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A novel technique for estimating the compact myocardium in fishes reveals surprising results for an athletic air-breathing fish, the Pacific tarpon

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A method for quickly assessing the relative proportion of compact myocardium in the ventricle of teleosts is introduced and used in juvenile Pacific tarpon *Megalops cyprinoides*, a member of the only air-breathing elopomorph teleost genus. The proportion of compact myocardium increased with body mass, reaching up to 60% of the ventricular mass. The finding for tarpon was a surprising discovery since recent literature has suggested that air breathing evolved primarily as means of supplying oxygen to the fish heart during activity. The present data, which represent the first quantitative assessment of the compact myocardium for any air-breathing fish, suggest that myocardial oxygen supply in the tarpon is supplemented by the coronary circulation associated with compact myocardium during exercise, while air breathing is important during aquatic hypoxia. Compact myocardium was also measured as a point of reference in an extant representative from a more ancient fish lineage than the elopomorphs, the water-breathing spiny dogfish *Squalus acanthias* and found to be only 9% of ventricular mass. In conclusion, the presence of a coronary circulation in extant elasmobranchs may mean that the coronary circulation evolved well before air breathing in fishes and, for tarpon at least, the coronary oxygen supply to the ventricular myocardium has not necessarily been superseded by air breathing.

Key words: air-breathing fish; coronary circulation; exercise; heart; *Megalops*; oxygen.

INTRODUCTION

Atlantic and Pacific tarpon *Megalops atlanticus* Valenciennes and *Megalops cyprinoides* (Broussonet) are the only elopomorph teleosts that breathe air (Graham, 1997). Juvenile tarpons reside in freshwater systems that typically become hypoxic, locally or seasonally (Townsend *et al.*, 1992). They regularly breathe air in hypoxic water and when metabolic rate is increased with exercise

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(Seymour *et al.*, 2004). Because they rarely breathe air in normoxic water, however, juvenile tarpons are generally regarded as facultative air breathers, although forced submersion at 22–26° C is reported to result in a deflated air-bladder, negative buoyancy and death after 10 days (Geiger *et al.*, 2000). Tarpons are also the only air-breathing fishes that spend their adulthood in the pelagic marine environment (Graham, 1997; Geiger *et al.*, 2000), where they are prized as sport fishes. Consequently, the athletic tarpon is a good model to examine the suggestion made by Farmer (1997) that the primary selection force for the evolution of air breathing among fishes has been the need to supply oxygen to the myocardium during exercise, rather than aquatic hypoxia.

Farmer's (1997) suggestion builds on two facts. First, the most primitive form for the ventricle in vertebrates has an entirely trabecular myoarchitecture, as found in cyclostomes, and the myocardial oxygen supply is derived entirely from the systemic venous return (termed a type I heart; Tota, 1983; Davie & Farrell, 1991). Thus, for water-breathing fishes, the type I heart is the last organ to receive oxygen from the circulatory system, a situation that contrasts diametrically with mammals, where the heart is the first organ to receive oxygenated blood *via* a coronary circulation. Second, when the bowfin *Amia calva* L. and the garfish *Lepisosteus oculatus* Winchell swim in normoxic water, they progressively increase their rate of lung ventilation with swimming speed (Farmer & Jackson, 1998).

There are two problems, however, with Farmer's (1997) suggestion. One is that extant elasmobranchs possess a coronary circulation (Santer, 1985), which introduces the possibility that the coronary circulation evolved in fishes well before air breathing. This possibility weakens, but does not negate Farmer's (1997) suggestion. A second problem relates to the effect of air breathing on myocardial oxygen supply during either aquatic hypoxia or exercise, which depends on the presence or absence of a coronary circulation. During aquatic hypoxia in water-breathing fishes, oxygen partial pressures both the arterial and venous blood decrease. Air breathing then boosts the oxygen partial pressure in venous blood. Oxygenated blood leaving the air-breathing organ (ABO) will certainly benefit type I hearts if the venous drainage from the ABO returns directly to the heart, which is the case for a large number of air-breathing fishes (Satchell, 1976; Graham, 1997). Nevertheless, such benefits are diminished if air-breathing fishes possess a coronary circulation, which provides a secondary, oxygen-rich blood supply directly to the heart from the gills. Furthermore, the arterial partial oxygen pressure is maintained during exercise, but not during aquatic hypoxia.

Despite the importance of the coronary circulation in air-breathing fishes, nothing quantitative is known about the extent of the coronary oxygen supply to the heart. For fishes in general, Tota (1983) used three additional classifications for the ventricular myoarchitecture to categorize the presence of a coronary circulation supply to the outer compact layer of ventricular myocardium, which encases to varying degrees the inner spongy myocardium (Santer & Greer Walker, 1980). In type II hearts, oxygenated blood from the coronary supply only reaches the compact myocardium. Compact myocardium ranges from 26 to 47% of the ventricular mass for pike *Esox lucius* L., longnose suckers *Catostomus catostomus* (Forster), Atlantic salmon *Salmo salar* L., Arctic grayling *Thymallus arcticus arcticus* (Pallas) and rainbow trout *Oncorhynchus mykiss* (Walbaum), increases with fish age, and varies with water temperature and sexual maturation (Poupa *et al.*, 1974; Cameron, 1975; Farrell *et al.*, 1988; Franklin & Davie, 1992). Thus, if a type II heart was present in an air-breathing fish, the oxygen derived from the ABO would benefit only the spongy myocardium. If an air-breathing fish were to have a type III or IV heart, where the coronary circulation can also perfuse some of the trabeculae of the spongy myocardium (*i.e.* the Thebesian vessels of elasmobranchs and tunas; Tota, 1983), the benefits of oxygenated blood from an ABO would be further diminished.

Clearly, the percentage of compact myocardium in the ventricle represents a critical piece of information in determining the potential benefit of air breathing to myocardial oxygen supply. Qualitative visual observations suggest that the compact myocardium is minimal in the ventricle of the obligate air-breathing lungfishes, *Lepidosiren* sp. (Foxon, 1950) and *Protopterus* sp. (Icardo *et al.*, 2005). Therefore, in accordance with Farmer's (1997) suggestion, it is anticipated that the air-breathing tarpons should have either a type I heart or a type II heart with limited compact myocardium. The air-breathing tarpon, however, does possess a coronary artery with a cephalad origin, negating the possibility of a type 1 heart (pers. obs.), but quantification of the extent of the compact myocardium is lacking. Therefore, in the absence of quantitative information for any air-breathing fishes, the present study tested the prediction that air-breathing Pacific tarpon have a type II heart with a limited compact myocardium. Compact myocardium was also measured as a point of reference in the water-breathing spiny dogfish *Squalus acanthias* L., an extant representative of a more ancient fish lineage than the elopomorphs. Dogfishes also have a compact myocardial layer associated with their conus arteriosus (Santer, 1985), whereas the conus arteriosus is vestigial in tarpon (pers. obs.).

Estimating the compact myocardium is aided by muscle arrangements of the inner spongy and outer compact myocardium, which are histologically quite different. Therefore, relative proportions of compact and spongy myocardium can be assessed using image analysis techniques of varying complexity (Poupa *et al.*, 1974; Cameron, 1975; Santer & Greer Walker, 1980; Egginton & Cordiner, 1997; Clark *et al.*, 2004), but these methods can be time consuming. Dissection techniques (Bass *et al.*, 1973; Farrell *et al.*, 1988) have been used to advantage and can be faster than image analysis. Recently, Simonot (2005) discovered that instead of dissecting cardiac tissue either fresh or after traditional fixation with formaldehyde, the compact muscle layer can be rapidly and easily peeled off the spongy muscle, much like peeling an orange, after the heart is fixed in 70% ethanol. This dissection method to estimate compact myocardium, which takes advantage of the connective tissue layer between the spongy and compact myocardia, is introduced here.

MATERIALS AND METHODS

Juvenile Pacific tarpon were caught in local billabongs near to Darwin, Northern Territory, Australia, either by hook and line or with seines. The four largest Pacific tarpon were obtained from a local wildlife park. Pacific tarpon were transported to

Charles Darwin University where they were held outdoors in recirculating, filtered water at an ambient temperature of 26° C. Body mass of the Pacific tarpon ranged from 86 to 1345 g ($n = 40$ fish). Pacific spiny dogfish were caught using long-lines with baited hooks off Centre for Aquaculture and Environmental Research, West Vancouver, Canada, where they were held outdoors in flowing sea water at an ambient temperature of 10° C. Body mass of the spiny dogfish ranged from 946 to 2378 g ($n = 21$ fish). The fish were not fed during their 1 to 3 weeks in captivity.

The ventricle was removed after the fish had been killed by cranial percussion. Excess tissue (conus and atrium) were carefully trimmed from the ventricle and blood was removed by gentle squeezing and application of tissue paper. Body mass (M) and ventricle mass (M_V) were determined immediately, and the ventricle was placed into 70% ethanol overnight. To remove the compact myocardium from the spongy myocardium, the ventricle was sectioned longitudinally from apex to the ventricular-conal ostium, through the atrio-ventricular ostium. Using a dissecting microscope, the compact myocardium was grasped with fine, curved forceps and peeled away from the trabecular muscle. By starting the procedure in several places on the ventricle, it was often possible to peel off the entire compact myocardium in one piece from one half of the ventricle. The spongy myocardium was inspected for blood, which was removed if present. It was clear from the success of this procedure that the tarpon heart is most likely a type II, with connective tissue separating the compact and spongy myocardial layers. With the spiny dogfish, separation of the two muscle types was more difficult (possibly because of the Thebesian vessels with direct connections from the coronary vessels through the spongy layer into the lumen of the heart) and so smaller pieces of compact myocardium were progressively dissected off the spongy myocardium. As a result, there was probably greater error in the estimate of compact myocardium of spiny dogfish.

The two tissue types were placed in weighing pans and dried in a 60° C drying oven. Dry masses were measured to the nearest 0·0001 g. In seven spiny dogfish, the conus arteriosus was also removed, weighed and the amount of compact myocardium determined in a similar manner as the ventricle.

Relative ventricular mass was determined using wet mass (M_{RVW}) from wet ventricular mass (M_{VW}) and M: $M_{RVW} = 100 M_{VW} M^{-1}$ and from dry mass (M_{RVD}) from dry ventricular mass (M_{VD}) and M: $M_{RVD} = 100 M_{VD} M^{-1}$. Per cent ventricular compact mass ($M_{VC}\%$) was determined using the dry compact (M_{CD}) and spongy (M_{SD}) masses: $M_{VC}\% = 100 M_{CD} (M_{CD} + M_{SD})^{-1}$. For spiny dogfish, similar calculations were made for the conus arteriosus to calculate relative wet and dry conal masses (M_{RCOW} and M_{RCOD}) and per cent conal compact mass $M_{COC}\%$. Per cent moisture of the tissue was calculated from the difference between the dry and wet masses.

RESULTS

TARPON

Neither M_{RVW} nor M_{RVD} varied significantly over the range of M studied here. M_{RVW} averaged 0·0658 ± 0·0018% for tarpon (mean ± S.E., $n = 40$). M_{RVD} averaged 0·0111 ± 0·0003% and moisture was 83·0 ± 0·4% (range 77·1–87·5%).

$M_{VC}\%$ for tarpon ranged between 30 and 60%, increased with M and was described by a quadratic equation [Fig. 1(a)]. While the large fish from the wildlife park had the largest per cent compact myocardium, they also had the smallest M_{RVW} (0·046–0·054%), which could have resulted from them possessing a greater amount of abdominal fat tissue compared with the wild fish.

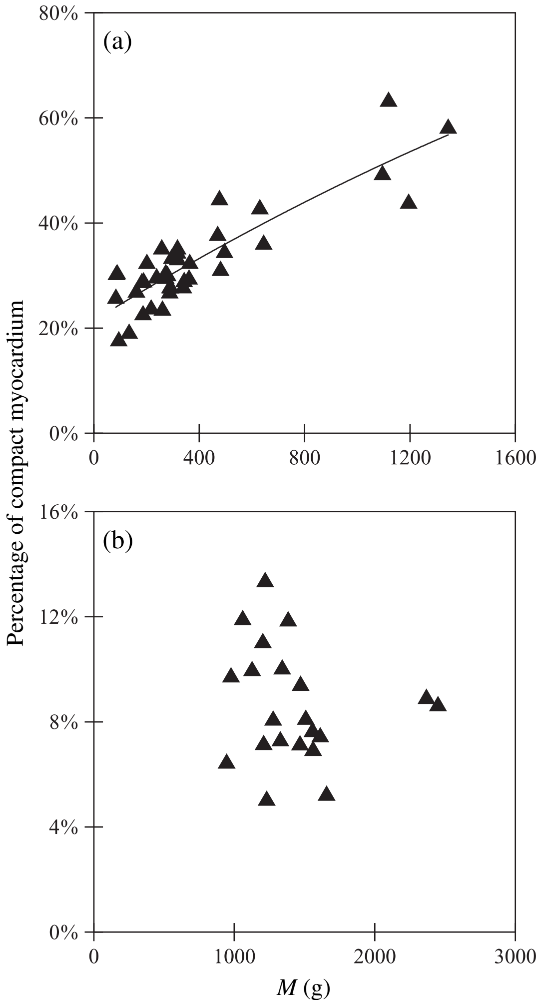

FIG. 1. Compact myocardium in the ventricle of (a) Pacific tarpon and (b) spiny dogfish over a range of body mass. The curve in (a) was fitted by: $y = 0{\cdot}031\ (\pm 0{\cdot}010)x - 3{\cdot}44\ (\pm 7{\cdot}4) \times 10^{-6}x^2 \pm 21{\cdot}42\ (\pm 2{\cdot}36)\ (r^2 = 0{\cdot}76)$. Values in parentheses are S.E.

SPINY DOGFISH

Neither M_{RVW} nor M_{RVD} varied significantly over the range of M studied here. M_{RVW} averaged 0·0836 ± 0·0038% for spiny dogfish weighing 946 to 2378 g ($n = 21$). M_{RVM} averaged 0·0105 ± 0·0003% and moisture was 86·9 ± 0·7% (range 80·4–90·8%).

M_{VC}% averaged 8·6 ± 0·5%, ranging between 5·1 and 13·3% for spiny dogfish weighing 946 to 2378 g, although this was not significantly related to M, unlike in tarpon [Fig. 1(b)].

For a sub-sample ($n = 7$) of spiny dogfish (M ranging from 1201 to 1612 g), M_{RCOW} averaged 0·015 ± 0·001%, M_{RCOD} averaged 0·0018 ± 0·0001% and moisture was 87·7 ± 0·6% (range 85·6–90·1%). Therefore, total conal mass was just under one-fifth (17%) of ventricular mass. M_{COC}% averaged 41·7 ± 0·7%, ranging between 28·1 and 47·8%. Combining the conal and ventricular tissues, the total per cent compact mass of these two tissues was 14·2 ± 0·9%, ranging from 10·5 to 16·2%.

DISCUSSION

METHODOLOGY

The peeling method used here provides a simple, rapid and precise method of estimating the percentage compact myocardium in fish with type II hearts such as tarpon (present study) and rainbow trout (Simonot, 2005). This peeling method was tried with formaldehyde-based fixatives with less success (unpubl. data). Why this is the case is unclear. The peeling method is less effective with type III hearts (*e.g.* the spiny dogfish) and requires more care and time to complete. In the present study, the estimates for type III hearts have more variability compared with type II hearts.

Both M_{RVW} and M_{RVD} were estimated here. The M_{RVD} estimate is obviously the more accurate of the two methods, but it is rarely reported in the fish literature (Bass *et al.*, 1973). An important piece of information gained here is the percentage moisture, which is an indirect measure of the error associated with estimating M_{RVW}. One source of error which will underestimate M_{RVW} is when small hearts begin to dry out in air before weighing. Conversely, it is difficult to remove all blood from the lumen of the heart quickly and consistently before fixation (blood fixed with ethanol is easy to remove) and this will lead to an overestimate of M_{RVW}. Per cent moisture for the ventricle was 83% for common carp *Cyprinus carpio* L. (Bass *et al.*, 1973), 77·1–87·5% for tarpon and 80·4–90·8% for spiny dogfish ventricle, and 85·6–90·1% for the conus of spiny dogfish. This range of variability may be acceptable for general comparisons such as this one, but for studies examining effects of temperature and sexual maturation, which are known to influence M_{RV} and coronary capillarization (Farrell *et al.*, 1988; Graham & Farrell, 1989; Franklin & Davie, 1992; Egginton & Cordiner, 1997; Clark *et al.*, 2004), M_{RVD} rather than M_{RVW} determinations might minimize variability.

M_{RVW} of tarpon (0·065%; range 0·045–0·100%) is at the lower end of the range for teleosts: 0·06–0·12% for rainbow trout, 0·09% for Arctic grayling, 0·08% for longnose sucker and 0·07% for burbot *Lota lota* (L.) (Cameron, 1975; Santer, 1985; Farrell *et al.*, 1988). Water temperature may play a role in these differences given that M_{RV} decreases with warm-acclimation in rainbow trout (Farrell *et al.*, 1988; Graham & Farrell, 1989) and striped bass *Morone saxatilis* (Walbaum) (Rodnick & Sidell, 1997). The M_{RV} of tarpon was slightly lower than that of the spiny dogfish, but tarpon had three-times more compact myocardium than spiny dogfish even when compact myocardium of the dogfish ventricle and conus were combined.

The percentage compact myocardium for spiny dogfish is close to the 14–24% reported for ectothermic sharks (Emery *et al.*, 1985), but lower than the 24·5% reported by Santer & Greer Walker (1980) for the same species and the 31–42% reported for sharks in general (Tota, 1989). Therefore, it may be that the peeling method underestimates compact myocardium in the type III elasmobranch heart.

COMPACT MYOCARDIUM IN AIR-BREATHING FISHES

This study tested the prediction that the air-breathing tarpon would have a type II heart with a small amount of compact myocardium. The per cent compact myocardium (averaging 33% over the *M* range), however, resembles that of similar-sized rainbow trout (30–50%; Farrell *et al.*, 1988), rather than being limited in amount. Furthermore, given the present relationship between compact myocardium and body mass, it is possible that adult tarpon routinely have >60% compact myocardium, values that have previously been associated with the most athletic teleosts [*e.g.* 61·7% for anchovy *Engraulis encrasicolus* (L.) 73·6% for bluefin tuna *Thunnus thynnus* (L.); Santer & Greer Walker, 1980]. A positive relationship between per cent compact myocardium and *M* has earlier been shown for common carp (Bass *et al.*, 1973) and rainbow trout (Farrell *et al.*, 1988), and compact myocardium increases from 5% in juvenile parr to over 30% in adult Atlantic salmon (Poupa *et al.*, 1974). Thus, in rejecting the present prediction, the present results also fail to support Farmer's (1997) suggestion that air breathing evolved among fishes to secure myocardial oxygen supply during exercise, rather than during aquatic hypoxia.

Air breathing in Pacific tarpons would boost oxygen supply to the spongy myocardium, but not necessarily to the remaining 50% of the ventricular muscle in larger individuals. Nevertheless, juvenile Pacific tarpons rarely breathe air in normoxic water whether they are resting (Shlaifer & Breder, 1940) or exercising moderately (Seymour *et al.*, 2004). In fact, during exercise coronary blood flow is expected to increase and enhance oxygen delivery to the heart, as it does with the two-fold increase in coronary blood flow in salmonids (Axelsson & Farrell, 1993; Gamperl *et al.*, 1995). More recent studies on the gas tensions in the ABO of Pacific tarpon under these conditions suggest that oxygen stored in the ABO is not even being removed under normoxic conditions or under moderate exercise and therefore cannot contribute significantly to the myocardial oxygen supply (R. S. Seymour, A. P. Farrell, K. Christian, M. B. Bennett, R. M. G. Wells & J. Baldwin, unpubl. data).

Instead, juvenile Pacific tarpon do breathe air during aquatic hypoxia and they increase air breathing frequency and oxygen uptake through the ABO when exercising under hypoxic conditions (Seymour *et al.*, 2004). This leads to the conclusion that the primary drive for air breathing in juvenile Pacific tarpon is aquatic hypoxia and exercise is a secondary drive. Furthermore, by having a very high percentage of compact myocardium, the Pacific tarpon heart benefits less from air breathing during hypoxia in terms of its myocardial oxygen supply. It is possible, of course, that the increased dependence of the ventricle on the coronary circulation as juvenile Pacific tarpon grow could reflect a decreased reliance on air breathing. This possibility will require further study with adult Pacific tarpon in the marine environment.

Primitive fishes share an ability to tolerate severely hypoxic environments, which is not really surprising considering the many environmental changes these lineages have survived. In terms of cardiac function, Farrell & Stecyk (2007) have argued that the large ventricle of hagfishes and its low myocardial power output results in a routine cardiac ATP demand that can be completely supplied through their glycolytic ATP production during anoxia (hagfishes have a low cardiac ATP demand rather than a greatly elevated cardiac glycolytic generating capacity compared with other fishes). As fishes evolved an increased scope for locomotory activity, the hagfish strategy for cardiac survival during hypoxia was rendered untenable because their myocardial ATP and oxygen demands increased as the ventricle generated a higher blood pressure. The evolutionary solutions to improve myocardial oxygen supply during aquatic hypoxia were either a coronary circulation, air breathing or some combination. The coronary circulation, however, apparently appeared before air breathing in the evolution of the vertebrates, being present in extant elasmobranchs and most ancient fishes. It has cephalad (the hypobranchial artery) as well as caudal (subclavian and coracoid arteries) origins (Parker & Davis, 1899; Grant & Regnier, 1926; Foxon, 1950; Davie & Farrell, 1991). Rays possess both caudal and cephalad coronaries, *Latimeria chalumnae* Smith and some chondrosteans have a caudal supply, other ancient fishes and sharks have a cephalad supply, and teleosts (including tarpons) possess a cephalad supply (although most lack a coronary circulation and a very few have both cephalad and caudal supplies, *e.g.* anguillids and marlins *Makaira* sp.). Whether all elasmobranchs possess a coronary circulation is unknown at this time, but those that have been studied to date do have one (Santer, 1985; Tota, 1989). Foxon (1950) considers the cephalad origin of the coronary circulation, which is the case for elasmobranchs, to be the more primitive vascular arrangement. In addition, elasmobranchs are characterized by a conus arteriosus that also receives a significant coronary supply. The rise of the teleosts saw the replacement of the conus arteriosus with a bulbus arteriosus that lacks a coronary circulation. The tarpons, like other ancient fish lineages, possess both a bulbus and vestigial conus. In fact, large specimens of *Protopterus* sp. and *Lepidosiren* sp. retain a coronary circulation, but one that is more visible on the conus than the ventricle (Foxon, 1950). Thus, a coronary circulation of some form is found in all extant lungfishes and elasmobranchs examined to date.

To conclude, the extent of the ventricular compact myocardium and its associated coronary supply is high in Pacific tarpon by any standards for a fish, and unusually so if oxygenated venous return from the ABO was to be the main means of supplementing venous oxygen supply to the ventricle. The present data suggest that a coronary circulation would benefit the Pacific tarpon heart during exercise in aquatic normoxia, while oxygenated venous return, largely from the ABO, would benefit the Pacific tarpon heart during aquatic hypoxia.

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