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### **Response to 'Crossbridge recruitment by stretching does not invalidate force spectroscopy experiments in living skeletal muscle**

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## LETTERS

## Reply from Vincenzo Lombardi

In his Letter to The Editor Giovanni Cecchi argues that the rapid recruitment of new myosin cross-bridges following a stretch, described in a recent publication in *The Journal of Physiology* by Fusi *et al.* (2010), has no consequence on the validity of the method used by Colombini *et al.* (2007) to estimate both the length of the actomyosin bond and the rate constant of its dissociation. According to Cecchi, the extent of stretches (>13 nm per half-sarcomere (hs)) used by Colombini *et al.* and the range of stretch durations (0.25–4 ms) ensure that the cross-bridge recruitment always attains saturation. Under these conditions the rupture force of the single bond  $F^*$  should still be obtained from the ratio  $F_t/N$ , where  $F_t$  is the rupture force of the cross-bridge ensemble and  $N$  is the number of cross-bridges, provided that the increased (but constant) value of  $N$  is considered. Cecchi concludes that the consequent downward shift of the relation between the rupture force and the logarithm of the loading rate  $r$  (Fig. 4 in Colombini *et al.*) does not affect the bond length  $x_\beta$  (estimated by using eqn (3) of Colombini *et al.* reported in Cecchi's letter as eqn (1)), while it changes the detachment rate constant  $k_0$ , that is, however, a parameter subjected to relatively large errors.

I cannot confirm these conclusions, as detailed here. For simplicity, eqn (3) of Colombini *et al.* can be rewritten as:  $F^* = m \cdot \ln(r) + q$ , where  $m = k_B T/x_\beta$  and  $q = k_B T/x_\beta \ln(x_\beta/(k_0 k_B T))$  are the slope and the intercept, respectively, of the original equation. It follows:  $x_\beta = k_B T/m$  and  $k_0 = (x_\beta \exp(-q \cdot x_\beta/k_B T))/(k_B T)$ . The assumption that the stretch-dependent recruitment doubles the number of cross-bridges implies that both the force and the loading rate per cross-bridge are halved, corresponding to halving the values on the ordinate and shifting the values on the abscissa by subtracting the term  $\ln(2)$ . The slope  $m'$  and intercept  $q'$  of the new relation are  $m' = 1/2 m$  and  $q' = 1/2 q + 1/2 m \cdot \ln(2)$ . It

follows by simple calculus that the change of the number of cross-bridges from  $N$  to  $2N$  increases the bond length  $x_\beta$  by a factor of 2 and does not affect the detachment rate constant  $k_0$ , in contradiction to Cecchi's conclusions.

The practical result of this new analysis is that the bond length calculated from the data of Colombini *et al.* should be 2.5 nm. However, it seems likely that the force ( $F_0$ ) of 10.8 pN attributed by Colombini *et al.* to an isometric force-generating cross-bridge at the temperature of these experiments (14°C) is overestimated because the fraction of attached myosin heads is assumed to be as low as 0.15. In fact, using the relation between  $F_0$  and temperature determined by Decostre *et al.* (2005) for the same frog species used by Colombini *et al.* (*Rana esculenta*),  $F_0$  would be 7.7 pN. If  $F_0$  is smaller by a factor of 1.4 than that assumed in Colombini *et al.*, the correct bond length becomes ( $2.5 \times 1.4 =$ ) 3.5 nm, exceedingly large even in comparison to the values reported from single molecule measurements (Nishizaka *et al.* 2000).

The finding that the relation between  $F^*$  and  $\ln(r)$  determined by Colombini *et al.* in an active muscle fibre is linear and thus resembles the response of an array of parallel elastic bonds is *per se* considered a reason sufficient to assess the validity of the method to estimate the actomyosin bond parameters. However, as admitted by Cecchi, many factors influence the relation. An analytical treatment of these influences, given its complexity, is presently not possible in my hands, but a qualitative discussion on the role of the main factors mentioned by Cecchi is attempted here. The rate of force rise during a ramp stretch should increase, as the stiffness increases with the recruitment of new cross-bridges, and the absence of evidence for this effect in the records published by Colombini *et al.* could be due, according to Cecchi, to the masking effect of the quick force recovery following a length step (Huxley & Simmons, 1971). Indeed, the frequency range of the rate of quick recovery ( $\sim 1000 \text{ s}^{-1}$ ) is adequate to produce a reduction of the force rise in response to a stretch for the range of stretch rates used by Colombini *et al.* However, this

effect cannot simply counteract the increase in the elastic response expected from the recruitment of new cross-bridges because, while the recruitment would be the same for all ramp stretches used, the truncation of force by the quick recovery would be larger the lower the stretch rate. This would produce an overestimate of the slope of the relation of rupture force *versus*  $\ln(r)$  and correcting for this effect would give a bond length even larger than 3.5 nm. More generally, the presence of a quick recovery described by Huxley and Simmons (1971), that readjusts the stress on the myosin cross-bridge for different strains, excludes the possibility to approximate the array of cross-bridges in the half-sarcomere to an ensemble of parallel elastic bonds.

This response is the result of the discussion of Cecchi's letter with Luca Fusi, Gabriella Piazzesi and Massimo Reconditi.

Laboratory of Physiology, Department of Evolutionary Biology, University of Florence Via G. Sansone 1, I-50019 Sesto, Fiorentino Italy

Email: vincenzo.lombardi@unifi.it

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