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The effect of stimulation frequency on the transmural ventricular monophasic action potential in yellowfin tuna *Thunnus albacares*

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Monophasic action potentials (MAPs) were recorded from the spongy and compact layers of the yellowfin tuna *Thunnus albacares* ventricle as stimulation frequency was increased. MAP duration decreased with increase in stimulation frequency in both the spongy and compact myocardial layers, but no significant difference in MAP duration was observed between the layers. © 2011 The Authors

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Key words: compact myocardium; endocardium; epicardium; heterogeneity; interval–duration relationship; spongy myocardium.

The ventricles of active fish species have a morphologically distinct outer compact myocardium and an inner spongy myocardium. Although the relative proportion each layer contributes to total myocardial mass has been quantified in a number of species and described in great morphological detail (Santer *et al.*, 1983; Tota *et al.*, 1983; Icardo *et al.*, 2002), there has been little comparison of their electrical properties. In the tunas (family Scombridae), the heart is relatively large and has a high proportion of compact myocardium (40–70%) that is morphologically distinct from the spongy myocardium (Agnisola & Tota, 1994). This makes large scombrids such as tunas excellent models to investigate regional variations in myocardial electrical properties. Differences in action potential duration (APD) between various ventricular regions (*e.g.* epicardium and endocardium) of the mammalian heart have been described, and exaggeration of these differences can lead to potentially lethal re-entrant arrhythmias (Antzelevitch, 2005). Myocardial layer-specific action potential characterization has not been made for the fish heart. The primary aim of this study was to investigate

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the transmural heterogeneity in the ventricular monophasic action potential (MAP) of the yellowfin tuna *Thunnus albacares* (Bonnaterre) and assess its modulation by stimulation frequency.

MAPs are waveforms recorded extracellularly that accurately reproduce the repolarization time course of the transmembrane action potential (Franz, 1999; Knollmann *et al.*, 2001). They differ from electrocardiograms (ECGs) because they only measure the electrical activity of a small group of cells under the active electrode and not the electrical activity of the whole heart. The MAP recordings described in this study were made by pressing a contact electrode against the epicardium or endocardium, while a second electrode acted as the reference electrode. The pressure exerted on the myocardium by the contact electrode deactivates sodium channels, reducing excitation and thereby providing a 'frozen' potential in contrast to the potential found in the unaffected adjacent cells. Therefore, a temporal electrical gradient is produced between the depolarized cells around the electrode and the adjacent cells. This electrical gradient reflects the repolarization time course of mammalian cardiac action potentials with high fidelity (Franz, 1999). The amplitude of a MAP recording, however, depends on contact pressure and tissue type and does not correspond to the amplitude of an intracellular action potential (Franz, 1999).

MAPs can be recorded from different regions and different layers of beating heart preparations and have been widely used in mammalian cardiac research (Babuty & Lab, 2001; Tsuburaya *et al.*, 2007). The use of MAPs as faithful representations of the transmembrane action potential time course in the heart of the rainbow trout *Oncorhynchus mykiss* (Walbaum) has been recently validated (Patrick *et al.*, 2010). In this study, MAP recordings were made from a scombrid (*T. albacares*) heart to assess the ventricular transmural electrical heterogeneity and the impact of pacing frequency.

Thunnus albacares ($n = 4$, mean \pm s.e. 3.9 ± 0.8 kg) were caught in the central north Pacific (in proximity to the Hawaiian Islands) using standard trawling gear and longline techniques. The experiments were performed in shipboard laboratory facilities. Fish were sacrificed by pithing, the heart was removed and placed in oxygenated physiological saline consisting of (in mmol l^{-1}): 185.7 NaCl, 1.1 MgCl_2 , 7.0 KCl, 3.22 CaCl_2 , 10 sodium pyruvate and 10 HEPES; pH of 7.7 with NaOH at 23°C . The majority of atrium was excised to remove myogenic stimulation but the atrioventricular node was left intact. The heart was placed in a modified Langendorff preparation with the bulbus arteriosus cannulated and perfused with oxygenated saline at 23°C . Flow was set to *c.* $25 \text{ ml min}^{-1} \text{ kg}^{-1}$ by varying input pressure. Output pressure was ambient (*i.e.* 0 kPa). The coronary artery was also cannulated and perfused with oxygenated saline. Platinum stimulation electrodes were positioned at the atrioventricular junction to ensure proximity to any remaining atrioventricular node tissue. The heart was stimulated to contract at various frequencies with a 10 ms square stimulating pulse 1.5 V above threshold strength (Student Stimulator, Grass Instruments; www.grasstechnologies.com). All hearts were paced at 0.6 Hz until a stable MAP reading was attained; stimulation frequency was then decreased to 0.4 Hz, returned to 0.6 Hz and then increased to 0.8 Hz. The stimulation frequency was then returned to 0.6 Hz to assess any change in the preparation over time. It was not possible to consistently pace the isolated heart at frequencies higher than 0.8 Hz, suggesting that under the conditions of the experiment the refractory period of the hearts was *c.* 1040 ms. Although

0.8 Hz is below the often reported range of Thunnini heart rates (1.5–4 Hz; Brill & Bushnell, 2001) and slightly below the *in situ* heart rate reported by Blank *et al.* (2002) (*c.* 1.1 Hz at 23° C), a recent *in vivo* study on free-swimming southern bluefin tuna *Thunnus maccoyii* (Castlenau) reported heart rates in the range of 0.34–0.84 Hz (Clark *et al.*, 2008). These data suggest that the frequencies of 0.4–0.8 Hz lie within a physiological range for *Thunnus* spp. at similar temperatures.

Two types of MAP electrodes were tested in this study. The first was a ‘suction’ electrode based on Runnalls *et al.* (1987). Briefly, the contact electrode was made from an Ag–AgCl pellet (Harvard Apparatus; www.harvardapparatus.co.uk) and fed into a microelectrode holder of slightly larger diameter (Harvard Apparatus). The reference electrode, also an Ag–AgCl pellet, was positioned in a hole cut into the side of the holder. Tubing was attached to the side arm and once pressed against the heart ‘suction’ was provided around the contact electrode by a syringe attached to the tubing. A three-way stopcock allowed ‘suction’ to be maintained at a constant level. The second type of electrode, based on Knollmann *et al.* (2001), was constructed from two silver wires (95% purity) 0.25 mm in diameter. The wires were insulated up to 1 mm from the tips by Teflon sleeves, twisted around each other and 3 mm of the distal ends bent by 90° to enhance flexibility. The reference electrode was placed 1 mm proximal from the contact electrode to avoid simultaneous contact with the myocardium. Electrode tips were chlorinated in bleach overnight to prevent direct current offset (Knollmann *et al.*, 2001).

To record MAPs with the ‘suction’ electrodes, a vacuum seal was created on the epicardial surface of the compact layer. Measurements recorded with the wire electrodes were obtained by pressing the contact electrode slightly into the cardiac tissue. Therefore, it is probable that both the pellet and wire electrodes were acting as contact MAP electrodes (Franz, 1999). Spongy myocardial readings were taken with both electrodes by feeding the electrode into the ventricle through the atrioventricular junction and pressing the electrode against the endocardial wall. The powerful contractions of the ventricle hindered the vacuum seal of the pellet electrodes, which prevented accurate recordings. The wire electrodes gave more stable MAP recordings and were used as the preferred method.

The electrodes were connected to an ml136 bio amp (ADInstruments Ltd; www.adinstruments.com) and Powerlab/4sp data acquisition system (ADInstruments Ltd). Groups of 12 MAPs from each stimulation frequency were averaged and the monophasic action potential duration (MAPD) at 25, 50 and 90% repolarization (MAPD25, MAPD50 and MAPD90) was calculated using Clampfit 9.0 (Molecular Devices; www.moleculardevices.com). Significant differences between tissue layers paced at different frequencies were assessed using two-way repeated measures ANOVA (RM ANOVA) followed by Student–Newman–Keuls *post hoc* tests. $P < 0.05$ indicated a significant difference.

Examples of MAPs recorded from both the compact and spongy myocardium at 0.4 Hz and 0.8 Hz are given in Fig. 1. The decrease in MAPD due to an increased pacing frequency can be clearly seen for both myocardial layers. Mean data for percentage change in MAPD25, MAPD50 and MAPD90 for each of the frequencies are given in Fig. 2. The raw data are provided in Table I. No significant difference in MAPD was found between the two myocardial layers at any level of repolarization or pacing frequency.



FIG. 1. Representative recordings of monophasic action potentials (MAPs) from the (a) spongy myocardium and (b) compact myocardium of the *Thunnus albacares* ventricle. Traces are the average of four recordings at a pacing frequency of 0.4 Hz (—) and 0.8 Hz (---). The y-axis has been normalized for both recordings.

The present investigation into the relationship between pacing frequency and MAPD in *T. albacares* found that MAPD is inversely proportional to pacing frequency. This phenomenon, known as the interval–duration relationship, has been previously described in both mammals and fishes (Liu & Antzelevitch, 1995; Harwood *et al.*, 2000; Hanton *et al.*, 2001). The exact mechanism underlying the interval–duration relationship is unknown, but may involve incomplete decay of the delayed rectifier currents (Katz, 2006). Both Vornanen *et al.* (2002) and Galli *et al.* (2009) found evidence for a strong delayed rectifier current (I_{kr}) in *O. mykiss* and pacific bluefin tuna *Thunnus orientalis* (Temminck & Schlegel) heart, respectively, and it is plausible that this current also underlies the interval–duration relationship in *T. albacares*. A similar decrease in APD with increased stimulation frequency has

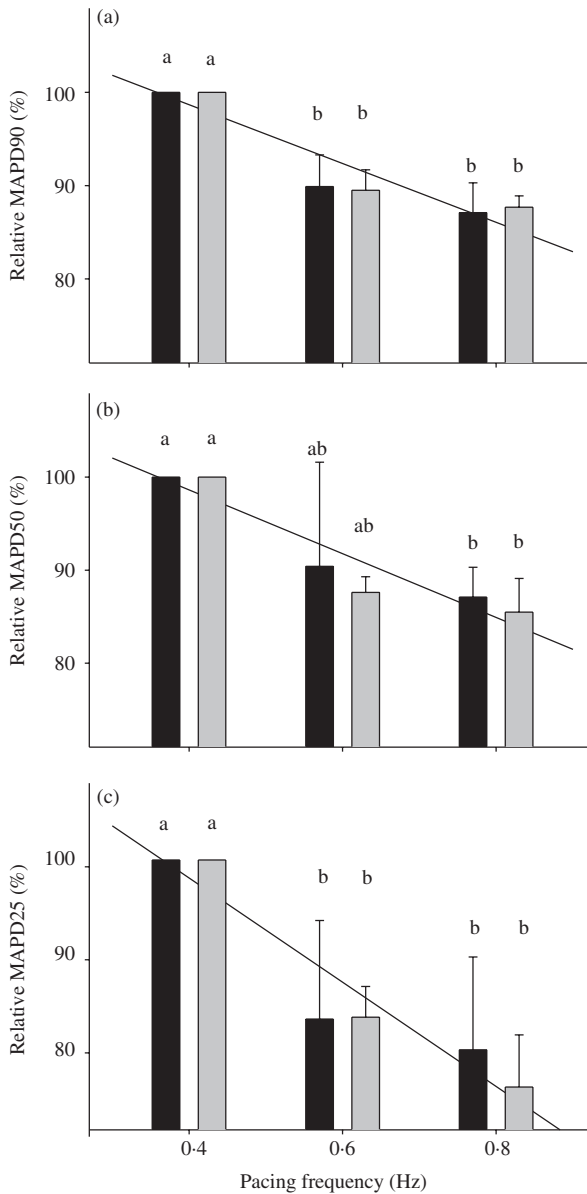


FIG. 2. The effect of frequency on monophasic action potential duration (MAPD) in the spongy (□) and compact (■) ventricular myocardium from *Thunnus albacares* ($n = 4$). The effect of frequency on MAPD is expressed as a percentage change from the normalized value at 0.4 Hz for (a) 25, (b) 50 and (c) 90% repolarization duration (MAPD25, MAPD50 and MAPD90, respectively). Differences were assessed using a two-way repeated measures ANOVA followed by Student–Newman–Keuls *post hoc* tests and are denoted by dissimilar lower case letters ($P < 0.05$). MAP recordings were taken while the heart was initially paced at 0.6 Hz, increased to 0.8 Hz, decreased to 0.4 Hz and then returned to 0.6 Hz to assess the fatigue of the preparation. No significant differences were found between the MAPDs recorded at 0.6 Hz at the start and end of the experiment, therefore these data were pooled into a single set for each heart in this analysis. There were no significant differences between myocardial layers at any given frequency.

TABLE I. The effect of pacing frequency on monophasic action potential duration (MAPD) at 25% (MAPD25), 50% (MAPD50) and 90% (MAPD90) repolarization in ventricular tissue from *Thunnus albacares*

Frequency (Hz)	Absolute duration (ms)					
	Compact myocardium			Spongy myocardium		
	MAPD25	MAPD50	MAPD90	MAPD25	MAPD50	MAPD90
0.4	131.8 ± 21.2	180.9 ± 22.6	260.8 ± 6.0*	136.2 ± 22.4	195.7 ± 21.4	254.4 ± 19.1*
0.6	100.5 ± 7.9	144.5 ± 8.3	212.2 ± 3.4	113.3 ± 16.5	169.3 ± 13.6	222.0 ± 7.6
0.8	90.0 ± 17.3	126.5 ± 18.0	211.3 ± 6.5	106.2 ± 30.7	159.8 ± 22.6	213.9 ± 14.4

Values are the mean ± s.e. of $n = 4$ fish. As there were no significant differences between the MAPDs recorded at 0.6 Hz at the start and end of the experiment the data was pooled into a single set for each heart.

*Significant difference within columns ($P < 0.05$, two-way repeated measures ANOVA with Student–Newman–Keuls *post hoc* analysis).

been shown in isolated myocytes from both *O. mykiss* and zebrafish *Danio rerio* (Hamilton) (Harwood *et al.*, 2000; Brette *et al.*, 2008). The former was related to a frequency-dependent decrease in the L-type Ca^{2+} current (Harwood *et al.*, 2000) and this may also contribute to the effect seen in *T. albacares* heart.

No significant difference was found between MAPD in the two myocardial layers of *T. albacares*, although transmural electrophysiological heterogeneities are common in mammalian hearts (Antzelevitch, 2005). The APD of the mammalian epicardial myocyte is shorter than that of the mammalian endocardial myocyte due to differences in the type and number of ion channels present in each tissue layer (Antzelevitch *et al.*, 1991; Nerbonne, 2000). The physiological relevance of transmural APD heterogeneity in mammals is unclear, but exaggeration of the transmural heterogeneity can lead to lethal re-entrant arrhythmias (Antzelevitch & Fish, 2001). The lack of transmural APD heterogeneity in the fish heart may contribute to its apparent resistance to arrhythmias (Cousins & Farrell, 1996; Patrick *et al.*, 2010).

The delayed rectifier current (I_{kr}), and the transient outward current (I_{to}) are thought to be important for setting the APD in mammalian myocytes and can explain transmural APD heterogeneity in a number of species (Liu *et al.*, 1993; Liu & Antzelevitch, 1995; Katz, 2006). The lack of a transmural change in MAP shape in the *T. albacares* heart may indicate little regional difference in repolarizing ionic currents. No evidence was found of an I_{to} -like notch in the MAP from either the compact or spongy *T. albacares* myocardium, despite the fact that MAP electrodes are capable of resolving action potential notches in mammalian tissue (Franz, 1999). Electrophysiological studies of isolated myocytes from *O. mykiss* (Vornanen *et al.*, 2002), *D. rerio* (Brette *et al.*, 2008) and *T. orientalis* (Galli *et al.*, 2009) also find no evidence of I_{to} , suggesting a relatively small or absent I_{to} in the fish heart.

The difficulties associated with maintaining large scombrids in captivity have limited electrophysiological investigations of their ionic currents. Only two such papers are known in the literature (Shiels *et al.*, 2004; Galli *et al.*, 2009). Galli *et al.* (2009) showed that whole-cell current-clamped action potentials from isolated *T. orientalis* myocytes at 23°C are similar in duration (248.0 ± 19.8 ms mean ± s.e.) to the

present MAP recordings from intact heart tissue. The upstroke of the MAP was slower than that of the intracellular action potential, which is consistent with previous comparisons of MAPs and intracellular action potentials from mammals (Babuty & Lab, 2001). The difference in upstroke velocity is thought to be related to the fact that the MAP is recorded from a small area of tissue around the MAP electrode, rather than a single myocyte, resulting in a sequential alteration in current flow across this area of tissue (Franz, 1999).

The principle findings of this study are (1) the MAP electrode can be successfully used to investigate transmural MAPDs in the hearts of large scombrids, (2) a negative interval–duration relationship exists in the *T. albacares* ventricle and (3) there is no difference in MAPD between the spongy and compact myocardial layers of *T. albacares*. Importantly, MAPs can be successfully measured at sea and therefore could be used to investigate integrated ion flow in fish hearts in the field.

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