



**HAL**  
open science

## Enhanced length-dependent $\text{Ca}^{2+}$ activation in fish cardiomyocytes permits a large operating range of sarcomere lengths

Simon Patrick, Anita Hoskins, Jonathan Kentish, Ed White, Holly Shiels,  
Olivier Cazorla

### ► To cite this version:

Simon Patrick, Anita Hoskins, Jonathan Kentish, Ed White, Holly Shiels, et al.. Enhanced length-dependent  $\text{Ca}^{2+}$  activation in fish cardiomyocytes permits a large operating range of sarcomere lengths. *Journal of Molecular and Cellular Cardiology*, 2010, 48 (5), pp.917 - 924. 10.1016/j.yjmcc.2010.02.008 . hal-01824348

**HAL Id: hal-01824348**

**<https://hal.umontpellier.fr/hal-01824348>**

Submitted on 12 Apr 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Enhanced length-dependent $\text{Ca}^{2+}$ activation in fish cardiomyocytes permits a large operating range of sarcomere lengths

Simon M. Patrick<sup>a</sup>, Anita C. Hoskins<sup>b</sup>, Jonathan C. Kentish<sup>b</sup>, Ed White<sup>c</sup>, Holly A. Shiels<sup>a,\*</sup>, Olivier Cazorla<sup>d</sup>

<sup>a</sup> Faculty of Life Sciences, University of Manchester, 46 Grafton Street, Manchester, M13 9NT, UK

<sup>b</sup> Cardiovascular Division, King's College London British Heart Foundation Centre, London, SE1 7EH, UK

<sup>c</sup> IMSB & MCRC, University of Leeds, Leeds, LS2 9JT, UK

<sup>d</sup> Inserm U-637 Physiopathologie Cardiovasculaire, CHU Arnaud de Villeneuve, 34295 Montpellier, France

## ARTICLE INFO

### Keywords:

Frank-Starling mechanism  
Titin  
Length-tension relationship  
Skinned cardiac myocytes  
Phosphorylation  
Myosin binding protein C (MyBPC)  
Myosin light chain-2 (MLC-2)  
TnI  
TnT

## ABSTRACT

Fish myocytes continue to develop active tension when stretched to sarcomere lengths (SLs) on the descending limb of the mammalian length-tension relationship. A greater length-dependent activation in fish than mammals could account for this because the increase in  $\text{Ca}^{2+}$  sensitivity may overcome the tendency for force to fall due to reduced cross-bridge availability at SLs above optimal myofilament overlap. We stretched skinned fish and rat ventricular myocytes over a wide range of SLs, including those on the descending limb of the mammalian length-tension relationship. We found that fish myocytes developed greater active tension than rat myocytes at physiological  $\text{Ca}^{2+}$  concentrations at long SLs as a result of a higher  $\text{Ca}^{2+}$  sensitivity and a steeper relationship between  $\text{Ca}^{2+}$  sensitivity and SL. We also investigated the diastolic properties of fish and rat myocytes at long SLs by measuring titin-based passive tension, titin isoform expression and titin phosphorylation. Fish myocytes produced higher titin-based passive tension despite expressing a higher proportion of a long N2BA-like isoform ( $38.0 \pm 2\%$  of total vs 0% in rat). However, titin phosphorylation in fish myocytes was lower than in rat, which may explain some of the difference in passive tension between species. The high level of titin-based passive tension and the differential phosphorylation of sarcomeric proteins in fish myocytes may contribute to the enhanced length-dependent activation and underlie the extended range of *in vivo* stroke volumes found in fish compared with mammals.

## 1. Introduction

During the evolution of the vertebrate heart, the main modulator of cardiac output has shifted from stroke volume to heart rate [1]. In many fish, cardiac output is modulated primarily by changes in stroke volume: for example, during intense swimming, the salmonid rainbow trout (*Oncorhynchus mykiss*) can increase stroke volume by up to 300%, whereas heart rate increases by only 50% [2]. By comparison, during maximal exercise in humans, stroke volume doubles while heart rate increases three-fold. The large increase in stroke volume in fish is likely to cause the myocytes to stretch past the optimum overlap of the thick and thin filaments [3]. However, when intact fish myocytes are stretched past sarcomere lengths (SLs) reported as optimal for mammalian active tension development [4], active tension continues to increase [3]. Thus, the fish heart is specialized for large extensions during diastolic filling and for active tension development during systolic emptying from a wide range of SLs. This modification of the Frank-Starling mechanism is advanta-

geous physiologically because fish maintain an ejection fraction close to 100% over the whole range of stroke volumes [5].

The mechanisms responsible for this wide range of functional SLs in fish myocardium are unknown. The morphology of the salmonid cardiac sarcomere is similar to that of the mammalian sarcomere with thin filament length being  $\sim 0.95 \mu\text{m}$  in both rat and salmonid ventricular myocytes [3]. There is no increase in the magnitude of the  $\text{Ca}^{2+}$  transient when fish myocytes are stretched [3], so it is likely that the processes responsible for the wide range of working SLs reside within the myofibrils. Thus, the fish heart represents a good model for studying modulation of cardiac contraction by stretch. Moreover, there is growing use of the zebrafish as a genetically tractable model for vertebrate cardiogenesis [6] and vertebrate cardiac remodelling [7]. Thus, a mechanistic understanding of the intrinsic control systems of the fish heart is important for extending this model to understanding the regulation of contraction in the mammalian heart.

In mammalian hearts, the rise in active tension produced by an increase in SL over the normal SL range (ascending limb of the length-tension relationship) is mainly due to an SL-dependent increase in the  $\text{Ca}^{2+}$  sensitivity of the myofilaments, known as length-dependent activation (LDA) (for review see [8,9]). Despite intense research, the

\* Corresponding author. Tel.: +44 161 275 5092.

E-mail address: holly.shiels@manchester.ac.uk (H.A. Shiels).

mechanisms of LDA remain unclear. It may be partly due to the stretch-induced decrease in myofilament spacing, which encourages the recruitment of force-producing cross-bridges between the thick and thin filaments [10,11], although the precise role of filament spacing is controversial [12]. Whether an SL dependence of  $\text{Ca}^{2+}$  sensitivity also occurs in the fish heart is unknown. A greater LDA in fish than in mammals could overcome the tendency for force to fall due to the reduced number of potential cross-bridges above the optimum for overlap of thick and thin filaments [3]. Alternatively, mammalian and fish myofibrils could show differences in the SL-tension relationship as a result of differences in physical factors (e.g., filament overlap) unrelated to changes in  $\text{Ca}^{2+}$  sensitivity. To examine these possibilities, it is important to test whether fish myocytes show both increased myofilament  $\text{Ca}^{2+}$  sensitivity and increased force production at SL past optimum filament overlap.

To understand the full cardiac cycle, it is necessary to also consider the passive tension developed within the myocyte. The main protein responsible for the development of passive tension in mammalian myocytes is titin [13]. The elasticity of the titin molecule is provided by specific sequences in the I-band region. Of particular importance are two unique sequences named the N2A and N2B regions, the latter being cardiac-specific. Adult mammalian hearts coexpress two main isoforms of cardiac titin: a shorter, stiffer isoform (N2B) and a longer, more compliant isoform (N2BA) (for review, see Linke [14]). The ratio of titin N2B/N2BA has been shown to vary between species and to be a determinant of the stiffness of cardiac muscle: the higher the ratio, the stiffer the muscle [15]. Titin-based stiffness is also modulated by phosphorylation of the N2B element by PKA or PKG, which leads to a decrease in passive force [16]. In addition to its involvement in passive properties, recent studies have suggested that titin also plays a role in LDA by sensing stretch and promoting actomyosin interaction [17,18].

To test the hypothesis that LDA is greater in fish than mammalian myocardium, we compared both the active and the passive properties of rat and fish permeabilized ventricular myocytes over a wide range of SLs, which includes the mammalian descending limb. We found that fish myocytes were more  $\text{Ca}^{2+}$ -sensitive and had a greater LDA. Fish myocytes also developed more titin-based passive tension than rat myocytes at each SL, which could account for the greater LDA.

## 2. Methods

For detailed methods, please see the [Supplementary data](#).

### 2.1. Sarcomere length and force measurements

Skinned myocytes from the trout (*O. mykiss*) ventricle and Wistar rat left ventricle were obtained by mechanical dissociation [19]. A skinned myocyte was attached to a piezoresistive strain gauge and a stepper-motor-driven micromanipulator, as described previously [19]. Experiments were carried out at 22 °C. Force was normalized to cross-sectional area for each cell [19]. The  $p\text{Ca}$  ( $-\log [\text{Ca}^{2+}]$ )-tension relationships were established at four SLs: 2.0, 2.3, 2.5 and 2.7  $\mu\text{m}$ . Steady-state passive tension recordings were also taken at these four SLs before the first activation, and in additional experiments, we measured the passive tension of fish myocytes incubated with the catalytic subunit of PKA. The relative tension- $p\text{Ca}$  relationship was fitted to the modified Hill equation [20] and the  $p\text{Ca}$  for half-maximal activation,  $p\text{Ca}_{50}$ , was calculated as a measure of  $\text{Ca}^{2+}$  sensitivity.

### 2.2. Myofilament protein analysis

Frozen tissue samples of rat, trout, and human biopsies were crushed in a precooled pestle and mortar, homogenized in Laemmli buffer (pH 6.8), and heated for 3 minutes at 100 °C. Titin was separated on agarose-strengthened 2% SDS-polyacrylamide gels

(modified from Hu et al. [21]) at 4 mA for 16–18 h, and myofibrillar proteins of  $M_w$  20–250 kDa were separated on discontinuous 7.5%/15% gradient gels. Phosphorylation and total protein were measured after staining the gels with Pro-Q and SYPRO Ruby (Molecular Probes, Eugene), respectively. Owing to the unequal loading of protein, phosphorylation as assessed with Pro-Q is expressed as a percentage of total protein in the corresponding band as assessed with SYPRO. Intensity of the bands was analyzed using a G:BOX/GeneTools analysis system (Syngene, MD, USA).

### 2.3. Statistics

Data are presented as mean  $\pm$  SEM. One-way ANOVA with Student–Newman–Keul's *post-hoc* (Sigmastat 3.5, Systat software, USA) was used to test for the effect of SL. A *t*-test was used to compare the linear regressions of the  $p\text{Ca}_{50}$  vs. SL data and an *F*-test (Origin Pro 8; OriginLab Corporation, Northampton, MA) was used to compare the  $p\text{Ca}_{50}$  vs. passive tension data. Significance for all tests was taken as  $P < 0.05$ .

## 3. Results

### 3.1. Active tension production in rat and fish ventricular myocytes

Skinned myocytes were held under isometric conditions throughout the experiment at SLs of 2.0, 2.3, 2.5 or 2.7  $\mu\text{m}$ , monitored by video microscopy. Each cell was tested at a single SL because in preliminary experiments, we found considerable rundown in fish myocytes that had been subjected to two sets of activation (data not shown). In addition, activation of fish myofilaments was possible only if cells were isolated from fresh tissue. Slack SL before attachment did not differ between rat ( $1.98 \pm 0.01 \mu\text{m}$ ,  $n = 30$ ) and fish myocytes ( $1.99 \pm 0.01 \mu\text{m}$ ,  $n = 30$ ). Fish skinned myocytes were much thinner than rat myocytes ( $13.3 \pm 3.1 \mu\text{m}$  vs.  $26.6 \pm 2.4 \mu\text{m}$ , respectively; Fig. 1A).

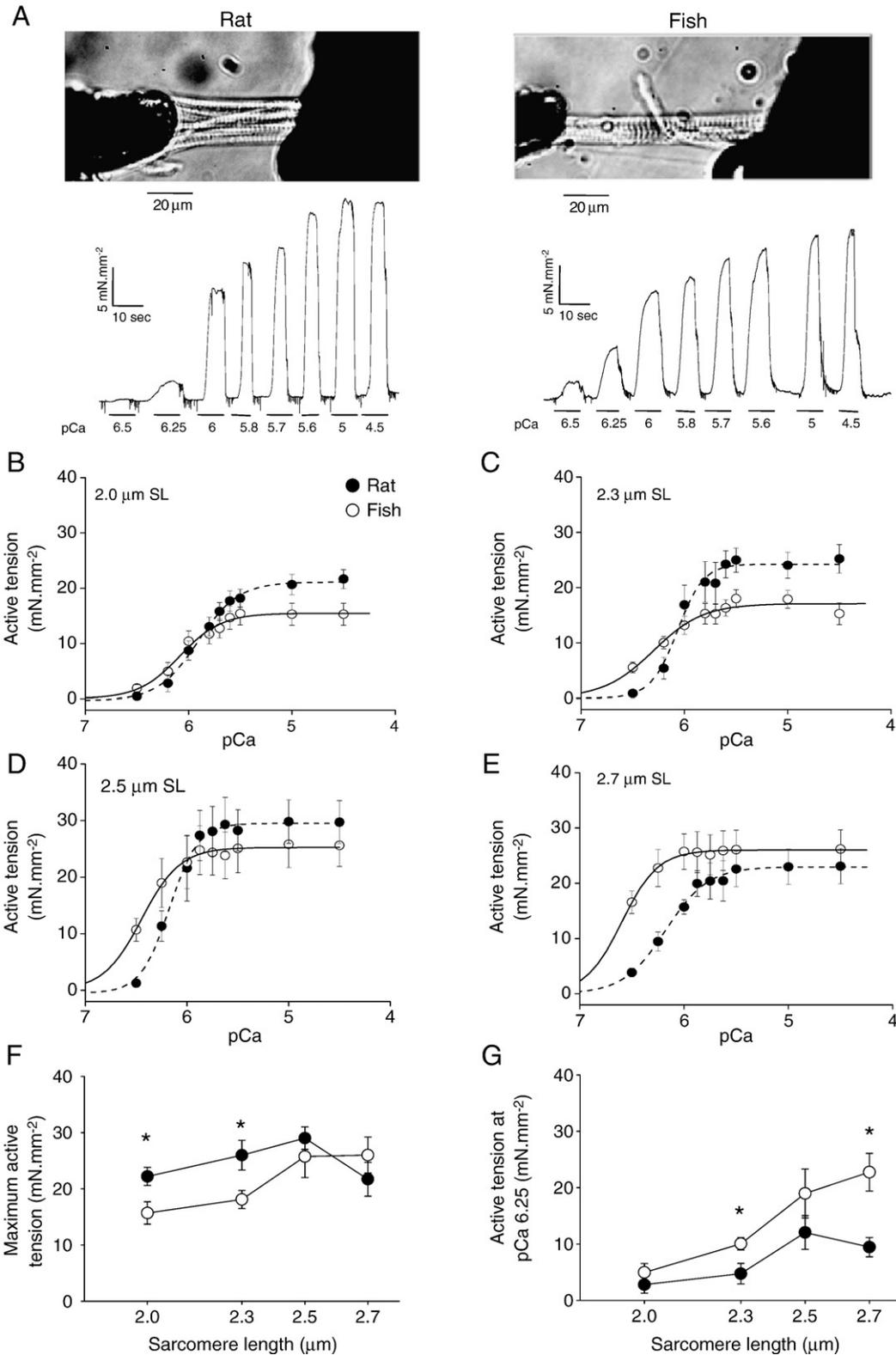
Maximum active tension was significantly greater in rat myocytes than in fish myocytes only at SLs of 2.0  $\mu\text{m}$  and 2.3  $\mu\text{m}$  (Fig. 1 and Table 1). However, maximum active tension continued to rise up to SL 2.7  $\mu\text{m}$  in fish myocytes, whereas that for rat tended to fall after 2.5  $\mu\text{m}$  SL (Figs. 1B–F). A similar trend was observed for the development of active tension at  $p\text{Ca}$  6.25, a physiological  $\text{Ca}^{2+}$  concentration for both the fish and the rat (Fig. 1G). However, at this  $[\text{Ca}^{2+}]$ , the fish myocytes produced significantly more active tension at SLs 2.3  $\mu\text{m}$  and 2.7  $\mu\text{m}$  (Fig. 1G), suggesting a higher myofilament  $\text{Ca}^{2+}$  sensitivity.

### 3.2. Myofilament calcium sensitivity at different sarcomere lengths

Individual data for both rat and fish myocytes were normalized to peak tension, fitted to the modified Hill equation and then averaged (Figs. 2A–D and Table 1). We found that the fish contractile machinery was significantly more sensitive to  $\text{Ca}^{2+}$  than the rat at each SL ( $P < 0.05$ ), as shown by the leftward shift of the fish tension- $\text{Ca}^{2+}$  curves (Figs. 2A–D) and the higher  $p\text{Ca}_{50}$  in fish at all SLs (Fig. 2E). The greatest difference in  $\text{Ca}^{2+}$  sensitivity was found at the longest SL (2.7  $\mu\text{m}$ ), where  $p\text{Ca}_{50}$  for the rat myocytes was  $6.19 \pm 0.07$   $p\text{Ca}$  units compared with  $6.54 \pm 0.03$  for fish myocytes (Fig. 2E and Table 1).

Both the rat and fish cells exhibit length-dependent increases in  $\text{Ca}^{2+}$  sensitivity (Fig. 2E). Fish myocytes exhibited significantly greater LDA than rat myocytes, as indicated by the higher gradient ( $\delta p\text{Ca}_{50}/\delta \text{SL}$ ) for fish ( $0.69 \pm 0.07$ ) compared with that for rat ( $0.41 \pm 0.02$ ;  $P < 0.05$ ) and the stretch-induced shift in myofilament  $\text{Ca}^{2+}$  sensitivity ( $\Delta p\text{Ca}_{50}$ ; Fig. 2F).

The Hill coefficient ( $n_H$ ), an index of cooperativity in the  $\text{Ca}^{2+}$ -activation process, was significantly different only at 2.3  $\mu\text{m}$  SL between rat ( $4.57 \pm 0.65$ ) and fish ( $3.13 \pm 0.88$ ,  $P < 0.05$ ; Table 1). In rat myocytes, the Hill coefficient increased with length from 2.0 to



**Fig. 1.** Active tension development. (A) Examples of a rat (left) and a fish (right) skinned myocyte attached to force transducers accompanied with representative traces of active tension generation at an SL of 2.0 μm. B–E: Ca<sup>2+</sup>-active tension relationships in myocytes from fish (open circles) and rat (closed circles) at 2.0 μm (B), 2.3 μm (C), 2.5 μm (D), and 2.7 μm (E) (*n* = 6–9). (F) Relationship between active tension and SL at maximal Ca<sup>2+</sup> (*p*Ca 4.5). (G) Relationship between active tension and SL at physiological Ca<sup>2+</sup> (*p*Ca 6.25). Note that when SL is increased from 2.5 to 2.7 μm, tension falls in rat but continues to increase in fish. \*Significant difference between species, *P* < 0.05.

2.5 μm SL, suggesting increased molecular cooperativity with length. In trout myocytes, *n<sub>H</sub>* decreased significantly from 2.0 to 2.3 μm SL; no difference was observed between the value at 2.0 and the longer lengths (Table 1).

### 3.3. Passive tension of rat and fish ventricular myocytes

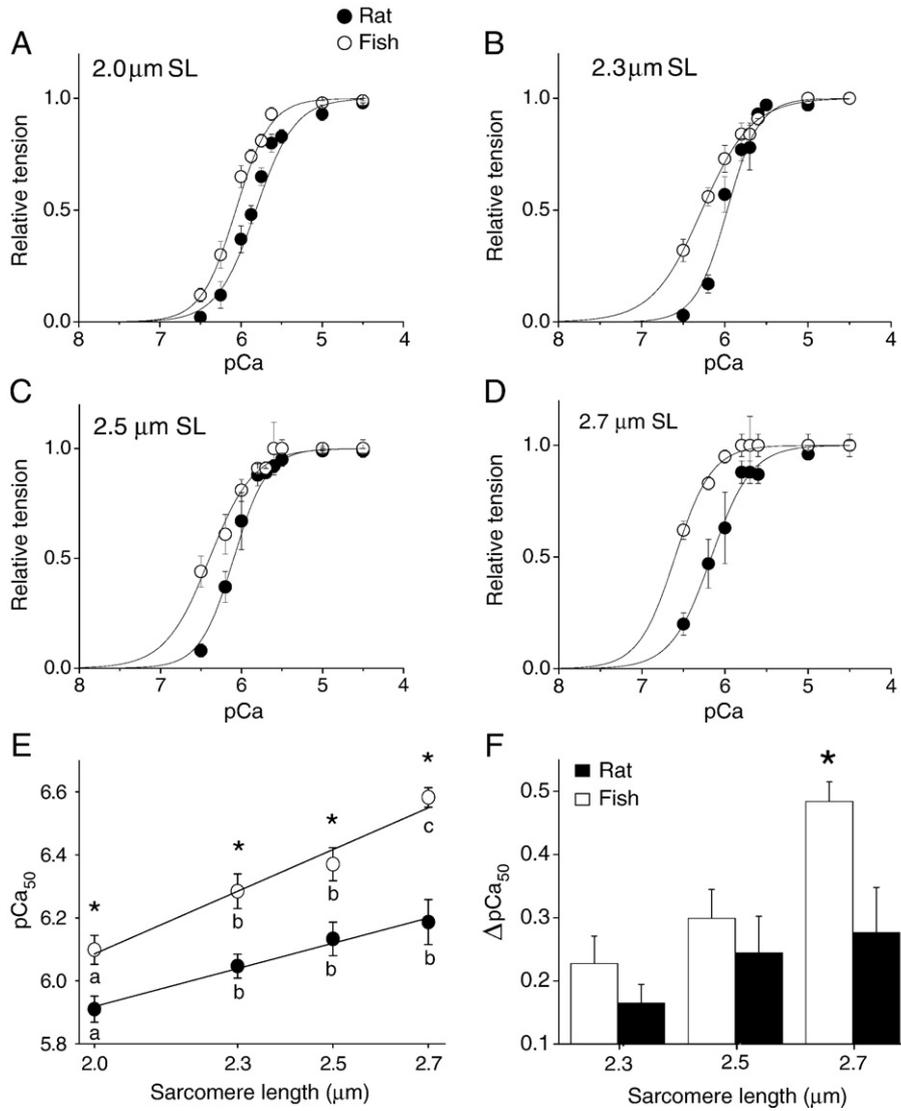
Steady-state passive tension was measured at *p*Ca 9. We found that fish myocytes produced significantly greater passive tension than

**Table 1**  
Mechanical characteristics of permeabilized cardiomyocytes from rat and fish.

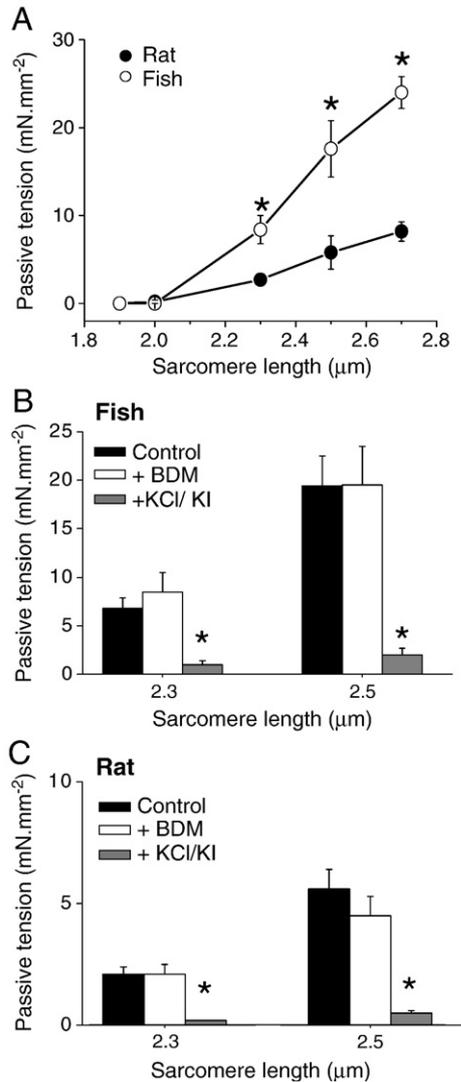
	<i>n</i>	$T_{pass}$	$T_{max}$	$pCa_{50}$	$n_H$
2.0 $\mu$ m SL					
Rat	7	<sup>a</sup> 0.2 ± 0.2	22 ± 1.6	<sup>a</sup> 5.91 ± 0.04	<sup>a</sup> 2.43 ± 0.21
Trout	8	<sup>a</sup> 0.0 ± 0.0	<sup>*</sup> 15.7 ± 2.0	<sup>a,*</sup> 6.10 ± 0.05	2.33 ± 0.13
2.3 $\mu$ m SL					
Rat	8	<sup>a,b</sup> 2.9 ± 0.3	26 ± 2.6	<sup>a,b</sup> 6.05 ± 0.04	<sup>b</sup> 4.26 ± 0.49
Trout	7	<sup>a,b,*</sup> 8.4 ± 1.6	<sup>*</sup> 18 ± 1.6	<sup>b,*</sup> 6.28 ± 0.06	<sup>*</sup> 1.64 ± 0.18
2.5 $\mu$ m SL					
Rat	7	<sup>b,c</sup> 5.8 ± 1.9	29 ± 2.0	<sup>b</sup> 6.13 ± 0.05	<sup>b</sup> 4.57 ± 0.65
Trout	7	<sup>b,c,*</sup> 18 ± 2.9	26 ± 3.7	<sup>b,*</sup> 6.37 ± 0.05	3.13 ± 0.88
2.7 $\mu$ m SL					
Rat	7	<sup>b,c</sup> 8.2 ± 1.1	22 ± 3.0	<sup>b</sup> 6.19 ± 0.07	<sup>a,b</sup> 3.35 ± 0.45
Trout	7	<sup>b,c,*</sup> 24 ± 1.8	26 ± 3.2	<sup>c,*</sup> 6.58 ± 0.03	2.47 ± 0.34

Average Hill ( $n_H$ ) fit parameters of the  $pCa$ -tension relationships (see [Methods](#)) obtained from myocytes at various SLs.  $T_{pass}$  and  $T_{max}$  are the passive tension and maximal active tensions, respectively (in  $mN/mm^2$ ). <sup>\*</sup>Significant difference between species; dissimilar letters represent significant differences within species between SLs ( $P < 0.05$ ).

rat myocytes at SLs above 2.2  $\mu$ m ([Fig. 3A](#)). For example, the passive tension in fish and rat myocytes at SL 2.7  $\mu$ m was  $24.0 \pm 1.8 mN/mm^2$  and  $8.2 \pm 1.1 mN/mm^2$ , respectively. Similar values were obtained when myocytes were stretched to SL 2.0 and 2.3  $\mu$ m at 15 °C rather than at 22 °C ([Supplemental Fig. S1](#)). The difference in passive tension was not due to residual cross-bridges in the relaxed state because passive tension was unaffected when myocytes were incubated for 10 minutes with 30 mM 2,3-butanedione monoxime (BDM), an agent that prevents the formation of strong-binding cross-bridges ([Figs. 3B and C](#)). The contribution of titin was determined after successive incubations with 0.6 M KCl (10 min) and 1 M KI (40 min) [22], which depolymerise thick and thin filaments, respectively [13]. The remaining passive tension is generated by the microtubule network. After KCl-KI treatment most passive tension disappeared and the remaining tension in rat and fish myocytes was similar ([Figs. 3B and C](#)). This indicates that in the fish heart, titin is the sarcomeric protein principally responsible for cellular passive tension development over the physiological SL range, as previously observed in the mammalian heart [13].



**Fig. 2.** Myofilament calcium sensitivity. (A–D) Active tension values normalized to maximal tension from fish (open circles) and rat (closed circles) at 2.0  $\mu$ m (A), 2.3  $\mu$ m (B), 2.5  $\mu$ m (C), and 2.7  $\mu$ m (D) ( $n = 6–9$ ). (E) Myofilament  $Ca^{2+}$  sensitivity, as indexed by the  $pCa_{50}$  values, obtained from relative tension– $pCa$  curves for fish (open circles) and rat (closed circles). Linear regressions were calculated from a scatter plot for both fish and rat. The gradients for fish ( $y = 4.76 + 0.66x$ ,  $r = 0.99$ ) and rat ( $y = 5.11 + 0.40x$ ,  $r = 0.99$ ) were significantly different ( $P < 0.05$ ). <sup>\*</sup>Significant difference between species; dissimilar letters reflect significant difference across SL within a species,  $P < 0.05$ . (F) Stretch-induced modification of myofilament  $Ca^{2+}$  sensitivity, measured as the change in  $pCa_{50}$  between 2.0  $\mu$ m SL and the stretched SL (2.3, 2.5, or 2.7  $\mu$ m). <sup>\*</sup>Significant difference between species,  $P < 0.05$ .



**Fig. 3.** Passive tension. (A) Myocytes from fish (open circles) and rat (closed circles) were stretched in relaxing solution to different sarcomere lengths. (B, C) The source of passive tension for fish (B) and rat (C) myocytes measured before (black bar) and after myocytes were incubated with 30 mM BDM (white bar) to prevent the formation of cross-bridges and the residual active tension component. The myocytes were then incubated with KCl/KI (gray bars) to remove thick and thin filaments and thus titin-based passive tension. \*Significant difference between species in (A) and significant difference from control in (B and C),  $P < 0.05$ .

### 3.4. Myofilament protein analysis

To examine whether the differences in passive tension development could be due to differential titin isoform expression, we examined isoform expression in fish and rat ventricles ( $n = 4$  hearts each). Human ventricular biopsies ( $n = 4$ ) were used as reference for N2B and N2BA isoform expression (Fig. 4A). While the rat heart expresses almost exclusively the stiffer N2B isoform (Fig. 4A), fish myocardium expressed two titin isoforms with mobilities similar to the N2B and N2BA isoforms expressed in human myocardium. The proportion of the long N2BA-like isoform in fish ( $38.0 \pm 2\%$  relative to total full-length titin) was similar to that in human donor myocardium (Fig. 4C). There was no evidence in fish myocardium for titin isoforms longer than N2BA or shorter than N2B that would explain the greater passive tension in trout. The expression of the more compliant N2BA-like isoform in fish, but not in rat, makes it unlikely that the higher passive tension in fish myocytes compared with rat myocytes can be attributed to differential titin isoform expression.

Previous studies reported that passive tension is decreased by titin phosphorylation of the N2B element [16,23]. To examine if a reduced level of titin phosphorylation could contribute to the increased passive tension in fish myocytes, we compared titin phosphorylation in skinned myocytes from fish and rat (Fig. 4D). Relative to the total titin signal, the level of titin phosphorylation in fish myocytes ( $0.44 \pm 0.05$ ; Pro-Q band intensity relative to total protein band intensity) was significantly lower than in rat myocytes ( $0.66 \pm 0.11$ ,  $P < 0.005$ ), which would tend to increase the stiffness of the fish myocytes relative to rat myocytes. Similar results were found using samples of unskinned myocardium (Supplemental Fig. S2), i.e., skinning of fish or rat myocytes did not affect titin isoform ratio or phosphorylation.

To explore whether the lower titin phosphorylation in trout myocytes could account for the higher passive tension, skinned trout myocytes were incubated with the catalytic subunit of PKA to phosphorylate titin *in vitro* (Fig. 4E). Passive tension was reduced by  $\sim 50\%$  at all SL, after PKA treatment. However, this reduction only accounted for one-half of the difference in passive tension between rat and fish.

The phosphorylation levels of MyBPC and TnT (Fig. 5) were not significantly different between skinned fish and rat myocytes. Phosphorylation of MLC<sub>2</sub> was greater in fish myocardium (phosphorylated MLC<sub>2</sub> intensity/total MLC<sub>2</sub> intensity =  $1.27 \pm 0.27$ ) than in rat ( $0.48 \pm 0.08$ ,  $P = 0.03$ ), whereas TnI phosphorylation was smaller in fish myocardium ( $0.25 \pm 0.04$ ) than in rat ( $0.60 \pm 0.09$ ,  $P = 0.003$ ).

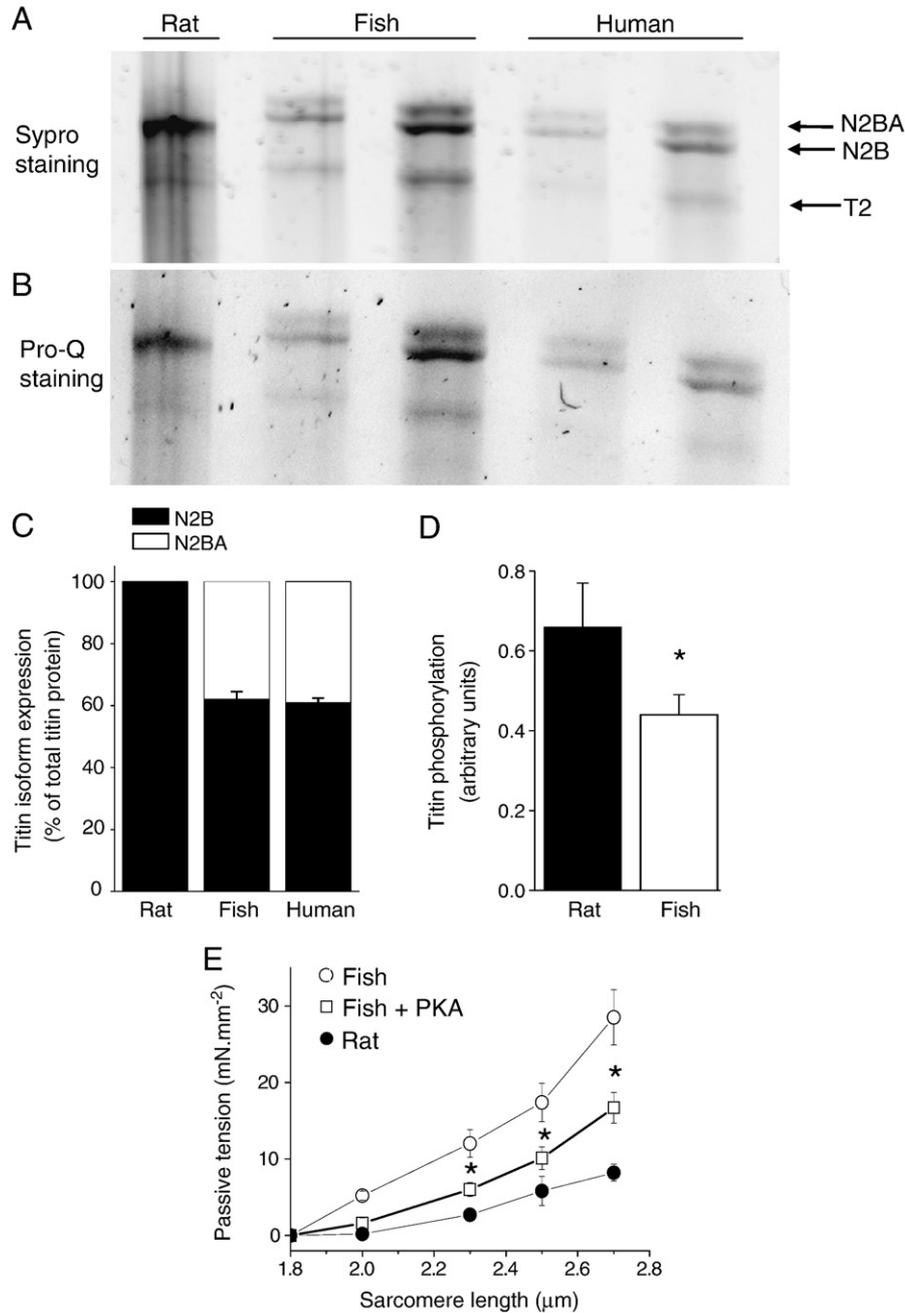
### 3.5. Passive tension dependency of myofilament calcium sensitivity

Titin-based passive tension has been implicated in the LDA mechanism. Thus, the higher passive tension developed by fish myocytes could contribute to the higher myofilament Ca<sup>2+</sup> sensitivity following stretch. Fig. 6 shows  $p\text{Ca}_{50}$  as a function of passive tension independent of SL. We found a positive correlation between titin-based passive tension and myofilament Ca<sup>2+</sup> sensitivity of active tension for both rat and fish myocytes (Fig. 6). This is in agreement with previous studies in the myocardium of mouse [17], rat [18], and guinea pig [20]. These results support the hypothesis that SL-dependent activation is a function of passive tension generated by titin—even in nonmammalian vertebrates.

## 4. Discussion

The key findings of this study are as follows: (1) the contractile machinery of skinned fish ventricular myocytes was more Ca<sup>2+</sup>-sensitive than those of skinned rat myocytes over a wide range of SLs; (2) the Ca<sup>2+</sup> sensitivity of skinned fish myocytes increased as SL increased past optimum myofilament overlap while the Ca<sup>2+</sup> sensitivity of skinned rat myocytes reached a plateau at 2.3  $\mu\text{m}$  SL; (3) LDA (length-dependent activation; SL dependence of Ca<sup>2+</sup> sensitivity) was greater in fish than in the rat myocytes; (4) titin-based passive stiffness of skinned fish myocytes was high, which may contribute to the enhanced LDA; (5) unlike rat, trout expresses a larger (and presumably more compliant) N2BA-like titin isoform, but total titin phosphorylation was lower in fish than in rat; and (6) phosphorylation levels of sarcomeric regulatory proteins, MLC<sub>2</sub> and TnI, were increased and decreased, in fish compared with rat, respectively.

We show that although maximum Ca<sup>2+</sup>-activated tension in fish myocytes was lower than in rat myocytes at short SLs, fish myocytes produced greater active tension at physiological Ca<sup>2+</sup> concentrations ( $p\text{Ca}$  6.25) over the entire SL range (Fig. 1). Our data agree with previous studies that show both cardiac troponin C (cTnC) and skinned muscle preparations from the fish heart are more Ca<sup>2+</sup>-sensitive than those of the mammalian heart [24,25]. The higher myofilament Ca<sup>2+</sup> sensitivity in fish in our study may be partly due to the higher Ca<sup>2+</sup> affinity of fish cTnC [25] but also to the lower phosphorylation of TnI and the higher MLC<sub>2</sub> phosphorylation. Because

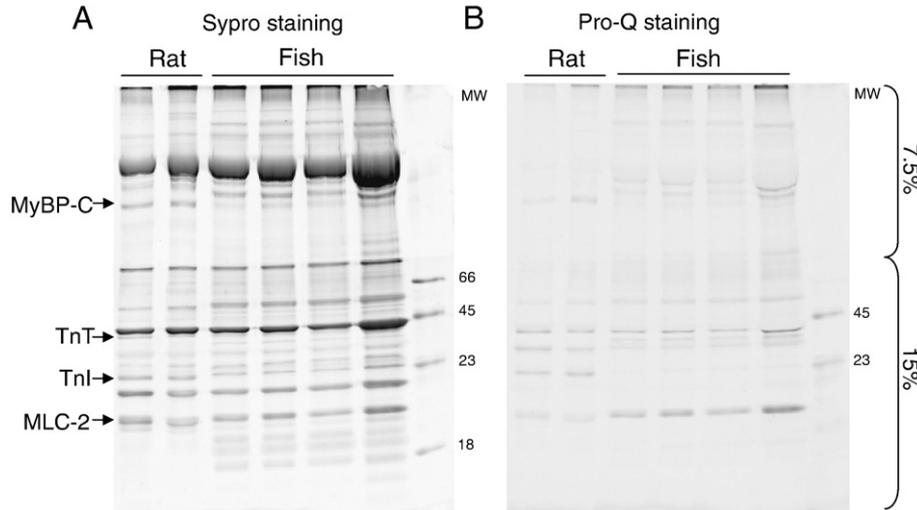


**Fig. 4.** Titin isoform expression and phosphorylation. (A) Example of titin isoform expression in rat, fish, and a human donor heart (determined by SYPRO staining). Note that protein loading is not homogenous between lanes. T2, titin degradation band. (B) Titin phosphorylation (determined by Pro-Q staining). (C) Titin isoform expression in rat, trout, and human donor myocardium. Data are an average of 4 hearts/species. (D) Total titin phosphorylation in intact rat and trout hearts. Titin phosphorylation levels were normalized to total titin protein. a.u., arbitrary units. \*Statistical difference between species,  $P < 0.01$ . (E) Passive tension developed by fish myocytes (open circles) and fish myocytes incubated with active PKA. \* Significant difference between treated and non treated trout myocytes,  $P < 0.05$ . Rat data from Fig. 3A shown for comparison.

the fish heart has to work at lower temperatures (5–22 °C), it is probable that it needs to develop higher active tensions at physiological  $Ca^{2+}$  concentrations to overcome the negative effect of cold on myofilament  $Ca^{2+}$  sensitivity and on the transsarcolemmal  $Ca^{2+}$  influx available for contraction [24,26].

The present study shows that LDA is more pronounced in the fish heart than the mammalian heart. Our data are in accordance with a previous study showing a steep relationship between active tension and length for intact fish myocardium [27]. Studies on skinned cardiac muscle have suggested that LDA is associated with a stretch-induced increase in the  $Ca^{2+}$  affinity of TnC [28,29]. Fish cTnC has 13 sequence differences from that of mammals [30]. A recent study [31] found that, in skinned mouse ventricular myocytes, substitution of native cTnC

with a cTnC containing 4 mutations corresponding to the fish cTnC led to an increase in  $Ca^{2+}$  sensitivity but a decrease in the length dependence of  $Ca^{2+}$  sensitivity. Our results do not confirm this latter finding because, under our conditions,  $Ca^{2+}$  sensitivity in fish myocytes increased over the full range of SLs investigated (up to 2.7  $\mu$ m SL) and displayed a higher SL dependence of  $Ca^{2+}$  sensitivity than rat myocytes (Fig. 2E). TnI has also been reported to be important in determining LDA because its phosphorylation by PKA was associated with enhanced LDA [32] and the presence of cTnI-Pro144 (such as in skeletal muscle) greatly diminished length-dependent properties of the sarcomere [33]. In contrast, we found that the lower TnI phosphorylation in fish was associated with an enhanced LDA. It remains to be determined if, and how, phosphorylation of the various



**Fig. 5.** Sarcomeric protein expression and phosphorylation. (A) A 15%/7.5% acrylamide discontinuous gradient gel showing myofilament proteins. (B) Phosphorylated myofilament proteins from rat and trout ventricles. Relative proportions of 15% and 7.5% acrylamide are shown along with the positions of MyBP-C, TnT, Tnl, and MLC<sub>2</sub>. Protein loading is not homogenous between lanes.

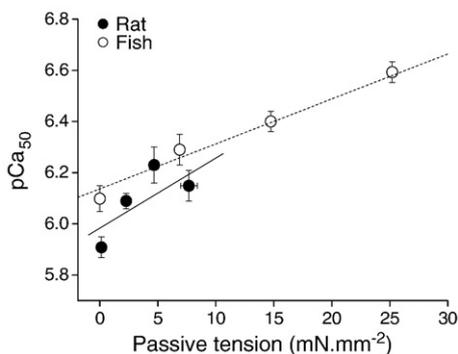
sites in Tnl and of MLC<sub>2</sub> may modulate the SL dependence of Ca<sup>2+</sup> sensitivity of the myocyte.

In this study, the passive tension generated in the fish myocytes was titin-based (Fig. 3B) and significantly higher than in the rat myocytes (Fig. 3A). Our results concur with those of Harwood et al. [27], who found that muscle strips taken from the compact (subepicardial) layer of the fish heart were twice as stiff as rat papillary muscle at muscle lengths optimal for contraction. Our work suggests that the higher stiffness of fish ventricle, compared with rat, may be due in part to the myocytes themselves. This difference between fish and rat was not due to different titin isoforms because fish myocytes expressed a mixture of N2B-like and N2BA-like isoforms (Fig. 4), similar to that found in healthy humans [34], and thus would be expected to be more compliant than the rat myocytes (100% N2B). There was, however, a reduced total phosphorylation of titin in the fish compared with rat myocytes. If titin in fish behaves as in mammals [16,23], the lower level of phosphorylation should enhance the stiffness of fish myocytes relative to rat. To test this hypothesis, we incubated skinned fish myocytes with active PKA. The significantly lower passive tensions measured in these experiments strongly suggests that phosphorylation decreases the passive tension of fish myocytes. However, phosphorylation accounted for no more than one-half of the difference between rat and fish passive tension. Development of passive tension in titin results from the sequential unfolding of Ig domains, PEVK sequence and N2 sequences. Titin

composition is known to be highly variable between mammals owing to alternative splicing. Any variation in the amino acid composition and length of these sequences, which are unknown for trout titin, could lead to differential behaviour under stretch. The longer *T*<sub>50</sub> for stress relaxation observed in trout myocytes (Supplemental Fig. S1) is in favor of specificity of titin in fish. Alternatively, posttranslational modifications that were not investigated in the present study could increase passive tension such as increased oxidant stress [35] or PKC phosphorylation of titin [36]. It is also important to note that because the amino acid sequence of trout titin is unknown, we cannot be sure whether a decrease in phosphorylation is due to less phosphorylation of specific sites or due to fewer proteins being phosphorylated.

A high diastolic stiffness is important in the mammalian heart because it prevents the myocytes from entering the descending limb of their length–tension relationship, upon which the mammalian heart does not function optimally in the steady state [4]. High diastolic stiffness also contributes to elastic recoil during early systole, which may be useful for animals with high heart rates [22]. However, because the fish heart operates at low frequencies and over a larger range of SLs, it seemed likely that the diastolic compliance of the fish myocyte would be low [3]. We found that the trout myocyte was stiffer than the rat myocyte, which differs from our previous findings in intact cells [3]. While the reasons for this discrepancy are unknown, they could include the use of different isolation procedures (in particular, the use of collagenase and proteases for the dissociation of the intact myocytes) and, possibly, a different myocardial origin of the myocytes. It is unlikely that the skinning procedure itself altered the passive stiffness of the myocytes because skinning did not change the isoform ratio or the phosphorylation level of titin (Supplemental Fig. S2). The passive tension values reported for skinned rat myocytes in the present study are lower than previously reported [15,19]. This discrepancy most likely arose because we measured the steady-state passive tension after allowing time for stress relaxation to occur, whereas in our previous reports, we used a ramp stretch. In addition, resting SL was ~1.98 μm for both rat and fish myocytes in the current study, which is longer than that found previously for rat (~1.8 μm), meaning that stretches to similar lengths here would result in lower titin-based passive tension.

Previous evidence suggests that titin may be intimately involved in the SL dependence of Ca<sup>2+</sup> sensitivity [17–19]. We found a positive correlation between titin-based passive tension and Ca<sup>2+</sup> sensitivity of active tension for both rat and fish myocytes (Fig. 6). The enhanced passive tension in fish could therefore offer an explanation for the greater SL dependence of Ca<sup>2+</sup> sensitivity in fish myocytes compared



**Fig. 6.** Passive tension–myofilament Ca<sup>2+</sup> sensitivity relationship. Values of pCa<sub>50</sub> have been averaged according to clusters of passive tension (0–1, 1–3, 5–10, 10–20, higher than 20 mN.mm<sup>-2</sup>). Linear regressions were calculated on a scatter plot for both fish ( $y = 6.14 + 0.018x$ ,  $r = 0.79$ ) and rat ( $y = 5.98 + 0.027x$ ,  $r = 0.58$ ).

with rat (Fig. 3). These results support the notion that SL-dependent activation is a function of passive tension generated by titin and extends to nonmammalian vertebrates.

In conclusion, our data show that skinned fish ventricular myocytes are more  $\text{Ca}^{2+}$ -sensitive than skinned rat ventricular myocytes over a wide SL range. We also show that fish myocytes display a greater SL dependence of  $\text{Ca}^{2+}$  sensitivity (LDA), which may account for the ability of fish to increase force past optimal thick and thin filament overlap at physiological  $\text{Ca}^{2+}$  concentrations. This may offer a sarcomeric mechanism for the exquisite stretch sensitivity of the fish heart [2] and explain the large capacity for stroke volume regulation of cardiac output in these vertebrates.

## Disclosures

None.

## Acknowledgments

This work was funded by the Biotechnology and Biological Sciences Research Council, the Fisheries Society of the British Isles, and the British Heart Foundation (PG/07/067/23323). We gratefully acknowledge Dr David McKenzie for his assistance in acquiring the rainbow trout and allowing us to hold them at the Station Méditerranéenne de l'Environnement Littoral throughout the study.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at

## References

- [1] Shiels HA, White E. The Frank-Starling mechanism in vertebrate cardiac myocytes. *J Exp Biol* 2008;211(13):2005–13.
- [2] Kiceniuk JW, Jones DR. Oxygen-transport system in trout (*Salmo gairdneri*) during sustained exercise. *J Exp Biol* 1977;69:247–60.
- [3] Shiels HA, Calaghan SC, White E. The cellular basis for enhanced volume-modulated cardiac output in fish hearts. *J Gen Physiol* 2006;128(1):37–44.
- [4] Weiwad WKK, Linke WA, Wussling MHP. Sarcomere length-tension relationship of rat cardiac myocytes at lengths greater than optimum. *J Mol Cell Cardiol* 2000;32(2):247–59.
- [5] Franklin CE, Davie PS. Dimensional analysis of the ventricle of an in situ perfused trout heart using echocardiography. *J Exp Biol* 1992;166:47–60.
- [6] Stainier DYR. Zebrafish genetics and vertebrate heart formation. *Nat Rev Genet* 2001;2(1):39–48.
- [7] Sun X, Hoage T, Bai P, Ding Y, Chen Z, Zhang R, et al. Cardiac hypertrophy involves both myocyte hypertrophy and hyperplasia in anemic zebrafish. *PLoS ONE* 2009;4(8):e6596.
- [8] Allen D, Kentish J. The cellular basis of the length-tension relation in cardiac muscle. *J Mol Cell Cardiol* 1985;9:821–40.
- [9] Calaghan SC, White E. The role of calcium in the response of cardiac muscle to stretch. *Prog Biophys Mol Biol* 1999;71(1):59–90.
- [10] Konhilas JP, Irving TC, de Tombe PP. Myofilament calcium sensitivity in skinned rat cardiac trabeculae: role of interfilament spacing. *Circ Res* 2002;90(1):59–65.
- [11] Pearson JT, Shirai M, Tsuchimochi H, Schwenke DO, Ishida T, Kangawa K, et al. Effects of sustained length-dependent activation on in situ cross-bridge dynamics in rat hearts. *Biophys J* 2007;93(12):4319–29.
- [12] Farman GP, Walker JS, de Tombe PP, Irving TC. Impact of osmotic compression on sarcomere structure and myofilament calcium sensitivity of isolated rat myocardium. *Am J Physiol Heart Circ Physiol* 2006;291(4):H1847–55.
- [13] Granzier HL, Irving TC. Passive tension in cardiac muscle—contribution of collagen, titin, microtubules, and intermediate filaments. *Biophys J* 1995;68(3):1027–44.
- [14] Linke WA. Sense and stretchability: the role of titin and titin-associated proteins in myocardial stress-sensing and mechanical dysfunction. *Cardiovasc Res* 2008;77(4):637–48.
- [15] Cazorla O, Freiburg A, Helmes M, Centner T, McNabb M, Wu Y, et al. Differential expression of cardiac titin isoforms and modulation of cellular stiffness. *Circ Res* 2000;86(1):59–67.
- [16] Krüger Martina, Linke WA. Titin-based mechanical signalling in normal and failing myocardium. *J Mol Cell Cardiol* 2009;46(4):490–8.
- [17] Cazorla O, Wu Y, Irving TC, Granzier H. Titin-based modulation of calcium sensitivity of active tension in mouse skinned cardiac myocytes. *Circ Res* 2001;88(10):1028–35.
- [18] Fukuda N, Wu Y, Farman G, Irving TC, Granzier H. Titin-based modulation of active tension and interfilament lattice spacing in skinned rat cardiac muscle. *Pflügers Arch* 2005;449(5):449–57.
- [19] Cazorla O, Szilagy S, Le Guennec JY, Vassort G, Lacampagne A. Transmural stretch-dependent regulation of contractile properties in rat heart and its alteration after myocardial infarction. *FASEB J* 2004;19(1):88–90.
- [20] Ait mou Y, Le Guennec JY, Mosca E, de Tombe P, Cazorla O. Differential contribution of cardiac sarcomeric proteins in the myofibrillar force response to stretch. *Pflügers Arch* 2008;457(1):25–36.
- [21] Hu DH, Kimura S, Maruyama K. Sodium dodecyl sulfate gel electrophoresis studies of connectin-like high molecular weight proteins of various types of vertebrate and invertebrate muscles. *J Biochem* 1986 Apr 1;99(5):1485–92.
- [22] Wu YM, Cazorla O, Labeit D, Labeit S, Granzier H. Changes in titin and collagen underlie diastolic stiffness diversity of cardiac muscle. *J Mol Cell Cardiol* 2000;32(12):2151–61.
- [23] Yamasaki R, Wu Y, McNabb M, Greaser M, Labeit S, Granzier H. Protein kinase A phosphorylates titin's cardiac-specific N2B domain and reduces passive tension in rat cardiac myocytes. *Circ Res* 2002;90(11):1181–8.
- [24] Churcott CS, Moyes CD, Bressler BH, Baldwin KM, Tibbits GF. Temperature and pH effects on  $\text{Ca}^{2+}$  sensitivity of cardiac myofibrils—a comparison of trout with mammals. *Am J Physiol* 1994;267(1):R62–70.
- [25] Gillis TE, Marshall CR, Xue XH, Borgford TJ, Tibbits GF.  $\text{Ca}^{2+}$  binding to cardiac troponin C: effects of temperature and pH on mammalian and salmonid isoforms. *Am J Physiol Regul Integr Comp Physiol* 2000;279(5):R1707–15.
- [26] Shiels HA, Vornanen M, Farrell AP. Effects of temperature on intracellular  $[\text{Ca}^{2+}]$  in trout atrial myocytes. *J Exp Biol* 2002;205(Pt 23):3641–50.
- [27] Harwood CL, Young IS, Altringham JD. Influence of cycle frequency, muscle strain and muscle length on work and power production of rainbow trout (*Oncorhynchus mykiss*) ventricular muscle. *J Exp Biol* 1998;201(19):2723–33.
- [28] Hofmann PA, Fuchs F. Bound calcium and force development in skinned cardiac muscle bundles: effect of sarcomere length. *J Mol Cell Cardiol* 1988;20(8):667–77.
- [29] Wang YP, Fuchs F. Length, force, and  $\text{Ca}^{2+}$ -troponin C affinity in cardiac and slow skeletal muscle. *Am J Physiol Cell Physiol* 1994;266(4):C1077–82.
- [30] Gillis TE, Moyes CD, Tibbits GF. Sequence mutations in teleost cardiac troponin C that are permissive of high  $\text{Ca}^{2+}$  affinity of site II. *Am J Physiol Cell Physiol* 2003;284(5):C1176–84.
- [31] Liang B, Chung F, Qu Y, Pavlov D, Gillis TE, Tikunova SB, et al. Familial hypertrophic cardiomyopathy-related cardiac troponin C mutation L29Q affects  $\text{Ca}^{2+}$  binding and myofilament contractility. *Physiol Genomics* 2008;33(2):257–66.
- [32] Konhilas JP, Irving TC, Wolska BM, Jweid EE, Martin AF, Solaro RJ, et al. Troponin I in the murine myocardium: influence on length-dependent activation and interfilament spacing. *J Physiol* 2003;547(3):951–61.
- [33] Tachampa K, Wang H, Farman GP, de Tombe PP. Cardiac troponin I threonine 144: role in myofilament length dependent activation. *Circ Res* 2007;101(11):1081–3.
- [34] Borbely A, Falcao-Pires I, van Heerebeek L, Hamdani N, Edes I, Gavina C, et al. Hypophosphorylation of the stiff N2B titin isoform raises cardiomyocyte resting tension in failing human myocardium. *Circ Res* 2009;104(6):780–6.
- [35] Grutzner A, Garcia-Manyes S, Kotter S, Badilla CL, Fernandez JM, Linke WA. Modulation of titin-based stiffness by disulfide bonding in the cardiac titin N2-B unique sequence. *Biophys J* 2009;97(3):825–34.
- [36] Hidalgo C, Hudson B, Bogomolovas J, Zhu Y, Anderson B, Greaser M, et al. PKC phosphorylation of titin's PEVK element a novel and conserved pathway for modulating myocardial stiffness. *Circ Res* 2009;105(7):631–8.