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**Title:** Evidence of functional deficits at the single muscle fiber level in experimentally-induced renal insufficiency

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## ABSTRACT

Chronic kidney disease patients present with metabolic and functional muscle abnormalities, called uremic myopathy, whose mechanisms have not yet been fully elucidated. We investigated whether chronic renal insufficiency (CRI) affects skeletal muscle contractile properties at the cellular level. CRI was induced surgically in New Zealand rabbits (UREM), with sham-operation for controls (CON), and samples were collected at 3 months post-surgery, following euthanasia. All protocols had University Ethics approval following national and European guidelines. Sample treatments and evaluations were blinded. Maximal isometric force was assessed in 382 permeabilized psoas fibers (CON, n=142, UREM, n=240) initially at pH7, 10°C ('standard' conditions), in subsets of fibers in acidic conditions (pH6.2, 10°C) but also at near physiological temperature (pH7, 30°C and pH6.2, 30°C). CRI resulted in significant smaller average CSA (~11%) for UREM muscle fibers (vs CON,  $P < 0.01$ ). At standard conditions, UREM fibers produced lower absolute and specific forces (i.e. normalized force per fiber CSA) (vs CON,  $P < 0.01$ ); force increased in 30°C for both groups ( $P < 0.01$ ), but the disparity between UREM and CON remained significant. Acidosis significantly reduced force (vs pH7, 10°C  $P < 0.01$ ), similarly in both groups (in UREM by -48% and in CON by -43%,  $P > 0.05$ ). For the first time, we give evidence that CRI can induce significant impairments in single psoas muscle fibers force generation, only partially explained by fiber atrophy, thus affecting muscle mechanics at the cellular level.

## 25 **Introduction**

26 Chronic renal insufficiency (CRI) is a major global health problem expected to affect  
27 40-50% of EU and USA populations (Grams et al., 2013; Zoccali et al., 2010) as well  
28 as millions in Asia (Abraham et al., 2016) and Latin America (Cusumano and  
29 González Bedat, 2008). Skeletal muscle is heavily compromised by CRI (Kaltsatou et  
30 al., 2015; Sakkas et al., 2003a, 2003b) with patients presenting with muscle atrophy  
31 (Kouidi et al., 1998), muscle weakness, limited endurance and fatigue intolerance  
32 (Campistol, 2002), only partly explained by inactivity (Sakkas et al., 2003b).  
33 Collectively described as *uremic myopathy* (Campistol, 2002), this muscular  
34 dysfunction spectrum contributes to patients' high cardiovascular and metabolic  
35 morbidity and mortality (Johansen et al., 2007; Pereira et al., 2015) and inhibits the  
36 efficacy of rehabilitative interventions.

37 Muscle atrophy occurs in various clinical conditions but also in health under  
38 sedentary/unloading or weightless conditions; its functional manifestations and the  
39 evidence of underlying mechanisms are influenced by the study model and the  
40 timeframe of sampling (Malavaki et al., 2015; Riley, 2005). In rat hindlimb  
41 suspension, within 1-4 days, a rapid loss of myofibrillar proteins (Munoz et al., 1993)  
42 is associated with force deterioration. However, after the acute phase, especially when  
43 disease is implicated, it is difficult to delineate whether force reduction may be solely  
44 due to a reduced fraction of contractile proteins within muscle fibers, or because the  
45 available contractile proteins are compromised, or both [for a discussion on 'muscle  
46 quality' see (Fragala et al., 2015)]. Moreover, apart from a differential time-course of  
47 signaling and phenotypic changes as the atrophy-inducing conditions continue  
48 (Malavaki et al., 2015), some ultrastructural and functional changes observed early on

49 [e.g. reduction in myofilament packing (Riley, 2005)], may not be evident at a later  
50 stage [e.g. in human space flight study (Fitts et al., 2010)].

51         Additionally, disease mechanisms may impact function and metabolic  
52 properties without obvious muscle atrophy. In a progressive renal failure animal  
53 model, fast and slow muscle dysfunction occurred without global changes in muscle  
54 mass or physiological cross-sectional area; still, individual fast fiber atrophy was  
55 observed (Organ et al., 2016). However, in another, surgical, CRI animal model no  
56 fiber atrophy was observed but oxidative capacity was affected (Acevedo et al., 2015).  
57 Additional factors such as neuropathy, mitochondrial dysfunction and substrate  
58 availability have been implicated in uremic myopathy [for a review refer to (Adams  
59 and Vaziri, 2006)], indicating that atrophy alone may not fully account for the  
60 observed muscle dysfunction in CRI.

61         Fatigue intolerance in CRI has been also attributed to the development of rapid  
62 acidosis (low intracellular pH) (Johansen et al., 2005; Moore et al., 1993). Low  
63 intracellular pH inhibits muscle contraction at the sarcomeric protein level due to an  
64 effect of H<sup>+</sup> both on the interaction between the motor proteins and on calcium  
65 handling [e.g. (Allen et al., 2008; Fabiato and Fabiato, 1978; Karatzaferi et al., 2008;  
66 Nelson and Fitts, 2014)]. However, the force depressing effect of acidosis declines in  
67 magnitude with increasing temperature towards physiological levels [e.g. (Karatzaferi  
68 et al., 2008; Pate et al., 1995; Westerblad et al., 1997)]. Thus far it is not known if the  
69 uremic muscle's response to acidic conditions is similar to that of a control muscle.

70         Overall, the mechanisms underlying muscle dysfunction in CRI constitute a  
71 difficult aspect to precisely evaluate and are not yet clear. Various interventions  
72 implemented so far, while beneficial (Gordon et al., 2007; Johansen et al., 2006;  
73 Sakkas et al., 2003b) have not fully corrected the functional deficits. Research so far

74 has been mostly performed at the end-stage renal disease and key issues related to  
75 CRI progression and contractile mechanisms are still unanswered. Moreover, it is not  
76 clear whether basic contractile properties are affected, whether whole muscle and/or  
77 individual fiber atrophy is present or not.

78 To answer such questions and to avoid confounding factors encountered in  
79 human patient studies (e.g. comorbidities, dialysis years, pharmaceuticals etc.), we  
80 employed a surgically induced animal model of CRI, maintained with a special diet  
81 (Gotloib et al., 1982). We used permeabilized (skinned) single muscle fibers to assess  
82 the contractile machinery *per se* independently of metabolic and neural factors *in vitro*  
83 (Cooke and Bialek, 1979), under variable conditions of pH and temperature  
84 (Karatzaferi et al., 2008, 2004). We studied fibers from psoas muscle which is  
85 characterized by its homogeneity in fast IIX (II<sub>d</sub>) fibers (Aigner et al., 1993;  
86 Hämäläinen and Pette, 1993) because the fast/glycolytic fibers, especially the most  
87 powerful ones expressing the IIX myosin heavy chain isoform, are more prone to  
88 atrophy in renal disease (Sakkas et al., 2003a; Sawant et al., 2011).

89 We aimed to evaluate, for the first time, the effects of CRI on the contractile  
90 properties of isolated single muscle fibers, focusing on the function of muscle fibers  
91 *per se* independently of possible acute neurological and metabolic abnormalities. We  
92 examined, in a blind design, whether CRI affected the cell's ability to produce  
93 maximal isometric force and whether the contractile 'response' to acute acidosis was  
94 altered, at an earlier stage of renal insufficiency than that of the available patient data.  
95 Moreover, considering the importance of temperature in translating our *in vitro*  
96 findings to *in vivo* function, we examined contractile properties both at the commonly  
97 employed temperature of 10°C and at the closer to physiological temperature of 30°C.

98

99 **Methods**

100 Animal care and experimentation procedures were approved by the Ethics Committee  
101 of the University of Thessaly (decision 2-1/10-10-2012) and the Scientific Committee  
102 of the University Hospital of Larissa, Greece (decision 1/4-1-2012). Animals were  
103 under veterinary care, according to national and EU directives (Directive  
104 2010/63/EU).

105

106 *Animal model*

107 CRI was induced surgically in 6 (N=6) new Zealand young adult female rabbits  
108 (UREM group) using a surgical protocol modified from Gotloib et al. (Gotloib et al.,  
109 1982). Three age-matched animals underwent sham operation (CON group). To  
110 exclude the possibility that the special diet *per se* would affect muscle properties, both  
111 control and uremic animals consumed the special rabbit chow (see Supplement).  
112 Twelve weeks after surgery, animals were sacrificed by injection of sodium  
113 pentobarbital solution (50 mg/ml applied in a dosage of 100 mg/Kg BW) followed by  
114 bilateral thoracotomy. Immediately after cardiac arrest, blood samples were collected  
115 for subsequent determination of serum urea and creatinine using standard photometric  
116 protocols.

117

118 *Muscle Samples*

119 Psoas muscle samples from UREM and CON animals were rapidly excised and  
120 permeabilized as previously described (Karatzafiri et al., 2008) (see Supplement).

121

122 *Experimental setup for single fiber mechanics*

123 Single fibers were dissected from the muscle bundle under a stereomicroscope on a  
124 cold stage and the fiber ends were attached between two tissue mounts of a  
125 customized micro-dynamometer (SI Heidelberg/WPI). Data were continuously  
126 recorded and later exported for further analysis.

127 The micro-dynamometer system (see Supplement) allowed for rapid  
128 temperature-jumps (t-jumps). The advantage of the t-jump is that by initially fully  
129 activating a fiber at low temperatures (here, 10°C), the sarcomere arrangement was  
130 stabilized before being briefly exposed to a higher, near physiological temperature  
131 (here, 30°C), generating maximal isometric force with less possible damage to the  
132 sarcomere arrangement (Karatzaferi et al., 2004) (for an indicative experiment see  
133 Figure 1).

134

#### 135 *Experimental solutions*

136 Basic rigor buffer contained: 120mM KAc, 5mM MgAc<sub>2</sub>, 1mM EGTA and 50mM  
137 MOPS (pH7) or 50mM MES (pH6.2). Relaxing solution: with addition of 5mM ATP.  
138 Maximal calcium activation: with addition of 1.1mM CaCl<sub>2</sub> (see Supplement). The  
139 ionic strength of the solutions was ~0.2M.

140

#### 141 *Maximum Isometric Force measurements*

142 All assessments and initial data reductions were done in a blind fashion. Average  
143 diameter was determined for subsequent cross-sectional area (CSA) calculations  
144 assuming a cylindrical shape.

145 Maximum isometric force (Po) was first evaluated at standard resting  
146 conditions (pH7, 10°C), at resting sarcomere lengths (2.2-2.4 μm). A number of fibers  
147 were subsequently activated at pH7 and 30°C. A subset of fibers underwent an



148 assessment at 10°C, in both resting (pH7) and acidic (pH6.2) conditions while fewer  
149 fibers were also assessed at pH6.2, 30°C. To avoid an order effect, fibers were  
150 randomly assigned to be first activated in pH7 and then in pH6.2 and *vice versa*.  
151 Lastly, fibers were re-assessed in initial conditions (to fulfill criteria of stability, i.e.  
152  $\leq 10\%$  initial force decline). We also assessed velocity of contraction using the load-  
153 clamp method in a subset of fibers at 10 °C (see Supplement).

154

#### 155 *Statistical analysis*

156 Force data distribution was tested using Kolmogorov-Smirnov test of normality. Due  
157 to the normal data distribution, statistical analysis was performed using parametric  
158 tests. Descriptive (Mean $\pm$ SD) and Inferential (SEM and exact 95% CIs) statistics of  
159 absolute and specific forces, as well as percentage force values are reported. A  
160 General Linear Model (GLM) analysis was performed to examine main effects of  
161 independent variables and their interaction and also provided pairwise comparisons.  
162 To examine possible differences in the response to either temperature or pH changes,  
163 force change was calculated in percentages of initial standard conditions and the  
164 differences between groups were tested using t-test for independent samples. All  
165 statistical analyses were performed using a commercially available statistical package  
166 (SPSS 15.0). The significance level was set at  $P < 0.05$ .

167

#### 168 **Results**

169 Surgery procedures were well-tolerated and animals had a normal after-surgery  
170 recovery. Twelve weeks post-surgery, BW ranged between 1,970-4,585 and 3,500-  
171 4,965 gr for UREM and CON animals respectively ( $P > 0.05$ ), with higher serum

172 creatinine ( $2.67 \pm 1.15$  vs  $1.38 \pm 0.09$  mg/dl ,  $P < 0.05$ ) and urea levels ( $67.33 \pm 32.02$  vs  
173  $40.67 \pm 4.62$  mg/dl,  $P > 0.05$ ) in UREM vs CON.

174

#### 175 *Cross –sectional area*

176 Calculated cross sectional areas (CSAs) of UREM fibers ( $n=240$ ,  $5,040 \pm 1,189 \mu\text{m}^2$ )  
177 were significantly lower compared to CONs ( $n=142$ ,  $5,671 \pm 1,259 \mu\text{m}^2$ ),  $P < 0.001$ .

178 Thus, the results were analyzed for both absolute and specific force values (i.e. force  
179 values normalized for fiber CSA to appraise force data independently of fiber  
180 atrophy).

181

#### 182 *Contractile properties in resting conditions (pH7)*

183 Single psoas fibers (CON  $n=142$ , UREM  $n=240$ ) were maximally calcium-activated  
184 at  $10^\circ\text{C}$ , pH7 ('standard conditions'). Some fibers were also assessed at  $30^\circ\text{C}$ , pH7  
185 (CON  $n=41$ , UREM  $n=73$ ) using the t-jump method. Descriptive and inferential  
186 statistics are presented in Table 1, Figure 2 and in the text.

187 Whether on absolute or specific force values, GLM analysis indicated a  
188 statistically significant main effect of *group* [ $F(1,492)=83.6$ ,  $P < 0.001$  and  
189  $F(1,492)=33.1$ ,  $P < 0.001$  respectively], *temperature* [ $F(1,492)=114.6$ ,  $P < 0.001$  and  
190  $F(1,492)=108.2$ ,  $P < 0.001$  respectively] as well as a significant interaction *group x*  
191 *temperature* [ $F(1,492)=19.9$ ,  $P < 0.001$  and  $F(1,492)=10.5$ ,  $P=0.001$ ] for absolute and  
192 specific forces respectively.

193 The pairwise comparisons revealed consistent functional deficits in UREM  
194 fibers compared to CONs. At the standard conditions, absolute isometric force (Figure  
195 2A) of UREM fibers was significantly lower (vs CON,  $P < 0.001$ ). After normalizing

196 force values for fiber CSA, UREM fibers were also found to produce on average  
197 significantly lower specific force (vs CON,  $P<0.01$ ) (Figure 2B).

198 The t-jump caused an expected significant force rise in both groups ( $P<0.001$ ).  
199 Still, UREM fibers produced significantly lower forces ( $P<0.001$ ) than CON, at 30°C,  
200 pH 7, for both absolute and specific forces, (Figure 2). After expressing the force  
201 response to the t-jump as a percentage of a fiber's own baseline force at pH7, 10°C,  
202 UREM fibers appeared to gain more, as their average temperature-induced force  
203 increase was 2.2 fold that of CONs ( $+167\pm170\%$  vs  $+76\pm42\%$ ,  $P<0.005$ ) without  
204 however remedying the significant force disparity between groups.

205

#### 206 *Contractile properties in acidic conditions (pH6.2)*

207 Lowering the pH, from 7 to 6.2, caused an expected significant force reduction in both  
208 groups ( $P<0.005$ , in 25 CON and 48 UREM fibers). Specifically, absolute and specific  
209 forces at pH6.2, 10°C, were for UREM fibers,  $211\pm125\ \mu\text{N}$  and  $46\pm26\ \text{mN}/\text{mm}^2$  & for  
210 CON fibers,  $316\pm116\ \mu\text{N}$  and  $55\pm20\ \text{mN}/\text{mm}^2$ , respectively. Whether on absolute or  
211 specific force, GLM analysis indicated a statistically significant main effect of *pH*  
212 [ $F(1,451)=22.9$ ,  $P<0.001$ , and  $F(1,451)=21.7$ ,  $P<0.001$  respectively], with the main  
213 effect of *group* being again significant [ $F(1,451)=18.6$ ,  $P<0.001$  and  $F(1,451)=5.1$ ,  
214  $P<0.05$  respectively], but the interaction of *group*  $\times$  *pH* was non-significant  
215 [ $F(1,451)=0.003$ ,  $P>0.05$ , and  $F(1,451)=0.028$ ,  $P>0.05$  for absolute or specific force  
216 respectively].

217 The pairwise comparisons (using Bonferroni adjustments) showed that at  
218 10°C, pH6.2, the absolute isometric force (Figure 3A) of UREM fibers was  
219 significantly lower (vs CON,  $P<0.05$ ) roughly by approx. -33%. UREM fibers

220 produced somewhat lower normalized isometric forces, roughly by approx. -17%  
221 (Figure 3B) but non-significantly (vs CON,  $P>0.05$ ).

222 After expressing the force response to the change of pH as a percentage of a  
223 fiber's own  $P_o$  at standard conditions (pH7, 10°C) the average % force decline due to  
224 the pH change tended to be larger for UREM fibers, albeit non-significantly (-  
225  $48\pm14\%$  vs  $-43\pm9\%$ ,  $P=0.06$ ).

226 The temperature effect at pH6.2 was also assessed in a subset of fibers  
227 (UREM= 21, CON=13). Because it was not possible to lower the pH while at 30°C,  
228 these data were not included in the global statistical assessments mentioned above.  
229 The absolute and specific force values at pH6.2, 30°C, were for UREM  $550\pm248\ \mu\text{N}$   
230 and  $114\pm52\ \text{mN/mm}^2$  & for CON fibers  $700\pm238\ \mu\text{N}$  and  $117\pm33\ \text{mN/mm}^2$ ,  
231 respectively. The effect of the t-jump at pH6.2 was also expressed as percentage of  
232 force achieved at pH6.2, 10°C. The average temperature-induced force increase for  
233 UREM ( $+219\pm160\%$ ) and CON fibers ( $+143\pm62\%$ ) did not differ significantly  
234 ( $P>0.05$ ).

### 235 *Velocity of contraction*

236 When examining the force-velocity relationship at 10°C (see Supplement), UREM  
237 muscle fibers (n=32) produced slower velocities compared to CON (n=15) in both  
238 resting and acidic conditions. As a result,  $V_{\text{max}}$  of UREM fibers at either pH7 or  
239 pH6.2, was ~50% that of CON respectively.

240

## 241 **Discussion**

242 To the best of our knowledge this is the first study to examine the effects of  
243 renal insufficiency on the contractile properties of single skeletal muscle fibers, under

244 resting and acidic (fatigue) conditions. We used methodology that is not acutely  
245 confounded by pervasive neural or metabolic abnormalities but instead focuses on  
246 fiber function *per se*, independently from the extent of whole muscle atrophy. We  
247 found significantly impaired, absolute and specific, isometric force at the single fiber  
248 level, in muscle fibers from an animal model mimicking CRI. This functional deficit  
249 was thus only partially explained by fiber atrophy and was persistent under ‘resting’  
250 and ‘fatigue’ conditions, under near physiological temperature conditions indicating a  
251 lower ‘muscle quality’ in UREM fibers. We also observed some evidence of slower  
252 velocities of contraction in UREM fibers (see Supplement) albeit in a small number of  
253 fibers. We thus provide compelling evidence of CRI-induced effects on single fiber  
254 mechanical properties.

255         We observed significantly smaller CSAs in UREM psoas fibers compared to  
256 CON (by ~11%) in agreement to human studies. In end-stage disease, muscle atrophy  
257 is ~ 27% (Sakkas et al., 2003b) affecting mostly the fast type IIA and IIX muscle  
258 fibers (Sakkas et al., 2003a; Sawant et al., 2011). Recently Acevedo et al., (Acevedo  
259 et al., 2015) reported no evident atrophy in the tibialis cranialis (a mixed fast hindlimb  
260 muscle) of surgically-induced uremic rats. In contrast, Organ et al., (Organ et al.,  
261 2016) reported atrophy in all fiber types of the extensor digitorum longus (EDL,  
262 another, mixed, fast muscle) of Cy/+ uremic rats while the overall mass and  
263 physiological CSA of the whole muscles remained unchanged. Together these and our  
264 results [in a larger species and a muscle expressing >95% IIX(IIId) myosin, (Aigner et  
265 al., 1993; Hämmäläinen and Pette, 1993)] could indicate a muscle type specificity of  
266 fiber atrophy in the early stages of CRI. Our findings are consistent with human  
267 studies reporting muscle atrophy in advanced kidney patients (Johansen et al., 2003;  
268 Sakkas et al., 2003a; Sawant et al., 2011) and suggest that atrophy could appreciably

269 affect fast muscles which are normally tasked to provide high levels of muscle power,  
270 earlier during the disease progress.

271 Atrophy is expected to result in lower absolute muscle force as less available  
272 cross-bridges per fiber will be generating tension at any given time. That doesn't  
273 mean that muscle quality, which is the "physiological functional capacity of muscle  
274 tissue" (Fragala et al., 2015) would be necessarily affected. The force-generating  
275 capacity relative to the muscle's CSA may or may not remain the same (Fragala et al.,  
276 2015), depending on the extent of exposure to the atrophic stimulus and the ability of  
277 the organism to adapt on prevailing conditions (Malavaki et al., 2015). In a 2-week  
278 suspension and fixed muscle length animal model, a near 60% atrophy in individual  
279 soleus muscle fibers was accompanied by a 17% specific force reduction; the  
280 concomitant increase in shortening velocity was attributed to an altered thick and  
281 thin filaments' packing (Riley, 2005). The latter mechanism however, appeared to be  
282 a transient response during the early phase of disuse atrophy; in a recent human space-  
283 flight study, soleus atrophied by 20%, shortening velocity decreased, and myofilament  
284 packing density increased (Fitts et al., 2010). In the present study we hadn't assessed  
285 filament packing and the literature so far doesn't report whether disease-induced  
286 atrophy may be linked to alterations in filament packing. Additionally, ageing and/or  
287 disease mechanisms may impact function and metabolic properties without a direct  
288 link to atrophy, thus affecting overall muscle quality (Fragala et al., 2015).

289 We found that at resting conditions, UREM fibers produced significantly  
290 lower absolute and specific forces compared to CONs, by 25% and 14%, respectively  
291 at 10°C (a standard *in vitro* testing temperature), and by 40% and 28% respectively, at  
292 30°C (a closer to physiological temperature). Thus in our study the modest degree of  
293 atrophy of uremic fibers only partially accounted for the force deficit. Organ et al.,

294 (Organ et al., 2016) in a 35-week Cy/+ rat model, reported a 21% reduction in  
295 absolute ankle dorsiflexion torque despite unchanged whole muscle dimensions.  
296 However, they noted individual fiber atrophy (roughly 20%) in the studied EDL  
297 mixed fast muscle. Our finding of an 11% atrophy in psoas muscle fibers is similar.  
298 However, since no single fiber mechanics were assessed in that study, how the  
299 individual fiber's force-generating capacity may have been affected in their model, is  
300 unknown. Notably, no fiber type shift was observed in the Organ et al study, further  
301 highlighting the complexity of mechanisms underlying reduced muscle quality in  
302 disease.

303         Muscle contraction is temperature sensitive (Ranatunga, 2010) and in  
304 agreement to previous reports (Coupland et al., 2001; Karatzaferi et al., 2008, 2004;  
305 Pate et al., 1995) both UREM and CON fibers substantially increased force generation  
306 in response to a t-jump from 10°C to 30°C. At pH7, force rise in CON fibers was  
307 ~76% in agreement to others using rabbit psoas skinned fibers (Coupland et al., 2001;  
308 Pate et al., 1995). Although the temperature-induced force increase was percent-wise  
309 higher in UREM, force at 30°C remained significantly lower in UREM vs CON  
310 fibers. Thus, absolute and specific force deficits of the UREM fibers observed at  
311 standard *in vitro* conditions held also true at a near physiological temperature.

312         The deficit in UREM fibers' specific force could indicate a reduced capacity  
313 to generate force per myosin cross-bridge or a lower number of active cross-bridges  
314 (Fitts et al., 1991; Karatzaferi et al., 2004) and the slower velocities could indicate a  
315 slower cross-bridge cycle (see Supplement). Possible disturbances on force  
316 transmission across the sarcomeric arrangements may also be implicated, such as  
317 changes in viscoelastic properties [e.g. in human chronic heart failure (Miller et al.,  
318 2010)] or filament packing [e.g. in human space flight (Fitts et al., 2010)]. Other data

319 from our group show increased protein carbonylation and other redox disturbances  
320 (Poulianiti et al., 2015), which could foreseeably cause structural modifications  
321 affecting the actomyosin interaction. One way would be via glycation, as oxidative  
322 stress could also promote the formation of advanced glycation end-products, AGEs  
323 (Miyata et al., 1997), which in studies of reversible glycation (Ramamurthy et al.,  
324 2003, 2001), have been indicated to cause glycation-related structural alterations in  
325 myosin affecting the *in vitro* motility speed.

326         Fatigue intolerance in kidney disease is associated with the rapid development  
327 of acidosis (Johansen et al., 2005; Moore et al., 1993). Acidosis alone or in  
328 combination with other ‘fatigue’ metabolites, contributes to force reduction (Allen et  
329 al., 2008; Karatzaferi et al., 2008; Nelson and Fitts, 2014). Here, the drop of pH  
330 reduced force by approx. 45% for both groups, in agreement to others (Cooke et al.,  
331 1988; Karatzaferi et al., 2003; Pate et al., 1995). The acidosis effect may be less  
332 pronounced at near physiological temperatures in single fibers (Karatzaferi et al.,  
333 2008; Pate et al., 1995), but still significant. However, the functional consequence of  
334 an acidosis-induced force reduction, coupled with a slower contractile velocity (see  
335 Supplement), could prove worse for uremic muscles *in vivo*. One can fathom that in  
336 acidosis, even during rest or with low exercise intensities (Johansen et al., 2005),  
337 uremic muscles could be at a severe functional disadvantage.

338         Our study had some limitations. Despite implementing the same surgical  
339 approach, a large variability in uremic psoas fibers’ contractile properties was  
340 observed. Also, UREM fibers protein extracts could not be resolved in SDS-PAGE  
341 (data not shown). Moreover, in retrospect, UREM fibers were more difficult to dissect  
342 and handle; due to the blind design, a ‘positive’ bias was probably inadvertently  
343 introduced; given standard criteria for force data quality [e.g. (Karatzaferi et al., 2003;



344 Liang et al., 2008)], it later transpired that relatively more UREM fiber data were  
345 excluded from statistical analysis than CON. Such discarded UREM fibers could be  
346 described as ‘mussy’ and ‘sticky’. Based on the above, possible changes in passive  
347 elastic properties may warrant further study [e.g. changes in titin or nebulin may be  
348 implicated (Horowitz et al., 1986)], as skeletal muscle viscoelastic properties  
349 changes, such as reported in heart failure patients (Miller et al., 2010; Toth et al.,  
350 2012) could be possible in our model. Advanced glycosylation has also been  
351 associated with glycation of type IV collagen of endothelial cells in ESRD  
352 (Thornalley and Rabbani, 2009) and further changes in overall muscle elastic  
353 properties cannot be excluded.

354 Main strengths of our study included: the use of the single fiber technique,  
355 which allows the assessment of fiber function isolating factors such as muscle  
356 atrophy, energetics or excitation-contraction coupling issues; the blind design; the use  
357 of sham-operated controls; the t-jump approach (which provides physiological  
358 relevance). Moreover, our model developed CRI for 3 months, i.e. a sufficient period  
359 considering a rabbit’s lifespan, making our results more relevant to human chronic  
360 disease. In the future, the stretch-release force response, possible changes in  
361 viscoelastic properties, or post-translational modifications of key sarcomeric proteins,  
362 such as myosin, titin and nebulin, should be assessed and associated with further  
363 functional assessments.

364 In conclusion, experimentally-induced renal insufficiency led to significant  
365 functional impairments in single psoas fibers’ mechanics, only partly explained by  
366 fiber atrophy. Our observations, if verified in human tissue, could help explain key  
367 aspects of functional problems observed in patients.

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370 **Conflict of interest statement**

371 The authors declare no conflict of interest.

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**Figure 1.** Indicative example of single fiber force recording. A fiber initially immersed in a relaxing solution was transferred (first arrow) in an activating solution (pH6.2, 10°C); once a steady state force was reached, the fiber was transferred (second arrow) in another activating solution (pH7, 10°C) and was allowed again to reach a steady state force. Following the low temperature activation, the fiber was briefly transferred (third arrow) in a pH7 activating solution at 30°C (t-jump). The fiber was returned to a relaxing solution of the pH of interest and reactivated to verify stability (not shown). The order of exposure to different conditions was random. Up to five maximal activations were possible.

**Figure 2.** Isometric force for CON (open bars) and UREM (filled bars) psoas muscle fibers. Data collected at 10°C and 30°C pH7 are presented as Mean values with exact Upper and Lower 95% CIs for: A. absolute and for B. specific isometric forces. At pH7, UREM fibers produced lower isometric forces compared to CON in all conditions even after correcting for muscle atrophy \* Denotes significant difference from corresponding 10°C value ( $P < 0.001$ ); † Denotes significant differences from corresponding value of CON fibers ( $P < 0.01$ ).

**Figure 3.** Effect of pH on isometric force for CON (open bars) and UREM (filled bars) psoas muscle fibers. Data collected at resting (pH7) and acidic (pH6.2) pH, at 10°C, are presented as Mean values with exact Upper and Lower 95% CIs for: A. absolute and for B. specific isometric forces. Lowering of pH caused significant reductions in absolute and specific forces in both fiber groups; At pH 6.2 UREM fibers produced significantly lower absolute forces compared to CON but the difference between groups was non-significant after correcting for muscle atrophy \*

Denotes significant difference from corresponding pH7 value ( $P < 0.005$ ); † Denotes significant difference from corresponding value of CON fibers ( $P < 0.05$ ).

Figure 1  
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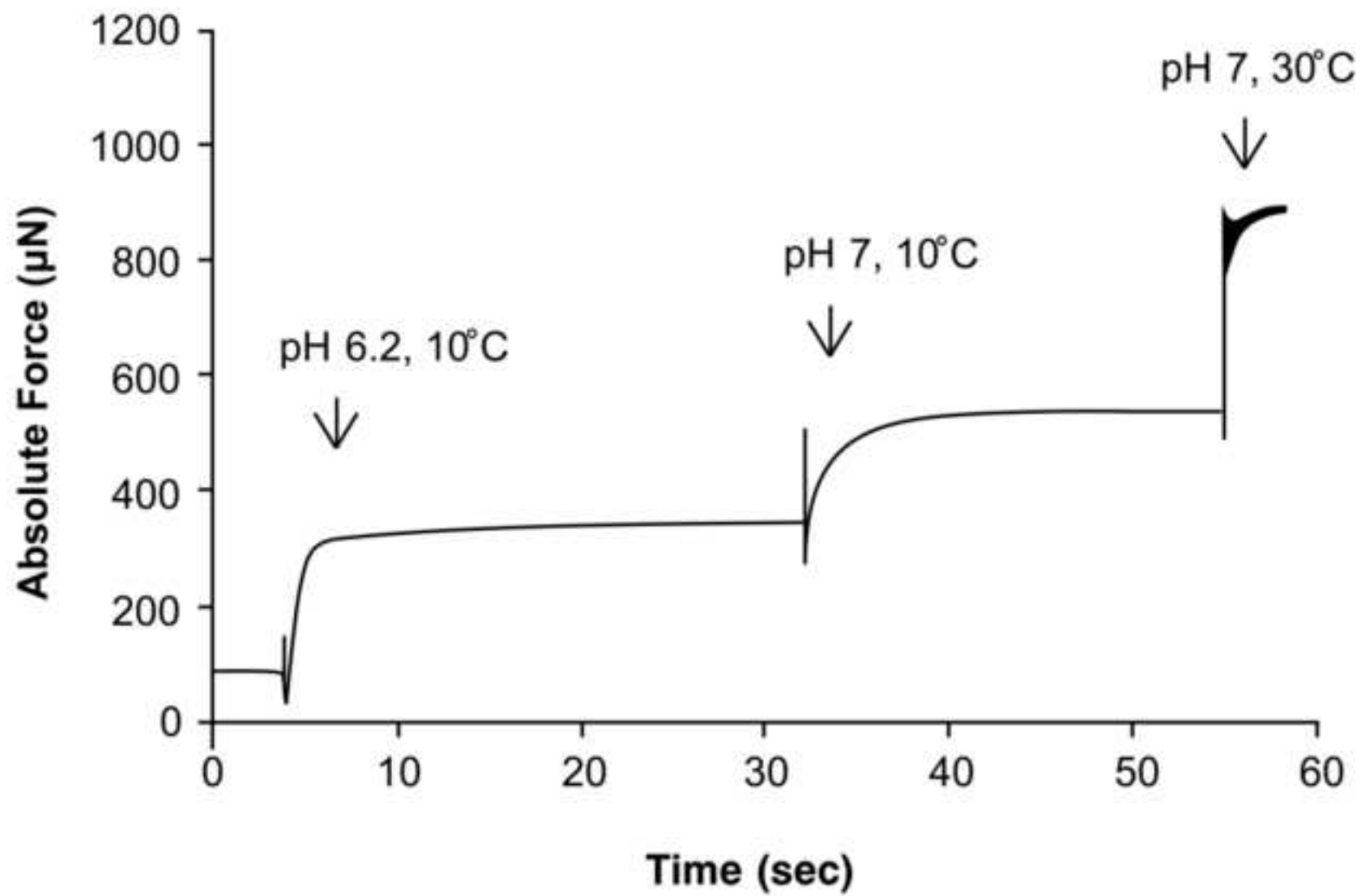


Figure 2  
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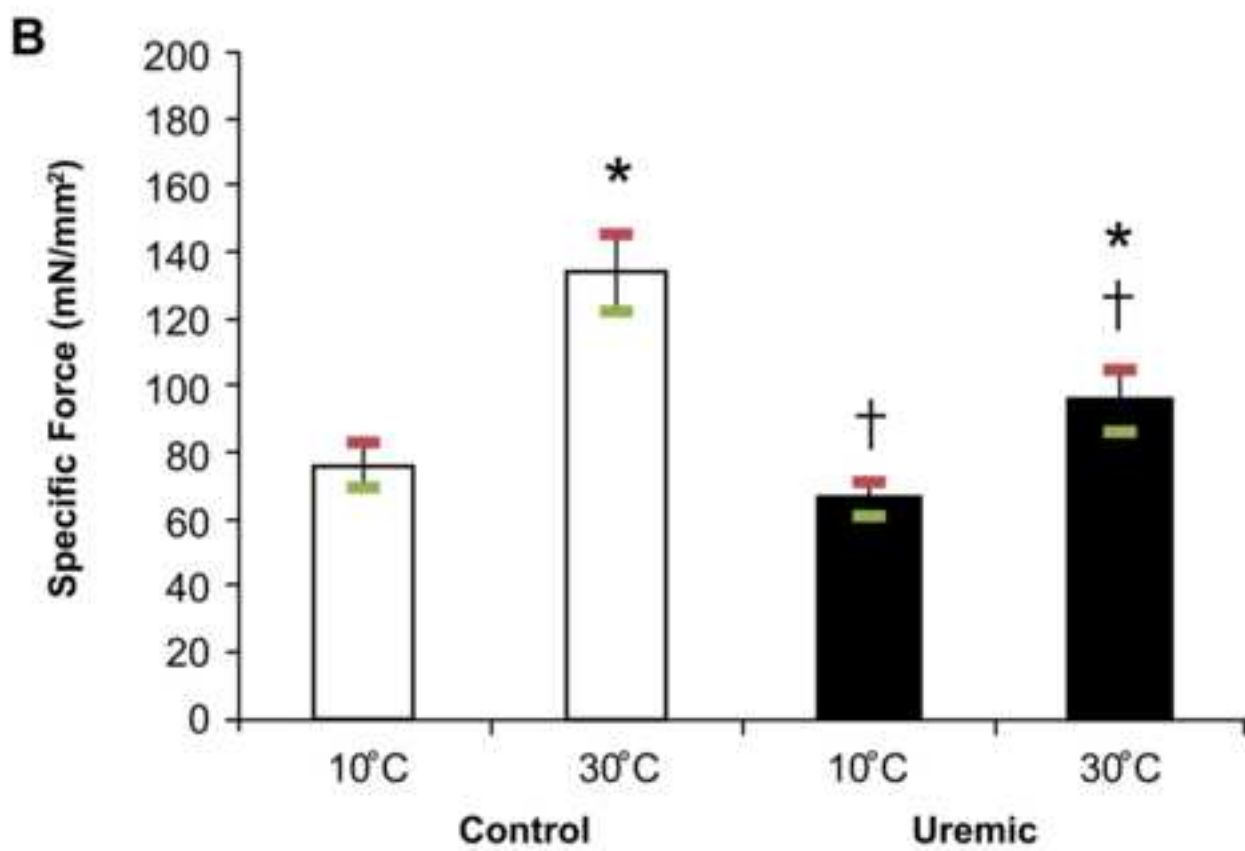
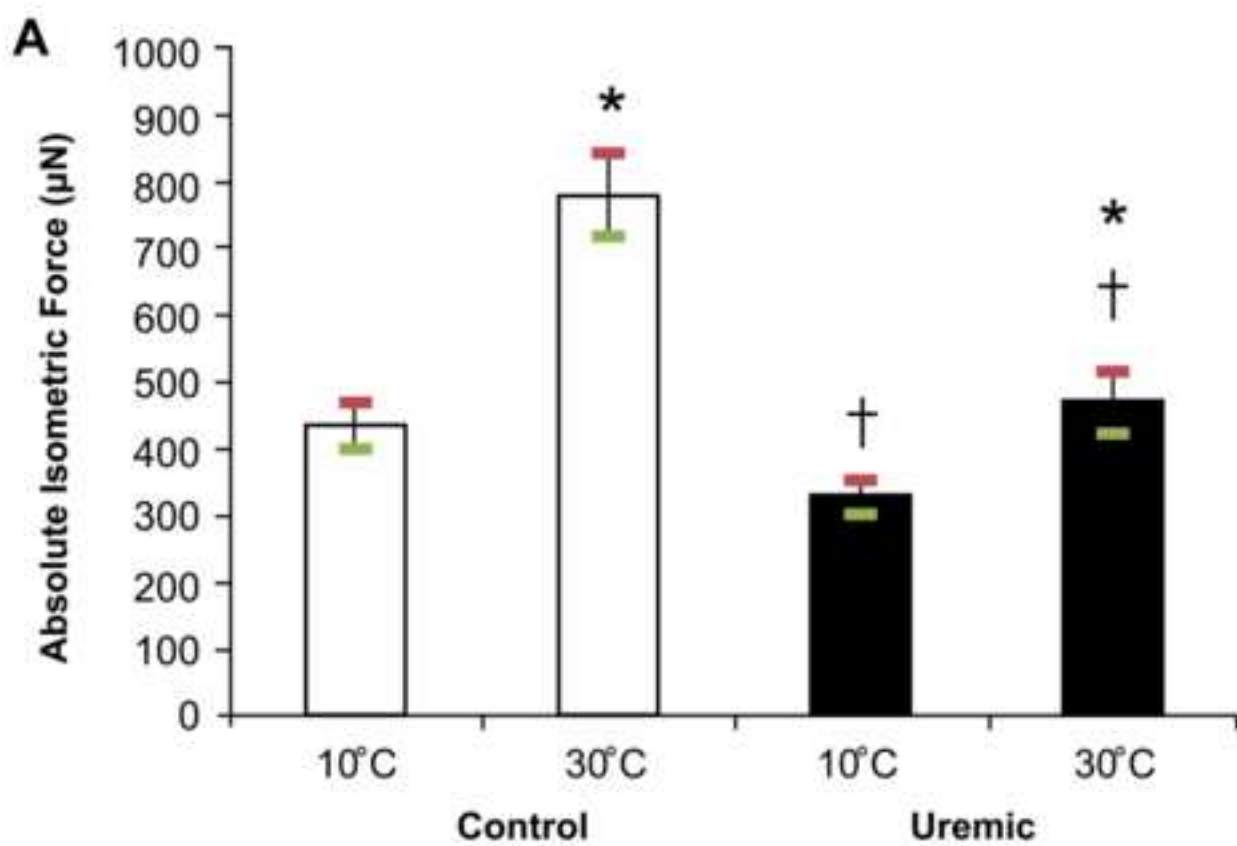
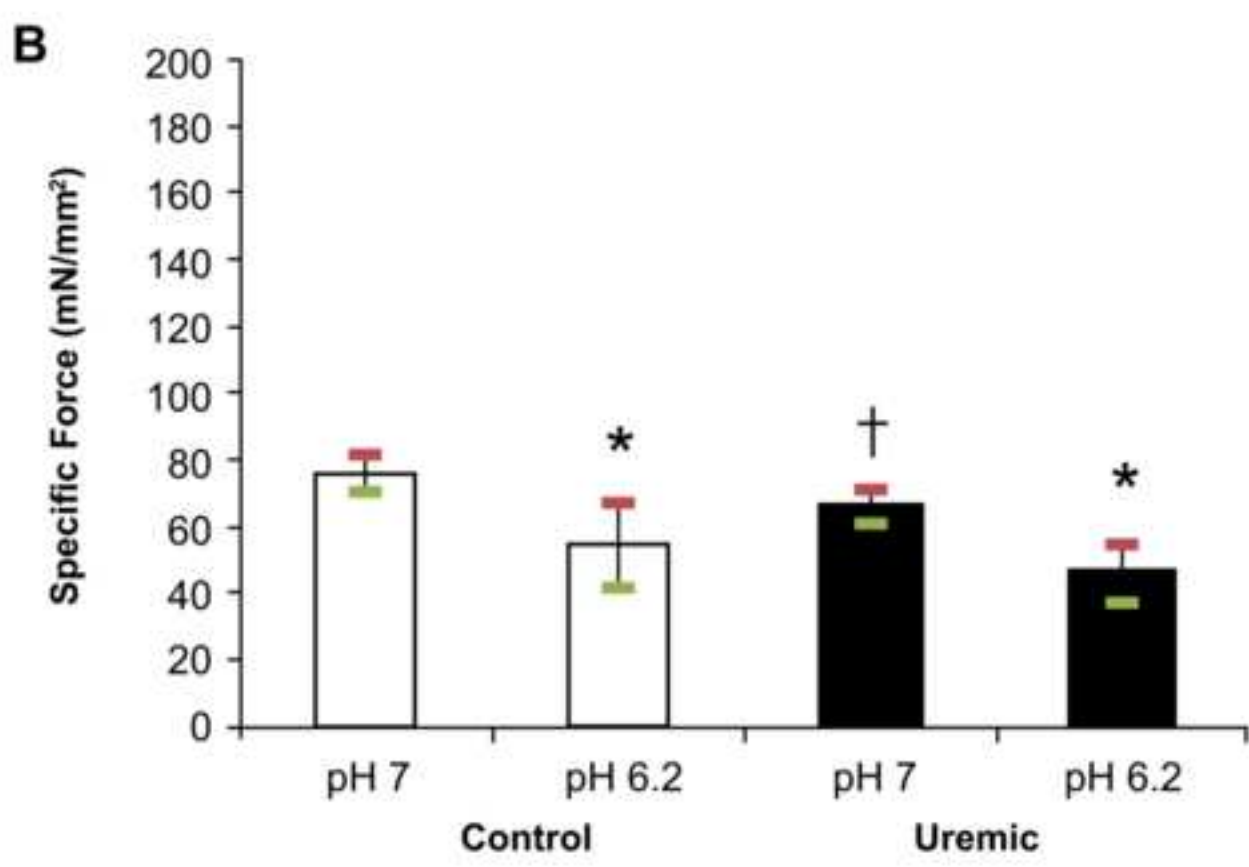
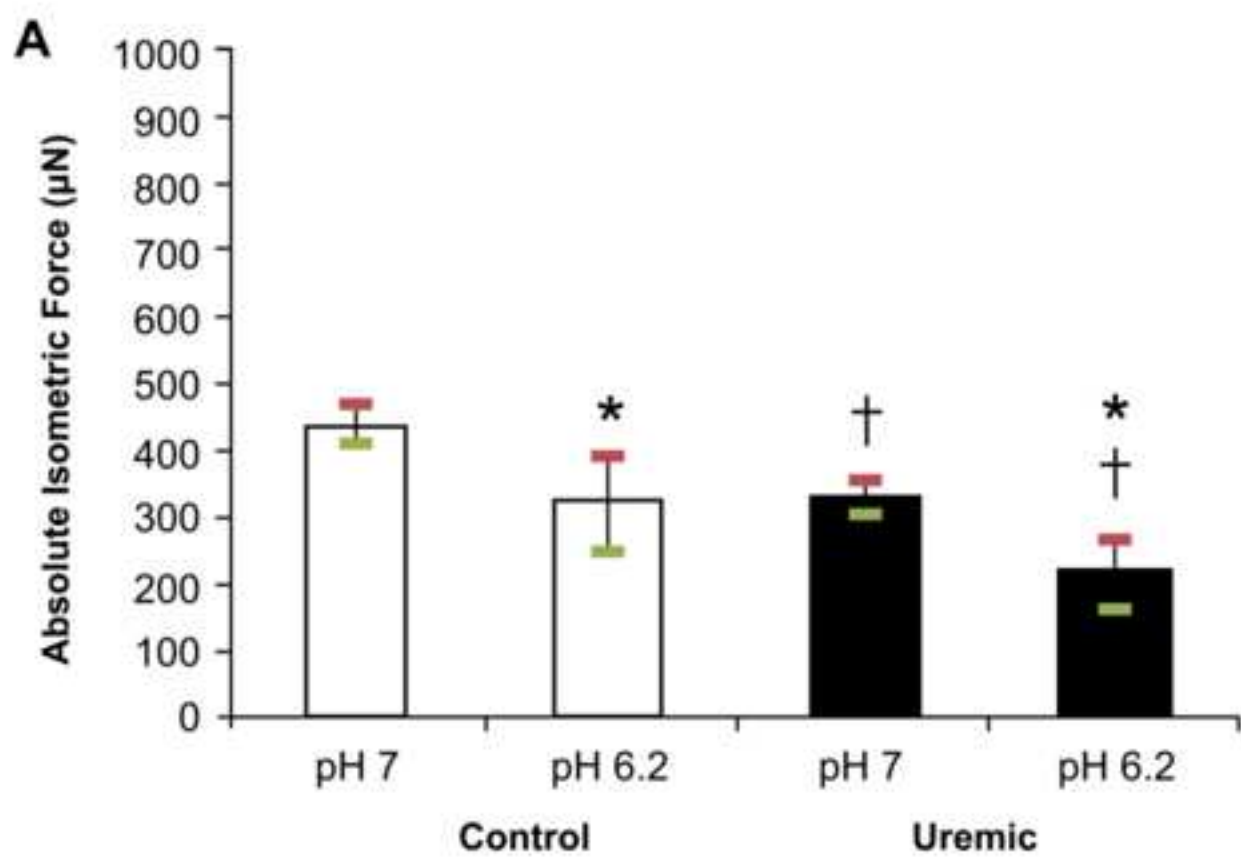


Figure 3  
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**Table 1.** Contractile properties of UREM and CON single psoas muscle fibers at 10°C and 30°C, pH7. Descriptive (Mean  $\pm$  SD) and Inferential statistics (SEM and exact 95% CIs) are reported for the collected isometric force data.

Dependent Variable	Group	Temperature (°C)	<i>n</i>	Mean	SD	SEM	95% Confidence Interval	
							Lower Bound	Upper Bound
Force ( $\mu$ N)	CONTROL	10	142	434	171	17	400	468
		30	41	780	216	32	717	844
	UREMIC	10	240	327	203	13	301	353
		30	73	470	263	24	423	517
Specific Force (mN/mm <sup>2</sup> )	CONTROL	10	142	76	25	3	70	83
		30	41	134	40	6	123	146
	UREMIC	10	240	66	38	2	61	71
		30	73	96	55	4	87	105

The value for *n* represents fibers assessed. GLM analysis indicated a significant main effect of either group, temperature and their interaction (group \* temperature) ( $P \leq 0.001$ ).