a**) in which RyR-positive foci are aligned along parallel lines that delimit the border of the narrow myocytes. In single ventricular (spongy layer) myocytes, antibody labeling shows that RyRs (**b**, *arrowheads*) are aligned only at the periphery of the cell, as in the compact layer. Two adjacent isolated atrial myocytes (**c**) show again RyR spots (*arrowheads*) aligned only at the periphery of the cells. As in the ventricle, RyRs are distributed along the longitudinal axes of the cell and follow the sarcomere pattern, being located close to, or at the level of, the Z-line. Bars 2  $\mu\text{m}$

is lower than that in the ventricular myocytes, as validated by the higher intercapillary distance ( $25.94 \pm 13.5 \mu\text{m}$ , mean  $\pm$  SD,  $n=60$ ). Within the capillaries, no differences between atrial and ventricular myocardium have been noted in the endothelial thickness of the capillary internal wall.

In transverse thin sections, the myofibrils have a subsarcolemma distribution and often have variable orientations (Fig. 5b). In contrast to the ventricular myocytes, both myofibrils and mitochondria are frequently surrounded by apparently “empty” spaces in which glycogen granules and rare Golgi complexes are visible (Fig. 5b, arrows). Electron-

dense lipid droplets ranging from 0.10 to 0.22  $\mu\text{m}$  in diameter are more common in atrial cells than in the ventricle where they are very rare. As in the two ventricular compartments, focal adhesions and desmosomes (Fig. 5b,d; double arrows) are also recognizable in tuna atrial myocytes. Numerous caveolae and small flask-shaped invaginations are sparsely distributed along the sarcolemma (Fig. 5c,d,f, white arrows) and follow a characteristic undulating shape.

Bluefin tuna atrial myocytes show a more extensive distribution of SR relative to the ventricular myocytes (Fig. 5d,e, arrows; see also quantitative data below). Atrial myofibrils are frequently surrounded by a loose network of SR (fSR; Fig. 5d,e, arrows) and by wide cisternae (jSR; Fig. 5f) bearing “feet” (RyRs) in association with the sarcolemma (Fig. 5f, arrowheads). In longitudinal sections, the apposition between the jSR and the sarcolemma forming peripheral couplings are numerous at the Z-line following an ordinated sarcomeric distribution, as noted in the ventricle. Much of the fSR is interfibrillar and is mostly situated in narrow gaps between the myofilaments (Fig. 5e, arrows).

Images from isolated myocytes (Fig. 4c) labeled with antibody against RyRs show foci (Fig. 4, arrowheads) aligned along parallel axes that delimit the borders of the cells. In the direction parallel to the fiber long axis, the RyR-positive foci are arranged in a sarcomere-related periodicity of  $\sim 2 \mu\text{m}$ , with a labeling pattern similar to that in the ventricular myocardium.

Being centrally located within the cells, mitochondria are often found in close apposition to the fSR (Fig. 5d, arrows) and contain a variety of cristae configurations that are closely packed one to another (Fig. 5b). The mitochondria are randomly distributed within the myocytes and, compared with those in the ventricular cells, appear less densely packed among the myofilaments. The internal membrane structure is not significantly different from that in the ventricle or from that described in mammalian atrial myocytes (McNutt and Fawcett 1969; Sommer and Johnson 1979). Within mitochondria of variable shapes ranging from ovoid to spherical profiles and sometimes projecting processes into the myofilament mass, long mitochondrial cristae run mainly transversely, occasionally exhibiting a spiral configuration or fusing one to each other.

#### Quantitative measurements

Among ventricular myocytes (Table 2), the volume of the SR is slightly higher in the spongy layer than in the compact layer. Within the compact layer, the content of SR relative to the total volume ( $0.022 \pm 0.008$ , mean  $\pm$  SD) or the SR volume relative to the cytoplasm ( $0.032 \pm 0.008$ , mean  $\pm$  SD) is lower when compared with the spongy layer ( $0.025 \pm$

**Table 2** Morphometric and stereologic measurements on Pacific bluefin tuna cardiac myocytes, All the values are expressed as mean±SD in experiments performed on eight individual animals; 70 cells from each tissue sample were considered for stereological measurements, except were indicated

Parameter measured	Ventricle		Atrium
	Compact layer	Spongy layer	
SR volume/total volume ( $V_{SR}/V_{tot}$ )	0.022±0.008*	0.025±0.01*	0.032±0.01*
SR volume/cytoplasmic volume ( $V_{SR}/V_{CYTO}$ )	0.032±0.008*	0.034±0.01*	0.044±0.01*
SR surface area ( $S_{SR}/V_{tot}$ ) ( $\mu\text{m}^2/\mu\text{m}^3$ )	0.25±0.1*	0.27±0.1*	0.37±0.1*
Mitochondrial volume/total volume ( $V_M/V_{tot}$ )	0.28±0.1*	0.25±0.1* <sup>#</sup>	0.25±0.11* <sup>#</sup>
Mitochondrial volume/cytoplasmic volume ( $V_M/V_{CYTO}$ )	0.29±0.1*	0.26±0.1* <sup>#</sup>	0.26±0.11* <sup>#</sup>
Mitochondrial inter-cristae distance (number of cristae/ $\mu\text{m}$ ); $n=30$ cells	13.34±1.7*	18.86±3.36* <sup>#</sup>	19.75±3.5* <sup>#</sup>

<sup>#</sup> Student's *t*-test,  $P>0.05$

\*Student's *t*-test,  $P<0.05$

0.01 and 0.034±0.01, mean±SD, respectively,  $P<0.05$ ). The significant difference between the two layers is also reflected in the surface-to-volume ratio where the values range from 0.25±0.05 in the compact layer to 0.27±0.05 in the spongy layer (mean±SD,  $P<0.05$ ).

As shown in Table 2, the total SR volume (fSR and jSR) in atrial cells is higher than that ventricular cells. The SR volume of atrial cells is significantly higher than that of ventricular cell types resulting in a volume-to-volume ratio of 0.032±0.01 and 0.044±0.01 (mean±SD,  $P<0.05$ ) for the total volume and myofibrillar volume, respectively. The surface density of the total SR membrane system in atrial cells is 0.37±0.1 (mean±SD), but only 1% is occupied by the jSR forming couplings with the sarcolemma. When comparing the densities of the internal fSR and the jSR in the ventricular myocytes of bluefin tuna, a small portion of the SR surface area (0.98% and 0.40% in the atria and ventricle, respectively) is occupied by peripheral couplings. Thus, fSR constitutes most of the volume of the visible membrane system.

Mitochondrial volumes (Table 2) are similar among all cardiac cell types. Ventricular myocytes of bluefin tuna show the highest volume of mitochondria in the heart. In particular, the volume of mitochondria from the compact layer (28%) is the highest among the three types of myocytes. The spongy layer shows the same mitochondria volumes (25%) as the atria, and the same trend is shown in the content of mitochondria relative to the myofibrillar volume (Table 2). Within ventricular and atrial myocytes, the density of the mitochondrial cristae varies in the three cardiac cell types (Table 2). Cells from the atrium display organelles with the highest cristae density (19.75±3.5, mean±SD,  $n=30$  cells), whereas, in the ventricle, the compact layer has a lower number of cristae than the spongy layer (13.34±1.7 and 18.86±3.36, mean±SD,  $n=30$  cells).

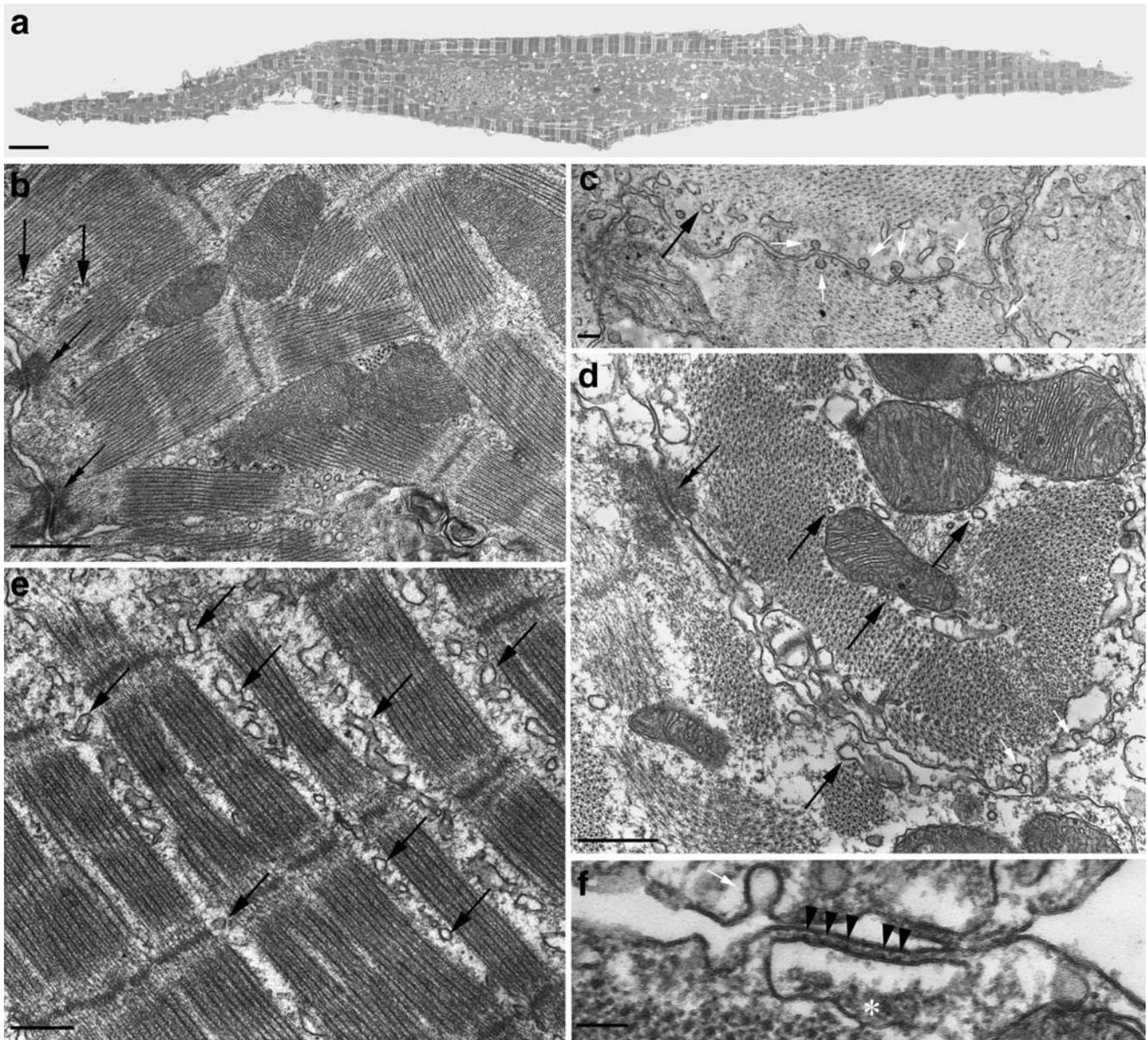
The relevant units of all the values given above are presented in Table 2.

## Discussion

### SR organization in bluefin tuna atrial and ventricular myocytes

In the present study, the heart of the Pacific bluefin tuna has been investigated by electron microscopy, with a particular focus on the content of the SR and mitochondria in atrial and ventricular myocytes. Bluefin tunas are strong continuous swimmers that cross oceanic basins (Block et al. 2005; Kitagawa et al. 2007) and have a high capacity for heat conservation and endothermy (Carey and Teal 1969; Block 1991; Block and Stevens 2001; Brill and Bushnell 2001). The myocardium must be capable of sustaining high levels of cardiac output over a wide range of ambient temperatures and, remarkably, supply an internal body temperature that is often 10–15°C or more elevated from the heart tissue temperatures.

Cardiac e-c coupling in all vertebrates depends on two factors. One is the entry of  $\text{Ca}^{2+}$  from the extracellular space, which follows two main pathways: the L-type  $\text{Ca}^{2+}$  channel (CaV1.2) and the  $\text{Na}^{2+}/\text{Ca}^{2+}$  exchanger, with a predominance of the former in most fishes (Vornanen 1998; Shiels et al. 2000, 2004). A second factor is the  $\text{Ca}^{2+}$  release from intracellular stores, presumably activated by the  $\text{Ca}^{2+}$  influx through CaV1.2 channels. In tuna, both mechanisms are highly represented. Voltage-clamp experiments on isolated ventricular myocytes have demonstrated that the value of the L-type  $\text{Ca}^{2+}$  channel current ( $I_{\text{Ca}}$ ) is species-specific in fish atrial and ventricular myocytes, with active fish, particularly tuna, having the highest  $I_{\text{Ca}}$  values (Tibbits et al. 1992; Vornanen 1997; Shiels et al. 2002a, 2002b, 2004; Vornanen et al. 2002a, 2002b). In the myocardium of most fishes, activator  $\text{Ca}^{2+}$  for cardiac contraction mostly derives from extracellular sources, and this is closely related to the scarcity of SR. From our observations in tuna myocardium, the SR has a relatively high



**Fig. 5** Atrial myocardium from Pacific bluefin tuna. **a** Isolated myocyte from acclimated Pacific Bluefin Tuna showing the overall internal organization of organelles along the longitudinal axis of the cells. **b** In *in situ* atrial myocytes, the myofibrils are frequently organized into an irregular and multidirectional manner, and glycogen granules are sometimes visible (*arrows*). Desmosomes are present at the junctional level of the cell (*double-arrows*). **c** Section stained with lead only highlighting “profiles” belonging to caveolae (*white arrows*

in **c, d**), frequently seen along the undulated plasmalemma of atria myocytes. **d, e** Cross and longitudinal sections, respectively, in which the fSR cisternae are readily recognizable as “empty” rounded profiles (*arrows* in **d, e**) or as a longitudinally oriented network running among myofibrils (*arrows* in **e**). **f** The jSR has a wide oval profile bearing “feet” (*arrowheads*) partially filled by calsequestrin (*white asterisk*). Bars 2  $\mu\text{m}$  (**a**), 1  $\mu\text{m}$  (**b, e**), 0.1  $\mu\text{m}$  (**c, d, f**)

abundance, supporting its functional relevance in the fiber activity cycle. We hypothesize that the extensive SR network in the cardiomyocytes of bluefin tuna indicates that internal  $\text{Ca}^{2+}$  cycling in these fish hearts may have evolved in relation to the high physiological demands of the elevated metabolic rate coupled with increasing tolerances to a wide range of environmental temperatures.

Previous physiological studies have demonstrated that bluefin tunas have a greater heart mass, higher cardiac output and heart rate, and higher concentrations of SERCA-2 than other teleosts (Poupa et al. 1981; Blank et al. 2002, 2004; Landeira-Fernandez et al. 2003; Castilho et al. 2007). This indicates that internal SR  $\text{Ca}^{2+}$  cycling must be of critical importance in the atrial and ventricle myocytes of

yellowfin, skipjack, and bluefin tuna (Keen et al. 1992; Shiels et al. 1999, 2004; Vornanen et al. 2002a, 2002b; Landeira-Fernandez et al. 2003; Blank et al. 2004). Bluefin tuna have the highest level of expression of SR proteins in association with their occupation of a higher latitudinal niche and cooler waters (Landeira-Fernandez et al. 2003). Our study provides morphological evidence for the content and distribution of the SR and indicates a well-developed internal SR system in bluefin myocardial cells. In addition, peripheral couplings represent sites at which this  $\text{Ca}^{2+}$  entry is translated into  $\text{Ca}^{2+}$  release, probably via the calcium-induced calcium-released mechanism (CICR; Fabiato 1983), indicative of bluefin using CICR. In bluefin tuna, peripheral couplings have an unusually large volume and a prominent content of calsequestrin and, thus, are designed to provide a high  $\text{Ca}^{2+}$  capacity. Moreover, the well-developed fSR adds extra pumping and storage capacity. The mutual cooperation between the transsarcolemmal  $\text{Ca}^{2+}$  entry and the internal SR  $\text{Ca}^{2+}$  release at peripheral couplings in bluefin tuna, in addition to the  $\text{Ca}^{2+}$  uptake capacity of the fSR, morphologically underlies the exceptional cardiovascular physiology of these unique oceanic predators. Of interest, the SR content is higher in atrial than ventricular cells in agreement with physiological observations showing a faster rate of contraction and relaxation of atrial compared with ventricular myocardium (Shiels et al. 1999, 2004). A related difference occurs in the amphibian heart in which the ventricle relies entirely on  $\text{Ca}^{2+}$  entry from the plasmalemma, whereas the atrium has a definite internal SR component and  $\text{Ca}^{2+}$  release channels (Niedergerke and Page 1981; Tijskens et al. 2003).

Bluefin tuna cardiac myocytes, like those of other fishes (Santer and Cobb 1972; Berge 1979), lack T-tubules; therefore, the two sources of activating calcium ( $\text{Ca}^{2+}$  entry and  $\text{Ca}^{2+}$  release from peripheral couplings) are both present at the fiber edge. Since the narrow and extended shape of the teleost heart cells decreases the diffusion distance from the sarcolemma to the center of the cell (Vornanen 1997), the small diameter and elongated shape of the bluefin tuna myocytes, as noted here, are of advantage in initiating contraction by limiting the diffusion distance for  $\text{Ca}^{2+}$ . From our observations, the high frequency of caveolae in the tuna myocardium contributes to the high surface-to-volume ratio of the cells, which also results from the small diameter, although it is not clear that they are related to e-coupling.

Despite being endowed with some internal SR, the myocardia of tuna and of other fishes have retained the ancestral functional cellular physiology of relying on a large entry of extracellular  $\text{Ca}^{2+}$  at each initiation of the contraction cycle. In the transition between primitive chordates and higher vertebrates, the myocardium remains in the primitive condition of extracellular  $\text{Ca}^{2+}$  entry,

whereas skeletal muscle acquires independence of extracellular  $\text{Ca}^{2+}$ , even in fish, which lie at the base of the vertebrate phylogenetic tree (Langille and Hall 1989). Tuna, in particular bluefins, have a recent common ancestry of 1.5 million years (C. Reeb, personal communication); thus, in these fishes, the evolution of the SR system and of more advanced myocardial structures are recent in vertebrates suggesting that common selective pressures underly the evolution of SR in birds, mammals, and endothermic fish.

As a comparison within the same taxa, the content of SR in yellowfin tuna myocytes from the ventricular compact layer is almost 10% lower than that in bluefin myocytes from the same layer (A. Di Maio, unpublished observations). This anatomical observation corresponds to the lower metabolic rates and lower SR  $\text{Ca}^{2+}$  ATPase of yellowfin in comparison with bluefin tuna (Blank et al. 2007; Landeira-Fernandez et al. 2003). Few teleost fishes have had quantitative studies carried out on the intracellular membrane systems in their myocardial cells. Among the electron-microscopic studies of the fish myocardium, only Bowler and Tirri (1990) have shown a distinct SR content in acclimated perch ventricular cells, indicating lower volumes of SR than those reported in this study. Thus, because of the lack of reports of stereological measurements of the cardiac SR in wild fish, the content of the internal membrane system of tuna myocardium studied here cannot be compared with that of other teleost species. On the other hand, all previous reports on the ultrastructure of fish myocardium have demonstrated that the internal membrane system of teleosts ranges from completely absent to a well-developed network in active fish (Table 3). In this study, we confirm that, with the evolution of the high metabolic rate and endothermy in the *Thunnus* lineage, there is a concomitant development of SR structure. This indicates that a high frequency heart and high metabolism are associated with the presence of a well-developed internal membrane SR in this active pelagic species.

#### Mitochondrial organization in bluefin tuna atrial and ventricular myocytes

The myocardium of athletic species possesses higher levels of mitochondria than that of sedentary species (Moyes and Hood 2003). Within the ventricle, our ultrastructure observations on mitochondria content show a slightly higher volume in the outer layer, but with a lower cristae density. This is in agreement with biochemical studies on the Atlantic bluefin tuna showing that the outer myocardial layer of juvenile *Thunnus thynnus* exhibits a higher oxidative activity than the inner layer (Modigh and Tota 1975). The high overall volume of mitochondria within the ventricle of the Pacific bluefin tuna is also consistent with

**Table 3** Comparison of the presence of sarcoplasmic reticulum (SR) in fish myocardium (- absent, + poorly developed, ++ well developed, NA not available)

Species	Cardiac SR		Reference
	Ventricle	Atrium	
<b>Teleosts</b>			
Pipefish ( <i>Syngnatus</i> sp.)	-	-	Kisch 1954
Plaice ( <i>P. platessa</i> )	-	NA	Santer and Cobb 1972
Hagfish	+	NA	Bloom 1962; Leak 1969
Icefish ( <i>C. rhinoceratus</i> )	+	NA	Feller et al. 1985
Striped bass ( <i>M. saxatilis</i> )	+	NA	Rodnick and Sidell 1997
Burbot ( <i>L. lota</i> )	+	NA	Shiels et al. 2006
Perch	+	NA	Bowler and Tirri 1990
Rainbow trout ( <i>S. gairdneri</i> )	+	NA	Santer 1974
Lamprey	+	NA	Bloom 1962
Pout ( <i>Gadiculus thori</i> )	+	+	Leknes 1980
<i>Onos cimbrius</i>	+	+	Leknes 1980
Haddock ( <i>M. aeglefinus</i> )	+	+	Leknes 1980
Pike ( <i>E. lucius</i> )	++	+	Midttun 1980
Crucian carp ( <i>C. carassius</i> )	++	NA	Vornanen 1989
Mackerel ( <i>S. scombrus</i> )	++	+	Midttun 1980
Albacore tuna ( <i>T. alalunga</i> )	++	NA	Breisch et al. 1983
Bluefin tuna ( <i>T. orientalis</i> )	++	++	Present study
<b>Elasmobranchs</b>			
<i>Chimaera monstrosa</i>	+	NA	Berge 1979
<i>Scyllium stellare</i>	+	NA	Helle et al. 1983

the measurement of high citrate synthetase activity, indicative of a high aerobic capacity (Blank et al. 2004). The higher density of mitochondrial cristae in the spongy compartment also confirms the elevated oxidative enzymatic capacity, which plays a major role in the catabolism of lactate, having a different oxidative activity and resistance toward acidosis (Basile et al. 1976; Gemelli et al. 1980; Greco et al. 1982). Together these findings indicate another structural specialization at the cellular level of the ventricular myocytes of bluefin tuna in relation to their aerobic capacity and high metabolic rates.

From our observations, atrial cells and spongy layer myocytes have a similar mitochondrial content and density of mitochondrial cristae, indicating equivalent metabolic properties, which is presumably attributable to the same metabolic zonation and regulatory advantages for the heart. Since the atrial and ventricular spongy layer are both perfused by luminal venous blood, the similarity in mitochondrial content between the two tissue compartments may reflect the same oxidative activity.

In agreement with previous reports on the higher enzymatic capacity of bluefin tuna hearts compared with those of yellowfin tuna (Blank et al. 2007), the ventricle of bluefin tuna (in both layers) has a higher mitochondrial volume than that of the yellowfin tuna (A. Di Maio,

unpublished data), confirming the high specialization of the cardiac muscle in the *Thunnus* lineage. Compared with other mammalian ventricular myocytes, the mitochondrial volume content in bluefin tuna cells lies between rabbit ventricle (24%; Barth et al. 1992) and mouse ventricle (37%; Barth et al. 1992; Knollmann et al. 2006). Moreover, comparative studies on ventricular cardiomyocytes of ectothermic vertebrates show a lower content of mitochondria volume (13% in frog and 25% in lizard; Bossen and Sommer 1984) than in bluefin tuna. Interestingly, these values are 5% higher in bluefin tuna atrial myocytes compared with the frog (Bossen and Sommer 1984) and ~1.2% higher than the mitochondria volume in mouse and lizard atrium (Bossen et al. 1978; Bossen and Sommer 1984). These findings suggest that tuna atrial myocardium represents an evolutionary step in the functionality and metabolic activity of cardiac cells, emphasizing the specialization of tuna ventricular fibers to their high metabolic rate. Furthermore, cold adaptation studies on fish demonstrate variations in the level of mitochondria content within the myocardium. Cold acclimated striped bass and icefish exhibit an increased volume of mitochondria at cold temperature (Tota et al. 1991; Rodnick and Sidell 1997), whereas the opposite considerations exist in acclimated perch (Bowler and Tirri 1990).

## Blood pressure

In tuna, more so than in other teleost, high blood pressure is necessary to compensate for energy losses within the prominent retial system and in the tissue vascular beds (Tota 1978). Tuna are challenged in providing blood to the capillary beds at a significant pressure to ensure adequate tissue fluid exchange. Thus, the dorsal aortic blood pressures in tuna, and hence the capillary pressure, are also higher than those in other fishes, and the capillary permeability of protein is reduced. Our findings of a higher inter-capillary distance in the ventricular spongy layer compared with the compact layer may rely on the different metabolism of the inner layer. In addition, since the development of the compact layer may relate to the swimming activity and lifestyle of the fish (Santer and Walker 1980), its volume relative to total ventricular muscle weight is higher than that in the spongy layer (Tota 1978; Breisch et al. 1983; Chugun et al. 1999). Our results confirm that the shorter distance between the capillaries within the outer ventricular layer increases the diffusion of metabolites maximizing the efficiency of contraction.

## Concluding remarks

The results from this electron-microscopic study reveal the high content of SR in the atrial and ventricular myocytes of the Pacific bluefin tuna. This anatomical study provides the morphological basis, in bluefin cardiac myocytes, for the presence of the CICR pathway, which assures a rapid delivery of  $\text{Ca}^{2+}$  to the myofibrils. The evolution of an internal membrane system within the atrial and ventricular myocardial cells contributing to a faster excitation-contraction-relaxation cycle underlies the high maximum heart rates, high cardiac output, and high metabolic rates observed in Pacific bluefin tuna. Additionally, the presence of the SR system may increase the thermal tolerance of the Pacific bluefin to low temperatures. The high specialization in SR function and ultrastructure, coupled with the elevated physiological processes and activity, may play a role in enhancing cardiac performances and permit higher heart rates and cardiac outputs. These factors are most likely crucial for the ecological success and niche expansion of Pacific bluefin tunas.

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