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1 **Gonad-related factors promote muscle performance gain during postnatal**  
2 **development in male and female mice**

3

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26 Abstract

27

28 In order to better define the role of male and female gonad-related factors (MGRF,  
29 presumably testosterone, and FGRF, presumably estradiol, respectively) on mouse hindlimb  
30 skeletal muscle contractile performance/function gain during postnatal development, we  
31 analysed the effect of castration initiated before puberty in male and female mice. We found  
32 that muscle absolute and specific (normalized to muscle weight) maximal forces  
33 were decreased in 6-month old male and female castrated mice, as compared to age- and  
34 sex-matched intact mice, without alteration in neuromuscular transmission. Moreover,  
35 castration decreased absolute and specific maximal powers, another important aspect of  
36 muscle performance, in 6-month old males, but not in females. Absolute maximal force was  
37 similarly reduced by castration in 3-month old muscle fibre androgen receptor (AR) -  
38 deficient and wild-type male mice, indicating that the effect of MGRF was muscle fibre AR  
39 independent. Castration reduced the muscle weight gain in 3-month mice of both sexes and  
40 in 6-month females but not in males. We also found that bone morphogenetic protein  
41 signaling through Smad1/5/9 was not altered by castration in atrophic muscle of 3-month old  
42 mice of both sexes. Moreover, castration decreased the sexual dimorphism regarding muscle  
43 performance. Together these results demonstrated that in the long-term MGRF and FGRF  
44 promote muscle performance gain in mice during postnatal development, independently of  
45 muscle growth in males, largely via improving muscle contractile quality (force and power  
46 normalized) and that MGRF and FGRF also contribute to sexual dimorphism. However, the  
47 mechanisms underlying MGRF and FGRF actions remain to be determined.

48

49 Keywords

50 Skeletal muscle; postnatal development; androgen deficiency; estrogen deficiency; maximal

51 force; maximal power, muscle fibre androgen receptor, muscle contractile quality.

52 Introduction

53

54 The postnatal growth of skeletal muscle is due to muscle fibre hypertrophy (71) resulting  
55 from a high protein synthesis rate (19). After 1 month of age, the increase in fibre diameter  
56 in mice occurs without addition of myonuclei provided by satellite cells (71). Male gonad-  
57 related factors (MGRF), in particular androgens (testosterone), are thought to play an  
58 important role in the postnatal development and maintenance of skeletal muscle mass, and  
59 sexual dimorphism of skeletal muscle. It is thought that the actions of androgens are mainly  
60 exerted through binding to the androgen receptor (AR), which directly modulates the  
61 transcription of target genes. In skeletal muscle, AR has been reported in satellite cells,  
62 muscle fibres and other cell lineages. Several animal studies reported that androgen  
63 deficiency resulting from castration of adult male animals causes variable levels of muscle  
64 atrophy (2, 9, 11, 30, 35, 37, 64), supporting the idea that MGRF play a role in the  
65 maintenance of muscle size. Less is known about the role of endogenous androgens, whose  
66 blood levels increase at puberty, on muscle contractile performance (function) gain during  
67 the postnatal development. Since muscle size is an important determinant of muscle  
68 performance, i.e. absolute maximal force and power, it is hypothesized that endogenous  
69 androgens contribute to the increase in muscle performance after puberty, but the target cells  
70 are unknown. Moreover, it remains largely unknown whether endogenous androgens affect  
71 specific maximal force and power (absolute maximal force or power/muscle weight) after  
72 puberty, i.e muscle contractile quality, another key determinant of muscle performance.

73

74 Several recent studies concluded that female gonad-related factors (FGRF), in particular  
75 estrogens (estradiol), positively regulate absolute maximal force in adult female mice (7, 25,  
76 40, 41, 49). Three estrogen receptors, ER $\alpha$ , ER $\beta$ , and the G-protein coupled receptor (Gper),

77 have been identified in skeletal muscles. [SEP]Moreover, it was reported that some beneficial  
78 effects of estrogens on muscle contractility can be very rapid (within 30 min) in adult female  
79 mice, suggesting a non-genomic mechanism and that estrogens can affect muscle quality  
80 (40). However, the roles of FGRF on muscle performance gain during the postnatal  
81 development are not well established in female mice. Indeed, it has been reported that  
82 during postnatal development, estrogens decrease absolute maximal force (67) or have no  
83 effect in female rats (42).

84

85 Despite recent developments, there is a tremendous lack of understanding of sex-based  
86 differences in muscle performance. Overall, evidence to date suggests that muscle  
87 performance is sex-dependent (15, 23, 27, 32–34, 62). Indeed, several studies reported that  
88 absolute maximal force and power are greater in adult male mice as compared to adult  
89 female mice (15, 33, 62), whilst others have not found such differences (23, 28). It is  
90 postulated that FGRF and MGRF contribute to the sexual dimorphism regarding muscle  
91 performance, however this remains to be firmly established.

92

93 In order to further characterize the role of MGRF and FGRF on postnatal development of  
94 muscle contractile performance, i.e. absolute isometric maximal force and absolute maximal  
95 power derived from force-velocity relationship, we analyzed in adult male and female mice  
96 the effects of castration initiated before puberty. Absolute isometric maximal force and  
97 power derived from force-velocity relationship are two important aspects of muscle  
98 performance during locomotion and muscular exercise, ie. to accomplish work, although  
99 they overestimate the force and power output of a muscle during in vivo dynamic muscle  
100 contractions (36). Our general hypothesis was that MGRF and FGRF play important roles in  
101 performance gain in male and female mice respectively, between the age of 1 month and 6

102 months. We also tested the hypothesis that castration before puberty decreases sexual  
103 dimorphism regarding muscle performance in the adult stage. Moreover, we analyzed the  
104 effect of castration before puberty in the absence of muscle fibre AR in order to determine  
105 whether AR mediates the potential role of MGRF in this cell type. To address this objective,  
106 we used male mice with loss of muscle fibre AR ( $AR^{skm-/y}$  mice) that were castrated before  
107 puberty or not. If it is the case, the effect of castration before puberty should be reduced in  
108 the absence of muscle fibre AR as compared with the presence of AR. We also analysed the  
109 effect of castration on several key functional, cellular and molecular determinants of muscle  
110 contractile performance that include muscle contractile quality, i.e. specific maximal force  
111 and power, neuromuscular transmission, fibre atrophy, fibre type composition, fibrosis and  
112 remodeling pathways involved in muscle growth and physiology (such as bone  
113 morphogenetic protein signaling, ubiquitin ligases, MSTN, IGF-1).

114

115



116 Materials and Methods

117

118 Mice

119

120 All procedures were performed in accordance with European legislations, in conformity  
121 with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and  
122 were approved by the Comité d'éthique en expérimentation animale Charles Darwin #5  
123 (Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche,  
124 France)(Autorisation de projet # 01361.03). Male and female wild type mice (C57BL/6  
125 background) were analyzed at the age of 1 month, 1.5 month, 3 months and 6 months. Body  
126 weights are shown in Table 1. Body weights were decreased in 3-month old male castrated  
127 mice and increased in 6-month old castrated female, as compared to age-and sex-matched  
128 intact mice ( $p < 0.05$ ). Therefore, these results indicate no reduction in muscle demand  
129 during standing and locomotion at the age of 6 months. We also used muscle fibre AR  
130 deficient male mice (referred to below as AR<sup>skm-/y</sup>)(on a C57BL/6 background). AR<sup>skm-/y</sup>  
131 mice were generated by breeding female AR<sup>L2/L2</sup> mice carrying “floxed” AR L2 alleles with  
132 male HSA-Cre transgenic mice, as described previously (9, 18). Sex matched wild-type  
133 littermates (AR<sup>L2/y</sup> mice) were used as controls. Male and female mice were castrated  
134 (ablation of gonads) at 1 month of age, before the onset of puberty (57).

135

136 Muscle contractile performance

137

138 Absolute isometric maximal force and power of the tibialis anterior (TA) muscle were  
139 evaluated by measuring the *in situ* muscle contractions in response to nerve stimulation, as  
140 described previously (22, 62, 69). Some plantaris muscles were also measured (18). Mice

141 were anesthetized using pentobarbital (60 mg/kg intraperitoneally). Body temperature was  
142 maintained at 37°C using radiant heat. The knee and foot were fixed with pins and clamps,  
143 and the distal tendon of the muscle was attached to a lever arm of a servomotor system  
144 (305B, Dual-Mode Lever, Aurora Scientific) using a non-elastic thread. The sciatic nerve  
145 was proximally crushed and distally stimulated by a bipolar silver electrode using  
146 supramaximal square wave pulses of 0.1 ms duration. Muscle was also directly stimulated  
147 after nerve stimulation at the frequency corresponding to maximal force in order to directly  
148 initiate muscle contraction in the case of neurotransmission failure (16). Stimulating  
149 electrodes were positioned on the midbelly of the muscle and the muscle was stimulated  
150 with a high strength voltage (80V). We measured the absolute maximal force that was  
151 generated during isometric contractions in response to electrical stimulation (frequency of  
152 75–150 Hz, train of stimulation of 500 ms). Absolute maximal force was determined at L0  
153 (length at which maximal tension was obtained during the tetanus). Absolute maximal force  
154 was normalized to the muscle mass as an estimate of specific maximal force, i.e. muscle  
155 contractile quality, a key determinant of muscle performance.

156

157 Force-velocity data were then obtained by eliciting contractions in response to sciatic nerve  
158 stimulation (500 ms, 125 Hz) at 6 different afterloads (over a range of approximately 10-  
159 50% absolute maximal force). The sciatic nerve was stimulated for 700 ms (125 Hz). A  
160 maximal isometric contraction of the muscle was initiated during the first 200 ms. Then, the  
161 muscle shortened during the last 300 ms against the load. Each contraction was separated by  
162 a 1 min rest period. The shortening velocity was measured during the first 20 ms of the  
163 shortening period. Absolute power was calculated (power = afterload x shortening velocity)  
164 and absolute maximal power was reported (mW). Specific maximal power (mW/g) was  
165 calculated by dividing maximal power by muscle weight, as another index of muscle

166 contractile quality and important determinant of muscle performance. After contractile  
167 measurements, the animals were sacrificed by cervical dislocation and muscles were  
168 dissected and weighed before being processed for downstream analyses.

169

170 Neuromuscular junction morphology

171

172 Neuromuscular junction (NMJ) analysis was performed on isolated muscle fibres as  
173 previously described (47, 59). Briefly, plantaris muscles were dissected and fixed in  
174 4%PFA/PBS for 30 min and rinsed with PBS at room temperature. Isolated muscle fibres  
175 were washed three times for 15 min in PBS, incubated for 30 min with 100 mM glycine in  
176 PBS and rinsed in PBS. Samples were permeabilized and blocked in blocking buffer (3%  
177 BSA/5% goat serum/0.5% Triton X-100 in PBS) for 4 hours at room temperature. They  
178 were then incubated overnight at 4°C with rabbit polyclonal antibodies against 68 kDa  
179 neurofilament (NF, Millipore Bioscience Research Reagents, 1:1000) and synaptophysin  
180 (Syn, Zymed, 1:200) in blocking buffer. After four 1-hour washes in PBS, muscles were  
181 incubated overnight at 4°C with Cy3-conjugated goat anti-rabbit IgG (Jackson  
182 Immunoresearch Laboratories, 1:500) and Alexa Fluor 488-conjugated  $\alpha$ -bungarotoxin ( $\alpha$ -  
183 BTX, Life Technologies, 1:1000) in blocking buffer. After four 1-hour washes in PBS,  
184 isolated muscle fibres were then flat-mounted in Vectashield (Vector Laboratories)  
185 mounting medium. Confocal images were acquired using Leica SPE confocal microscope  
186 with a Plan Apo 63x NA 1.4 oil objective (HCX; Leica). Confocal software (LAS AF;  
187 Leica) was used for acquisition of Z serial images, with a Plan Apo 63x NA 1.4 oil objective  
188 (HCX; Leica). Confocal images presented are single-projected image derived from image  
189 stacks. For all imaging, exposure settings were identical between compared samples and  
190 groups. Quantifications were done as previously (48), using ImageJ software (version

191 1.46m). AChR rich-endplate area per neuromuscular junction corresponds to the occupied  
192 area of  $\alpha$ -BTX fluorescent signal. More than 20 fibres from at least five different mice of  
193 each group were analysed.

194

195 Fibre size and type

196

197 Transverse serial sections (8  $\mu$ m) of TA muscles were obtained using a cryostat, in the mid-  
198 belly region. Some of sections were processed for histological analysis according to standard  
199 protocols (stained for Sirius red). Others were used for immunohistochemistry as described  
200 (17, 38). For determination of muscle fibre diameter and myosin heavy chain (MHC)  
201 analysis, frozen unfixed sections were blocked 1h in PBS plus 2% BSA, 2% sheep serum.  
202 Sections were then incubated overnight with primary antibodies against laminin (rabbit  
203 polyclonal, 1:300, Dako, Les Ulis, France) and myosin heavy chain (MHC) isoforms  
204 (Developmental Studies Hybridoma bank, University of Iowa, USA). After washes in PBS,  
205 sections were incubated 1 h with secondary antibodies (alexa fluor, Life Technologies, Saint  
206 Aubin, France). For morphometric analyses images were captured using a motorized  
207 confocal laser-scanning microscope (LSM 700, Carl Zeiss SAS, Le Pecq, France).  
208 Morphometric analyses were made using ImageJ software and a homemade macro. The  
209 smallest diameter (min Ferret) of all the muscle fibres of the whole muscle section was  
210 measured. For muscle fibre diameter and fibre typing analyses all of the muscle fibres of the  
211 muscle section were measured. The extent of fibrosis was assessed by Sirius red staining.

212

213 Remodeling pathways: protein

214

215 TA muscle was lysed in RIPA buffer [50 mM Tris pH 7.5, 1 % Nonident P40, 0.5 %  
216 Sodium Deoxycholate, 0.1 % SDS, 150 mM NaCl, 5 mM EDTA, 1 mM  
217 phenylmethanesulphonylfluoride (PMSF) and protease inhibitor cocktail (45 µg/mL, 11 873  
218 580 001, Roche)] with a potter at 4°C. Homogenates (100 µg of protein) were  
219 electrophoresed on 10 % polyacrylamid gels. Proteins were electroblotted to Hybond  
220 nitrocellulose membranes (Amersham Biosciences) and immunodetected using primary  
221 antibodies directed against Phospho-Smad1 (Ser463/465)/ Smad5 (Ser463/465)/ Smad9  
222 (Ser465/467) (#13820, Cell signaling, 1/1000), FoxO1 (#2880, cell signaling, 1/1000)  
223 Smad1/Smad9 (#ab108965, abcam, 1/1000), phospho-FoxO1 (Ser256) (#9461, cell  
224 signaling, 1/1000), FoxO3a (#12829, cell signaling, 1/1000), phospho-FoxO3a (Ser318/321)  
225 (#9465, cell signaling, 1/1000) and tubuline (IGBMC). Secondary antibodies conjugated to  
226 horseradish peroxidase (Amersham Biosciences) were detected using an enhanced  
227 chemiluminescence detection system (Pierce, Rockford, IL, 1/10000).

228

229 Remodeling pathways: mRNA

230

231 Total RNA from the TA muscle was isolated using TRIzol Reagent (Invitrogen). A total of 2  
232 µg of RNA was reverse transcribed to cDNA with SuperScript II reverse transcriptase  
233 (Invitrogen Life Technologies) and random hexamer primers according to the supplier's  
234 protocol. Quantitative RT-PCR was performed by using the SYBR Green 1 marker PCR kit  
235 (Roche) according to the supplier's protocol (18). The 18S ribosomal RNA was used as an  
236 internal control. Primers were shown in Table 2.

237

238 Statistical analysis

239

240 Groups were generally compared using 2 way-variance analysis (castration x age, sex x age,  
241 castration x genotype). If necessary, Bonferroni post-tests were also performed. For groups  
242 that did not pass tests of normality and equal variance, non-parametric tests were used  
243 (Kruskal Wallis and Wilcoxon). Values are means  $\pm$  SEM. Significance was set at  $p < 0.05$ .  
244

245 Results

246

247 1-Castration reduces both absolute maximal force and power gains in male mice

248

249 We measured **the absolute maximal force** of the TA muscle in response to nerve  
250 stimulation, an important aspect of muscle performance, in male mice. Castration performed  
251 at 1 month of age reduced the gain in absolute maximal force between 1 month and 6  
252 months. Indeed, absolute maximal force was decreased in 3- and 6-month old male castrated  
253 mice (-18% and -17% respectively), as compared to age-matched intact male mice ( $p <$   
254 0.05)(Figure 1A). The absolute maximal force was related to the specific maximal force  
255 (absolute maximal force/muscle weight), and the muscle weight (see below). We found that  
256 the increase in **specific maximal force** between 1 month and 6 months was reduced by  
257 castration. Specifically, specific maximal force was reduced in castrated male mice at 6  
258 months of age, as compared to age-matched intact male mice (Figure 1B)( $p < 0.05$ ).

259

260 **Absolute maximal power**, another important aspect of TA muscle performance, was also  
261 measured. The gain in absolute maximal power between 1 month and 3 or 6 months  
262 observed in intact male mice was reduced by castration. Absolute maximal power was  
263 decreased by 30% and 18% in 3- and 6-month old castrated male mice, respectively ( $p <$   
264 0.05), as compared to age-matched male intact mice (Figure 1C). Absolute maximal power  
265 was related to **specific maximal power**, and muscle weight (see below). We found that  
266 specific maximal power was reduced in 3- and 6 month old castrated male mice, as  
267 compared to age-matched male intact mice (Figure 1D)( $p < 0.05$ ).

268

269 We also measured TA **muscle weight**, because absolute maximal force and power are

270 proportional to muscle size (muscle cross-section area and volume/weight). The gain in  
271 muscle weight observed between 1 month and 3 months in intact male mice was reduced by  
272 castration in male mice. Muscle weight was decreased by -16% in male castrated mice at 3  
273 months of age ( $p < 0.05$ ), as compared to age-matched male intact mice (Figure 1E).  
274 However, at 6 months of age, muscle weight was similar in castrated and age-matched intact  
275 male mice (Figure 1E).

276

277 Together, our results indicate that castration before puberty decreases the gains in absolute  
278 maximal force and power between 1 month and 6 months of TA muscle in male mice. This  
279 is due to reduced gain in specific maximal force and power, i.e. two keys aspects of muscle  
280 contractile quality, and a delayed muscle growth (increase in muscle weight) in male mice.

281

282 2-Castration decreases absolute maximal force gain in female mice

283

284 Castration reduced the gain in TA **absolute maximal force** between 1 month and 3 or 6  
285 months in female mice such that values were decreased in 3- and 6-month old female  
286 castrated mice by -17% and -11% respectively, as compared to age-matched female intact  
287 mice ( $p < 0.05$ )(Figure 1F). Moreover, the gain in **specific maximal force** between 1 month  
288 and 6 months was reduced by castration since specific maximal force was lower in castrated  
289 female mice, at 3 and 6 months of age, as compared to age-matched intact female mice  
290 (Figure 1G)( $p < 0.05$ ).

291

292 Castration did not affect the gain in TA **absolute maximal power** between 1 month and 3 or  
293 6 months in female mice. Indeed, absolute maximal power was not different in 3- and 6-  
294 month old female between castrated and intact mice (Figure 1H). Similarly, castration did



295 not affect specific maximal power since specific maximal power did not significantly  
296 increase in 3- and 6 month old castrated female mice, as compared to age-matched female  
297 intact mice ( $p=0.07$ )(Figure 1I).

298

299 Castration reduced the gain in TA **muscle weight** between 1 month and 3 or 6 months in  
300 female mice. Indeed, female castrated mice demonstrated a reduction of 11 and 5% in  
301 muscle weight at 3 and 6 months of age, respectively, as compared to age-matched intact  
302 female mice (Figure 1J)( $p < 0.05$ ).

303

304 Taken together, our results indicate that castration before puberty decreases absolute  
305 maximal force of TA muscle in female mice, but not absolute maximal power. The reduced  
306 absolute maximal force results from the decrease of both specific maximal force, i.e. an  
307 aspect of muscle quality, and muscle weight.

308

309 3- Castration reduces sexual dimorphism regarding muscle performance

310

311 Sexual dimorphism was studied in both intact and castrated mice. We found first a sexual  
312 dimorphism concerning **absolute maximal force** of TA muscle in intact mice. The absolute  
313 maximal force of female intact mice was reduced (-10%) as compared to male intact mice  
314 (compare Figure 1F to Figure 1A)( $p < 0.05$ ). Secondly, in contrast, absolute maximal force  
315 of female and male castrated mice did not differ (compare Figure 1F to Figure 1A).  
316 Moreover, there was no sexual dimorphism regarding **specific maximal force** in intact and  
317 castrated mice. Indeed, specific maximal force of intact and castrated female mice were  
318 similar as compared to intact and castrated age-matched male mice (compare Figure 1G to  
319 Figure 1B).

320

321 **Absolute maximal power** of the TA muscle also differed between sexes in intact mice.  
322 Absolute maximal power of intact female mice was decreased (-18%), as compared to intact  
323 age-matched male mice (compare Figure 1H to Figure 1C)( $p < 0.05$ ). In contrast, the  
324 absolute power of 3-month old female castrated mice was increased as compared to age-  
325 matched male castrated mice (compare Figure 1H to Figure 1C)( $p < 0.05$ ). We also found a  
326 sexual dimorphism concerning **specific maximal power**, since female intact mice had a  
327 lower specific maximal power, as compared to age-matched male intact mice (compare  
328 Figure 1I to Figure 1D). In contrast, the specific maximal power of female castrated mice  
329 was increased, as compared to age-matched male castrated mice (compare Figure 1I to  
330 Figure 1D)( $p < 0.05$ ).

331

332 Finally, there was a sexual dimorphism concerning TA **muscle weight** in intact mice.  
333 Muscle weight of 3- and 6- month old female intact mice was reduced (-6%), as compared to  
334 age-matched male intact mice (compare Figure 1J to Figure 1E)( $p < 0.05$ ). Similarly, the  
335 muscle weight of 6-month old castrated female castrated mice, but not 3-month old castrated  
336 female mice, was decreased as compared to age-matched male castrated mice (compare  
337 Figure 1J to Figure 1E)( $p < 0.05$ ).

338

339 Together, these results indicate that in intact mice there is a sexual dimorphism concerning  
340 both absolute maximal force and power of the TA muscle. The reduced muscle performance  
341 in female mice is due to a decreased specific maximal force and power, i.e muscle quality,  
342 and a lower muscle weight. Moreover, castration in both sexes reduces the sexual  
343 dimorphism regarding absolute maximal force and power.

344

345 4- Deficiency in muscle fibre AR does not alter the effect of castration on muscle  
346 performance in male mice

347

348 To determine if muscle fibre AR mediates MGRF-induced performance gain, male AR<sup>skm-/y</sup>  
349 mice, in which muscle fibre AR is selectively ablated, as well as male AR<sup>L2/y</sup> (control)  
350 littermates, were castrated at 1 month of age, and analyzed at 3 months of age. In agreement  
351 with previous results (9), **absolute maximal force** of the TA muscle was lower in intact  
352 AR<sup>skm-/y</sup> mice than in AR<sup>L2/y</sup> mice (Figure 2A)(p <0.05). Interestingly, we found that  
353 absolute maximal force was similarly decreased in castrated male mice, as compared to  
354 genotype-matched intact male mice, in both genotypes (-29% for AR<sup>skm-/y</sup> mice and -28% for  
355 AR<sup>L2/y</sup> mice)(Figure 2A)(p <0.05). **Specific maximal force** was unchanged by castration in  
356 both genotypes (Figure 2B). Moreover, **TA muscle weight** was similarly reduced in  
357 castrated male mice (-33% for AR<sup>skm-/y</sup> mice and -29% for AR<sup>L2/y</sup> mice), as compared to  
358 genotype-matched intact male mice (Figure 2C)(p <0.05).

359

360 Together our results indicate that muscle fibre AR deficiency does not alter the effect of  
361 castration on TA muscle performance, suggesting that the action of MGRF is not mediated  
362 by muscle fibre AR.

363

364 5-Reduced muscle performance is not related to altered neuromuscular transmission in 3-  
365 month old castrated mice

366

367 It has been reported that androgens influence neuromuscular transmission (3). To determine  
368 whether **neuromuscular transmission failure** contributes to the reduced absolute maximal  
369 force in castrated mice, we also performed electrical stimulation of the TA muscle that can

370 directly initiate muscle action potentials, without the need of neuromuscular transmission (8,  
371 16, 51). We found that absolute maximal force in response to nerve stimulation was  
372 decreased by castration in 3-month old mice of both sexes (Figures 3A and B)( $p < 0.05$ ),  
373 confirming our previous results (Figures 1A and G). Interestingly, direct TA muscle  
374 stimulation with a high strength voltage did not improve absolute maximal force in 3-month  
375 old castrated mice of both sexes since there was no difference between nerve and muscle  
376 stimulations (Figures 3A and B), indicating no neurotransmission failure.

377

378 To complete the analysis of neuromuscular transmission, we checked that castration does  
379 not alter **neuromuscular junction morphology** in plantaris muscle fibres. Before that, we  
380 confirmed that absolute maximal force and weight of the plantaris muscle were decreased in  
381 3-month-old male castrated mice, as compared to age-matched intact male mice ( $p <$   
382  $0.05$ )(Figure 3C). In contrast, specific maximal force was unchanged by castration (Figure  
383 3C), indicating that the effects of castration on muscle performance were similar in plantaris  
384 and TA muscles, at least in 3-month old male mice. Plantaris muscle fibres isolated from 3-  
385 month-old castrated male mice were stained with  $\alpha$ -BTX to detect AChR clusters and with a  
386 mixture of antibodies against neurofilament and synaptophysin to label axonal branches and  
387 nerves terminals, respectively. The structure of the synapse in castrated mice was  
388 indistinguishable from intact ones. Indeed, all endplates analyzed formed a continuous  
389 branched postnatal topology and exhibited a typical and «pretzel-like» morphology (Figure  
390 3D). The fact that AChR-rich endplate area per NMJ was reduced by 30% in castrated mice  
391 ( $p < 0.05$ )(Figure 3E) could be explained by the decreased fibre size as shown below.  
392 Moreover, both in castrated and intact mice, axonal branches properly innervated the  
393 postsynaptic counterpart and nerve terminals were in perfect registry with AChR clusters.  
394 Quantitative analysis revealed that the synaptophysin area per NMJ (Figure 3F) as well as

395 the overlap area between pre- and postsynaptic elements (Figure 3G) were unchanged in  
396 castrated mice compared to intact ones.

397

398 Taken together, these observations demonstrate that castration does not disturb NMJ  
399 structure, in agreement with the observations that 3-month old castrated male mice exhibit  
400 normal neuromuscular transmission, excluding the possibility that reduced performance is  
401 explained by decreased muscle activation.

402

403 6-Reduced muscle performance is related to fibre atrophy and fibrosis in 3-month old  
404 castrated mice

405

406 As mentioned above, part of the reduction in muscle performance is related to decreased  
407 muscle weight in 3-month old castrated mice of both sexes. Therefore, we further analysed  
408 the **reduced TA muscle weight** in 3-month castrated mice of both sexes (Figure 4A), as  
409 previously shown (Figures 1E and J), and found that it was not associated with a decrease in  
410 **bone growth** in castrated mice of both sexes. Indeed, the length of the tibia was not changed  
411 by castration in both 3-month old male ( $17.8 \pm 0.3$  mm in castrated versus  $18.2 \pm 0.3$  mm in  
412 intact mice) and female ( $18.0 \pm 0.1$  mm in castrated versus  $18.3 \pm 0.2$  mm in intact mice)  
413 mice. Moreover, the reduced muscle weight in castrated mice was related to muscle **fibre**  
414 **atrophy** since histological analyses revealed a left shift in the fibre diameter distribution in  
415 both 3-month old castrated mice of both sexes (Figures 4BC). In line, there was an increase  
416 in **fibrosis** in 3-month old castrated mice ( $14.2 \pm 0.9$  % in castrated versus  $11.7 \pm 2.0$  % in  
417 intact mice)( $p < 0.05$ )(Figure 4D). We also determined whether fibre atrophy was  
418 accompanied by an increase in the **percentage of fibres expressing MHC-2a** that are fast  
419 fibres having small fibre diameter. We found that the percentage of fibres expressing MHC-  
420 2a was not modified by castration in 3-month old mice of both sexes, indicating no change

421 in fibre type composition in the muscle (Figure 4E).

422

423 Together, our results indicate that reduced muscle performance gain in 3-month old  
424 castrated mice of both sexes is associated with decreased muscle fibre growth and increased  
425 fibrosis but no change in fibre type composition.

426

427 7-Castration alters intramuscular remodeling pathways in 3-month old male mice

428

429 We first evaluated the activation of bone morphogenetic protein (BMP) signaling via  
430 Smad1/5/9, that is an important emergent pathway controlling muscle size and performance  
431 (61, 72). We investigated whether castration before puberty influences the BMP signaling  
432 axis in skeletal muscle. Castration in 3 month-old male mice altered neither the amount of  
433 phosphorylated Smad1/5/9 (Figures 5A and B), nor *activin-like kinase 3 (ALK3)* transcript  
434 levels (Figure 5C). *Smad4* transcript levels were decreased by castration (Figure  
435 5D)( $p < 0.05$ ), but those of the downstream factor *Id1* (inhibitor of DNA binding) were  
436 unaffected in castrated male mice (Figure 5E). Moreover, castration in 3-month-old female  
437 mice did not alter *ALK3* (Figure 5C), *Smad4* (Figure 5D), and *Id1* (Figure 5E) transcript  
438 levels ( $p < 0.05$ ). Together, these results suggest no major change in Smad1/5/9 signaling  
439 with castration in both 3-month old male and female mice.

440

441 We then determined the effect of castration on the **ubiquitin proteasome system** that plays  
442 an important role in muscle physiology and atrophic process (4, 44). Castration in 3-month  
443 old male mice decreased the levels of the protein phosphorylated (inactivated) form of  
444 Foxo3a (Figures 6A and B), without changing that of phosphorylated Foxo1 (Figures 6A  
445 and C), two transcription factors important for the regulation of E3 ubiquitin ligases.

446 Moreover, we found that the transcript levels of *Murf1* (Figure 6D) and *FbxO30* (Figure 6E)  
447 were reduced in 3-month old castrated male mice, as compared to age-matched intact male  
448 mice ( $p < 0.05$ ), whereas that one of *atrogen 1* was unchanged (Figure 6F). In contrast,  
449 castration did not affect the transcript levels of *Murf1*, *FbxO30* and *atrogen 1* in 3-month-  
450 old female mice (Figures 6D-F). Together, these results suggest that 3-month after castration  
451 E3 ubiquitin ligases (*atrogen 1*, *Murf1*, and *FbxO30*) might be less active in males and  
452 unchanged in females.

453

454 In addition, we measured the transcript levels of *IGF-1* and *MSTN* (myostatin), encoding  
455 proteins regulating muscle growth and function (44, 58, 66). In 3-month-old mice, castration  
456 increased the transcript level of *MSTN* in males, but did not affect it in females (Figure 6G).  
457 In contrast, the transcript level of *IGF-1* was unchanged in castrated males and increased in  
458 castrated females (Figure 6H).

459

460 Together, our results indicate that reduced muscle performance gain is associated with  
461 changes in the levels of ubiquitin ligases and MSTN in 3-month old male castrated mice, but  
462 not in Smad1/5/9 signaling.

463

464

465

466 Discussion

467

468 MGRF promotes long-term muscle contractile quality

469

470 Our results show that castration initiated before puberty decreased the performance of the  
471 TA muscle in 6-month old male mice. Thus, MGRF, between the age of 1 month and 6  
472 months, contribute to 29% and 38% of absolute maximal force and power gains,  
473 respectively (Table 3). The reduced absolute maximal force and power in 6-month old  
474 castrated male mice is due to a lower specific maximal force and power, but not a decreased  
475 muscle weight (Table 3). Therefore, our results support the original and important notion  
476 that endogenous androgens promote postnatal performance gain in 6-month old male mice  
477 via the improvement/maintenance in **muscle contractile quality**, i.e. specific maximal force  
478 and power, but not enhanced muscle growth. Concerning muscle growth, it is somewhat  
479 unexpected that the increase in muscle weight is only delayed by castration, nuancing the  
480 widespread view that androgens have an overall anabolising effect. Since in the present  
481 study we studied a fast-twitch muscle, it remains to be determined whether the contractile  
482 quality of a muscle with mixed fibre type composition (such as soleus muscle) is similarly  
483 reduced by removal of MGRF in 6-month-old male mice. MSTN encoding myostatin can  
484 also improve muscle contractile quality during postnatal development, but together with an  
485 inhibition of muscle growth (45, 52, 62, 65).

486

487 Our results indicate that increased fibrosis, but not neuromuscular transmission failure, can  
488 explain in part, the reduced specific maximal force and power in 6-month old castrated male  
489 mice. It is possible that the decrease in muscle quality is due to accumulation of  
490 nonfunctional proteins since we found that ubiquitin ligases are presumably less active in 3-



491 month old castrated male mice. It has been reported that decreased specific maximal force  
492 and power are associated with reduced levels of ubiquitin ligases (44). Our results does not  
493 however, relate to fibre type transition since we found no notable increase in the percentage  
494 of less powerful fibre expressing MHC-2a (20), at least in 3-month old castrated male mice.  
495 These data are in line with previous studies analyzing hypogonadal male mice (63). Finally,  
496 decreased phosphorylation of the myosin light chains could also contribute to the reduced  
497 specific maximal force, since it has been reported that acute androgen (dihydrotestosterone)  
498 administration increases both specific maximal force and phosphorylation of the myosin  
499 light chains (29).

500

501 MGFR are not the only factors involved in muscle performance gain

502

503 A finding of interest is that the contribution of MGRF to muscle performance gain is not  
504 predominant (Table 3) since 62 to 71% of the muscle performance gains between 1 and 6  
505 months are due to other factors. **Other endocrine factors** affecting muscle quality during  
506 muscle development may be considered. Thyroid hormones alter fibre transition that occurs  
507 during postnatal development (1, 26), and potentially affect specific maximal power since  
508 fast type fibres are more powerful than slow type fibres. In mice expressing dominant  
509 negative mutant IGF-1 receptors in skeletal muscle, there is a prevalence of fast fibres (68),  
510 suggesting a possible effect of endocrine or local IGF-1 on specific maximal power.  
511 However, we found that IGF-1 transcript levels were not modified in male castrated mice, at  
512 least at the age of 3 months. Concerning growth hormone, its direct effect on muscle is  
513 unlikely since muscle growth hormone receptor deficiency does not affect fibre type  
514 composition in postnatal muscle (70) and it has been reported that growth hormone does not  
515 alters specific maximal force (10).

516

517 The effects of MGRF on muscle performance and growth are not mediated by fibre AR and  
518 BMP signaling through Smad1/5/9 phosphorylation in 3-month old male mice

519

520 Interestingly, the effect of castration before puberty on absolute maximal force gain is not  
521 abolished in the **absence of muscle fibre AR**, at least in 3-month old male mice. At the age  
522 of 3 months, the reduced absolute maximal force in castrated male mice resulted from a  
523 **lower muscle weight** and fibre atrophy. These results suggest that the action of endogenous  
524 androgens on muscle performance gain and growth is not mediated by muscle fibre AR, at  
525 least in 3-month old male mice. These findings extend those of a previous study showing  
526 that 1 month-castration performed in the adult stage similarly decreased muscle weight in  
527 deficient or non-deficient muscle fibre AR male mice (9). In accordance, it has been  
528 reported that the postnatal development of hindlimb muscle is independent from fibre AR  
529 signaling in mice (9, 13, 55).

530

531 Many other cells express AR, in particular satellite cells. However, in the present study, the  
532 possibility that androgen effect on muscle weight is mediated via the AR of satellite cells is  
533 unlikely since satellite cells do not contribute to muscle growth after the age of 3 weeks.  
534 Indeed, there is no further myonuclei addition at this postnatal stage in mice (71). A  
535 possibility is that androgen effect on muscle growth can be mediated via AR localized in the  
536 brain. This hypothesis is supported by the facts that: (i) the level of voluntary exercise in  
537 male animals is negatively and positively modulated by castration and androgen  
538 administration, respectively (14, 35) and (ii) reduced activity alters muscle performance and  
539 size (31).

540

541 Another possibility is that other endocrine/paracrine factors mediate the effect of androgens  
542 on muscle weight. Indeed, testosterone can be converted to estrogens by aromatase, and  
543 estrogens are known to affect muscle physiology (FGRF, see below). GH and IGF-1 are  
544 unlikely since it has been reported that the circulating GH and IGF-1 are not mandatory for  
545 mediating the effect of androgens, at least in highly androgen responsible muscle from adult  
546 male mice (64). Several recent studies reported that androgens interact with MSTN, a  
547 member of the transforming growth factor-beta (TGF $\beta$ ) superfamily, in skeletal muscle (5,  
548 12, 46, 65). In line, we found that the transcript level of MSTN was increased by castration  
549 in 3-month-old male mice. Since inactivation of MSTN increases muscle growth (62, 66),  
550 the higher expression of MSTN in castrated male mice can explain muscle atrophy.

551

552 Another member of the (TGF $\beta$ ) superfamily, BMP signaling through Smad1/5/9  
553 phosphorylation is an emergent pathway controlling muscle size (61, 72). Indeed, it has been  
554 suggested that BMP signaling participates in postnatal muscle development, since the  
555 phosphorylation of Smad1/5/9 is lower in 6-month old (adult) mice as compared to younger  
556 mice (72). BMPs are proteins that bind to BMP receptor, such as ALK3, that in turn  
557 phosphorylates Smad1/5/9 proteins, promoting with *Smad4*, the regulation of target genes,  
558 in particular *Id1* and various processes regulating muscle size (60). However, our data  
559 provide initial insights that the delayed muscle growth in 3-month old castrated mice is not  
560 likely to be related to changes in BMP signaling through Smad1/5/9 phosphorylation. The  
561 **ubiquitin proteasome system** also plays an important role in the atrophic process (4).  
562 However, in contrast to increased MSTN expression, the likely less active ubiquitin ligases  
563 cannot explain the reduced weight in 3-month old castrated male mice.

564

565 Differential effect of FGRF versus MGFR on muscle performance gain

566

567 Another novel finding of our study is that, in contrast to MGRF in male mice, **FGRF does**  
568 **not contribute to maximal power gain** between 1 month and 6 months in female mice  
569 (Table 3). However **FGRF contribute to 20% of maximal force gain** in 6-month old  
570 female mice (Table 3), similarly to MGFR in male mice, and its action is irrespective of any  
571 change in neurotransmission. These results differ, for yet unknown reasons, from those of  
572 previous studies showing that castration increases or has no effect on absolute maximal  
573 force in growing female rats (42, 67). In line with our results, it has been shown that  
574 estrogens positively modulate absolute maximal force in adult female mice (24, 49, 50).  
575 Indeed, castration reduces **specific maximal** force and maximal calcium activated force of  
576 permeabilized fibres in adult female mice, and this effect is explained by a lower fraction of  
577 myosin heads strongly bound to actin (49, 50).

578

579 Together with the reduced specific maximal force, i.e muscle quality, a lower muscle weight  
580 explains the effect of castration on the absolute maximal force in 3- and 6-month old female  
581 mice. Thus, in contrast to MGRF, we found that FGRF also contributes to the **increase in**  
582 **muscle weight** during postnatal development, even though its contribution is rather small  
583 (12%)(Table 3). Our results also demonstrate that FGRF promotes the growth of muscle  
584 fibres, in agreement with a recent study (39). However, this fibre growth is not related to  
585 changes in MSTN, BMP signaling through Smad1/5/9 phosphorylation and ubiquitin  
586 ligases, at least in 3-month old female mice. The increased transcript level of IGF-1, a factor  
587 promoting muscle growth, in 3-month old castrated female mice could be a compensatory  
588 phenomenon. It is possible that impaired intrinsic function of satellite cells (39) contributes  
589 to the reduced muscle growth observed after castration in female mice.

590

591 It also remains to be confirmed whether the action of putative endogenous estrogens on  
592 absolute maximal force is mediated via estrogen receptor (ER) that exhibits different  
593 subtypes, ER $\alpha$ , ER $\beta$  and Gper. It was reported that estrogen effects on muscle are mediated  
594 in part via muscle ER $\alpha$  in mice (6, 54). In accordance, ER $\beta$  deficiency does not lead to  
595 significant change in absolute maximal force (23). However, a recent study demonstrated  
596 that estrogens have a rapid effect on muscle contractility via both ER $\beta$  and Gper (40), e.g.,  
597 the potentiated force was increased. There is a possibility that estrogen effects can be  
598 mediated by brain ER since estrogens increase the level of voluntary exercise (14, 21) which  
599 is known to modulate muscle performance and growth. In agreement, a recent study  
600 suggests that castration-induced muscle atrophy could result from the reduced level of motor  
601 activity in adult female mice (21). However, it has been reported that the benefits of  
602 estrogens is independent of physical activity, e.g. can be observed in inactive muscle (24). In  
603 summary, we demonstrate that FGRF play a role in maximal force gain and muscle mass  
604 development, contrasting the traditional view that estrogens have no impact on muscle  
605 postnatal development. The signaling axis through which these effects are mediated is still  
606 not well defined.

607

608 Sexual dimorphism concerning muscle performance is reduced by castration

609

610 We also report several differences between sexes concerning muscle performance at 6  
611 months of age, in intact mice. The reduced absolute maximal force in 6-month intact female  
612 mice, as compared to males, is explained by a **lower muscle weight**, in line with previous  
613 studies, without difference in specific maximal force (62). Our results suggest that the lower  
614 muscle weight in female mice is related to sex-based difference in *IGF-1* gene expression  
615 (lower transcript level in female) but not BMP signaling, ubiquitin ligases and *MSTN* gene

616 expression. Regarding absolute maximal power, we found that the lowered absolute  
617 maximal power in 6-month old female intact mice results from both reductions in **specific**  
618 **maximal power** and muscle weight, as previously shown (62). We report here that reduced  
619 specific maximal power is not related to an increased percentage of less powerful fibres  
620 expressing MHC-2a. It is possible that the increased fibrosis in female mice contributes, at  
621 least in part, to the reduced specific maximal power.

622

623 Another novel finding of our study is that castration before puberty reduces the sexual  
624 dimorphism concerning both absolute maximal force and power in 6-month old mice,  
625 indicating that MGRF and FGRF contribute to the sex-based differences regarding muscle  
626 performance. Concerning the **lower muscle weight** that explains the lower absolute  
627 maximal force and power in intact female mice, we found that castration does not fully  
628 eliminate this sex-based difference, suggesting that both endogenous sexual hormones and  
629 other additional factors can contribute to this aspect, such as MSTN (43) or IGF-1. In line,  
630 we found a sex-based difference in MSTN mRNA level in castrated mice. The **lower**  
631 **specific maximal power** in intact female mice is reversed by castration (increased specific  
632 maximal power in castrated females versus castrated males), suggesting that MGRF and  
633 FGRF have beneficial and detrimental actions on specific maximal power, respectively. Our  
634 results indicate that these effects cannot be attributed to a sex-based difference in fibre type  
635 specification in castrated mice, a finding that adds to an equivocal body of evidence  
636 regarding the respective effects of androgens and estrogens on muscle fibre type  
637 specification (2, 27, 39, 53, 56, 63).

638

639 Conclusion

640

641 In summary, our study indicates that MGRF promotes muscle absolute maximal force and  
642 power gains between 1 month and 6 months in male mice, mainly via promoting muscle  
643 contractile quality, and without affecting neuromuscular transmission. In 3-month old male  
644 mice, the effects of MGRF on muscle performance are not mediated by muscle fibre AR. In  
645 female mice, FGRF promotes absolute maximal force gain between 1 month and 6 months  
646 but not absolute maximal power gain. Here we provide preliminary insights that demonstrate  
647 that the effects of MGRF and FGRF in 3-month old mice are not related to alterations in  
648 BMP signaling through Smad1/5/9. However, our results suggest that the action of MGRF  
649 could be mediated via the upregulation of ubiquitin ligases in 3-month old male mice. Now,  
650 more protracted efforts are needed to define the signaling cascades responsible for the  
651 effects of sex-related hormones. We also show that MGRF and FGRF only marginally  
652 contribute to muscle performance gain between 1 month and 6 months of age in both sexes,  
653 indicating the existence of additional factors, endocrine or not. Finally, we have  
654 demonstrated that MGRF and FGRF contribute to the sexual dimorphism regarding muscle  
655 performance in adult mice. Thus, we provide evidence demonstrating that both MGRF and  
656 FGRF are required for the normal postnatal development of muscle performance in mice of  
657 both sexes.

658

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675

676

677 Declaration of interest

678

679 The authors declare that there is no conflict of interest that could be perceived as prejudicing  
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681

682 Author contributions

683

684 DM and AF conceived the research.

685 VUP, JM, ML, PR and AF performed experiments and analysed data.

686 AS, DJO, PN and OA provided expertise.

687 VUP, DM and AF wrote the manuscript.

688 All authors edited and approved the manuscript.

689

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- 918
- 919

920 Legends of figures

921

922 Figure 1. Muscle performance in castrated male and female mice (TA muscle).

923 A: Absolute maximal force in male mice. B: Specific maximal force in male mice. C:

924 Absolute maximal power in male mice. D: Specific maximal power in male mice. E: Muscle

925 weight in male mice. F: Absolute maximal force in female mice. G: Specific maximal force

926 in female mice. H: Absolute maximal power in female mice. I: Specific maximal power in

927 female mice. J: Muscle weight in female mice.

928 1m : 1-month old ; 1.5 : 1.5-month old ; 3m : 3-month old; 6m : 6-month old ; cas :

929 castrated.

930 c : Castrated mice different from corresponding intact mice ( $p < 0.05$ ).

931 s : Female mice different from corresponding male mice ( $p < 0.05$ ).

932 n=8-16/group;

933 The data in the figure were collected during the first set of measurements, in the same mice.

934

935 Figure 2. Muscle performance in 3-month old castrated male mice with deficiency in muscle

936 fibre AR (TA muscle).

937 A: Absolute maximal force. B: Specific maximal force. C: Muscle weight.

938  $AR^{L2/y}$  : Wild-type mice.  $AR^{skm/-y}$  : Mice with muscle fibre AR deficiency.

939 c : Castrated mice different from corresponding intact mice ( $p < 0.05$ ).

940 n=6-8/group

941 The data in the figure were collected during the second set of measurements, in the same

942 mice.

943

944

945 Figure 3. Neuromuscular transmission and neuromuscular junction morphology in 3-month  
946 old castrated mice.

947 A: Absolute maximal force in response to nerve or muscle stimulation in male mice (TA  
948 muscle). B: Absolute maximal force in response to nerve or muscle stimulation in female  
949 mice (TA muscle). C: Absolute and specific maximal forces and weight of plantaris muscle  
950 (male mice). D: Representative images of neuromuscular junction in castrated male mice  
951 (plantaris muscle). Scale bar = 20  $\mu\text{m}$ . E: AChR-rich endplate area (plantaris muscle, male  
952 mice). F: pre/post overlap (plantaris muscle, male mice). G: Synaptophysin area (plantaris  
953 muscle, male mice).

954 c : Castrated mice different from corresponding intact mice ( $p < 0.05$ ).

955 n=9-14/group for A-C; n=20/group for D-G.

956 The data in the figure were collected during the third set of measurements, in the same mice.

957

958

959 Figure 4: Muscle and fibre atrophy, and fibre type composition in 3-month old male and  
960 female castrated mice (TA muscle).

961 A: Muscle weight. B: Distribution of diameter (min ferret) of fibres in castrated male mice,  
962 using histological analysis. C: Distribution of diameter (min ferret) of fibres in castrated  
963 female mice. D: Fibrosis using histological red Sirius staining. E: Percentage of fibres  
964 expressing MHC-2a, using immunohistological staining.

965 c : Castrated mice different from corresponding intact mice ( $p < 0.05$ ).

966 s : Female mice different from corresponding male mice ( $p < 0.05$ ).

967 n=10-14 per group for A; n=3-4 per group for B-E.

968 The data in the figure were collected during the third set of measurements, in the same mice.

969



970 Figure 5. Intramuscular remodeling pathway: markers of BMP signaling through Smad1/5/9  
971 in 3-month old castrated male and female mice (TA muscle).

972 A : Representative images of Western blots (male mice). B : Protein levels of  
973 phosphorylated Smad1/5/9 (male mice). C: mRNA levels of ALK3. D: mRNA levels of  
974 Smad4. E: mRNA levels of ID1.

975 Int : intact ; Cas : castrated.

976 c : Castrated mice different from corresponding intact mice ( $p < 0.05$ ).

977 s : Female mice different from corresponding male mice ( $p < 0.05$ ).

978  $n=5-7$  per group.

979 The data in the figure were collected during the third set of measurements, in the same mice.

980

981 Figure 6. Intramuscular remodeling pathway: markers of the ubiquitin proteasome system,  
982 and IGF-1 and MSTN transcript levels in 3-month old castrated male and female mice (TA  
983 muscle).

984 A : Representative images of blots (male mice). B : Protein levels of phosphorylated Foxo3a  
985 (male mice). C : Protein level of phosphorylated Foxo1 (male mice). D: mRNA levels of  
986 Murf1. E : mRNA levels of FbxO30. F : mRNA levels of atrogen 1. G : mRNA levels of  
987 MSTN. H: mRNA levels of IGF-1.

988 Int : intact ; Cas : castrated ; IGF-1 : insulin growth factor 1 ; MSTN : myostatin.

989 c : Castrated mice different from corresponding intact mice ( $p < 0.05$ ).

990 s : Female mice different from corresponding male mice ( $p < 0.05$ ).

991  $n=5-7$ /group.

992 The data in the figure were collected during the third set of measurements, in the same mice.

993

994

995 Table 1. Body weights.

996 -----

997                                      Castrated                                      Intact

998 -----

999 3-month old

1000 Male                                      23.5±0.8<sup>c</sup>                                      27.8±0.1

1001 Female                                      23.2±0.5                                      22.9±0.4

1002

1003 6-month-old

1004 Male                                      30.9±1.1                                      30.0±0.6

1005 Female                                      30.4±1.5<sup>c</sup>                                      25.4±0.8

1006 -----

1007 c : significantly different from intact (p < 0.05).

1008 n=5-11/group

1009 The data in the Table 1 were collected during the first set of measurements.

1010

1011

1012 Table 2. Primers used.

1013 -----

1014 Name                      Sequence

1015 -----

1016 18S                      5'-TCGTCTTCGAAACTCCGACT-3'

1017                              5'-CGCGGTTCTATTTTGTGGT-3'

1018 ID1                      5'-CTCGGAGTCTGAAGTCGGGA-3'

1019                              5'-GAACACATGCCGCCTCGG-3'

1020 ALK3                      5'-CTCTGAGAATTCTGAAGAAAGCAGC-3'

1021                              5'-TCCTGCTGTCTCACTGGTGT-3'

1022 Smad4                      5'-GAATAGCTCCAGCCATCAGTCT-3'

1023                              5'-GAATGCACAATCGCCGGAGG-3'

1024 IGF                      5'-AGCAGCCTTCCAACCTCAATTAT-3'

1025                              5'-GAAGACGACATGATGTGTATCTTTATC-3'

1026 MuRF                      5'-TGAGGTGCCTACTTGCTCCT-3'

1027                              5'-GTGGACTTTTCCAGCTGCTC-3'

1028 MSTN                      5'-GCTACCACGGAAACAATCAT-3'

1029                              5'-CAATACTCTGCCAAATACCA-3'

1030 Atrogin                      5'-TCACAGCTCACATCCCTGAG-3'

1031                              5'-TCAGCCTCTGCATGATGTTC-3'

1032 FbxO30s                      5'-AGGGACGTTTGTGGCAGTTT-3'

1033                              5'-ACTGAATCGCCATACCTTCTC-3'

1034 -----

1035

1036

1037 Table 3. Contribution of male (MGRF) and female (FGRF) gonad-related factors to TA muscle  
 1038 performance gains and growth (increased weight) between 1 month and 3 or 6 months of age.

1039 -----

1040	Sex	Age	Force	Power	Weight
1041	-----				
1042	Contribution of MGRF				
1043	Male	3 month	26%	58%	31%
1044	Male	6 month	29%	38%	0%
1045	Contribution of FGRF				
1046	Female	3 month	32%	0%	23%
1047	Female	6 month	20%	0%	12%
1048	-----				

1049 Force: absolute maximal force; Power: absolute maximal power. The contribution of  
 1050 MGRF and FGRF to muscle performance gain was calculated as follow. For example,  
 1051 absolute maximal force gain in castrated and intact 6-month old male mice was 142.2% and  
 1052 101.3% respectively. Therefore, the contribution of MGF (%) to muscle P0 gain in 6-month  
 1053 old male mice was  $=100-(101.3/142.2)*100 = 28.8\%$ .

1054 The data in the Table 3 were collected during the first set of measurements.

1055

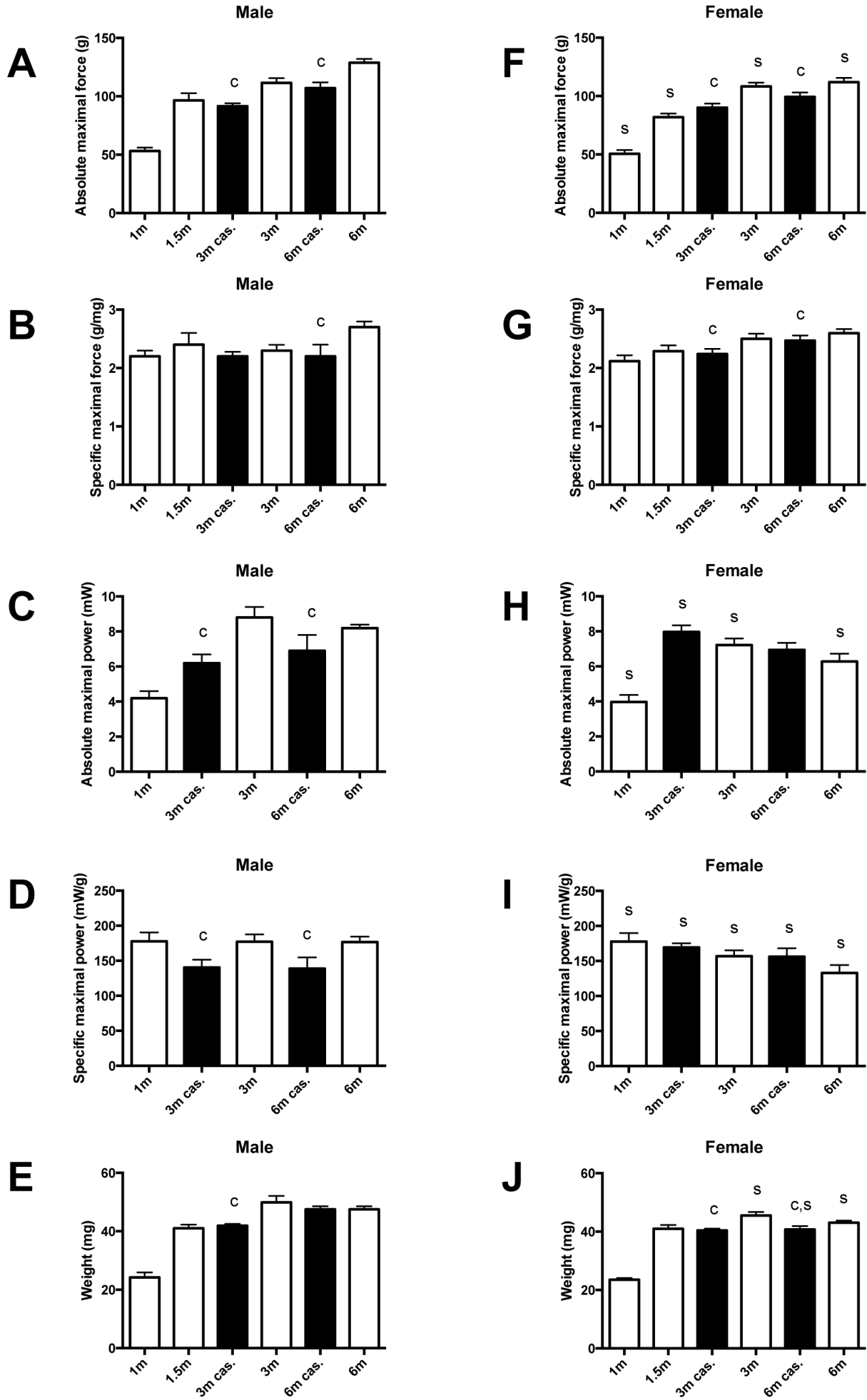
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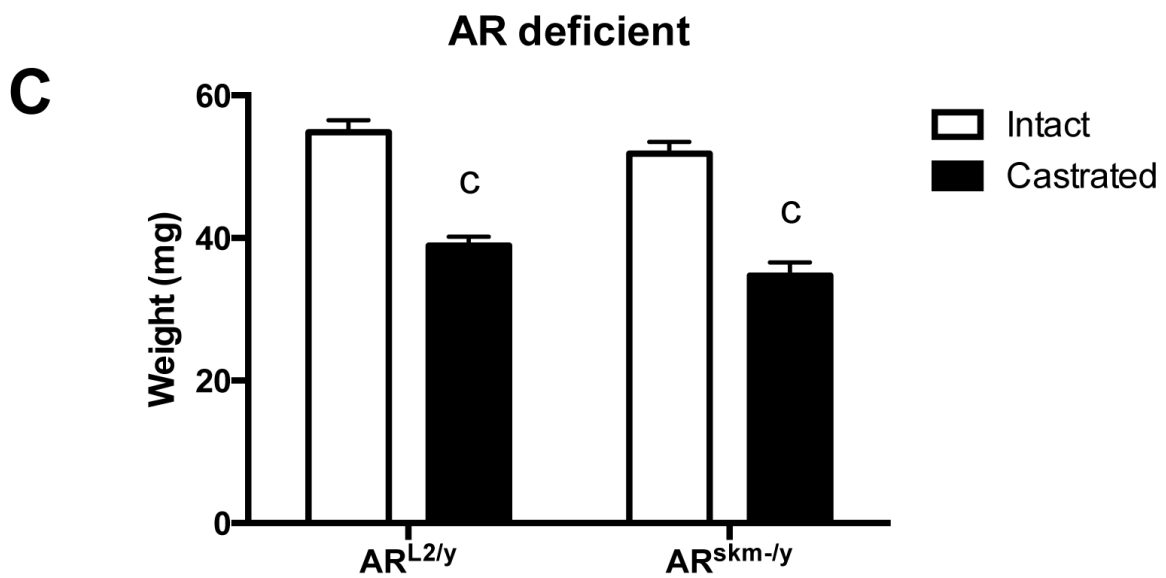
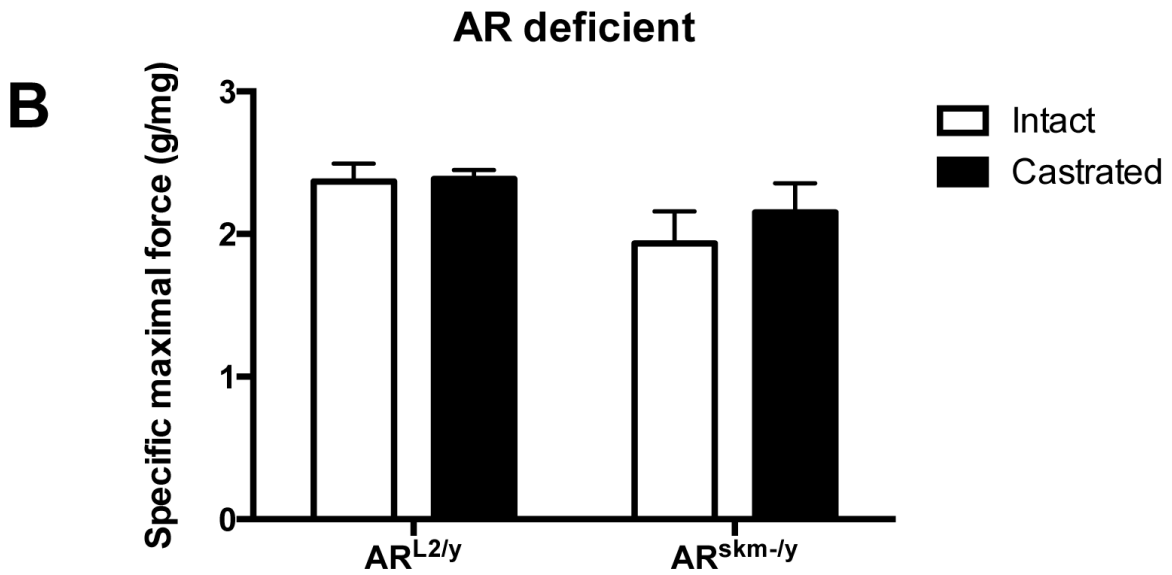
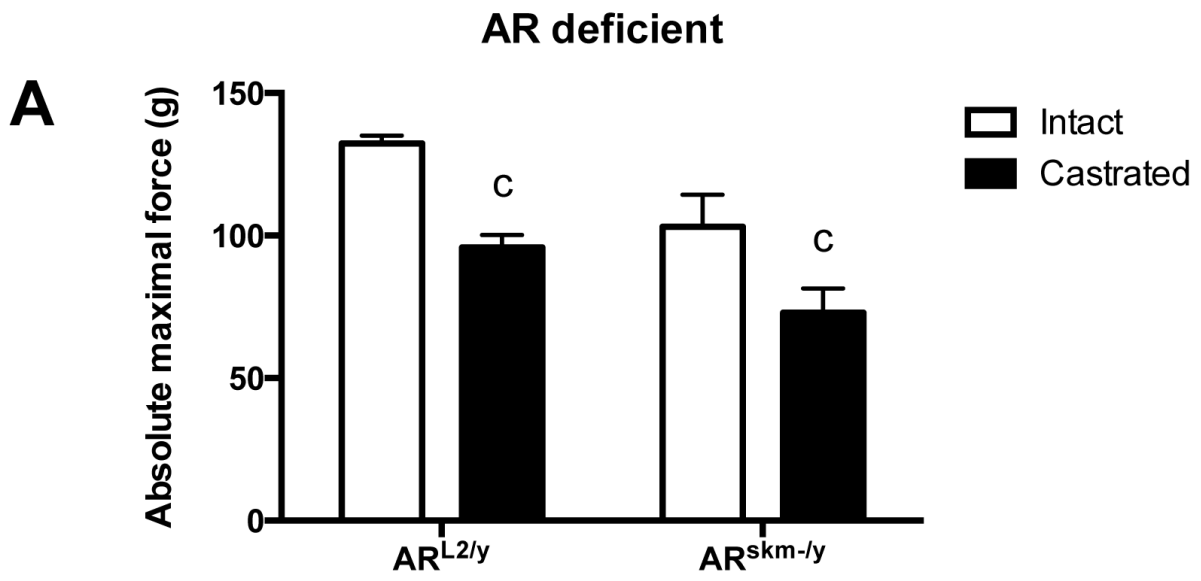
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# Figure 1

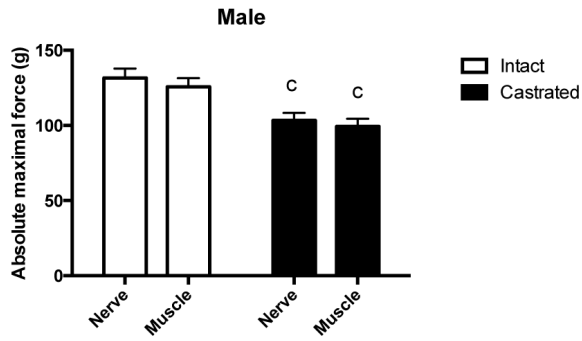


# Figure 2

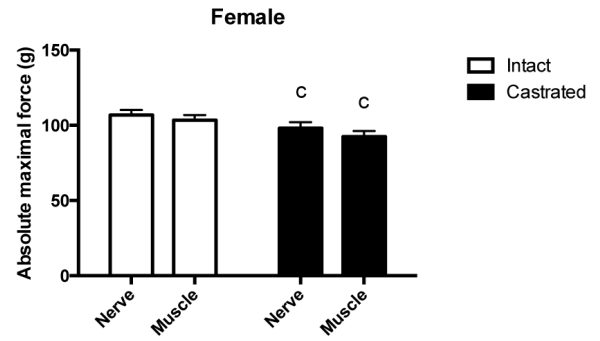


# Figure 3

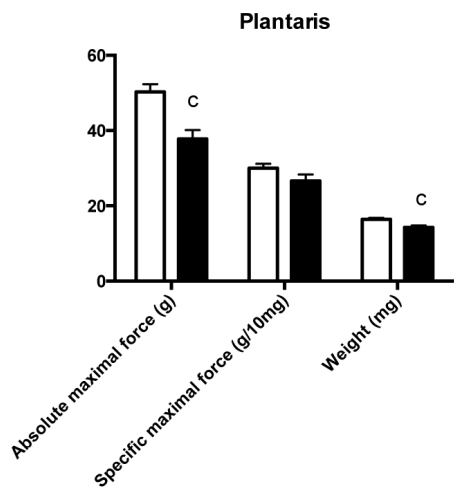
## A



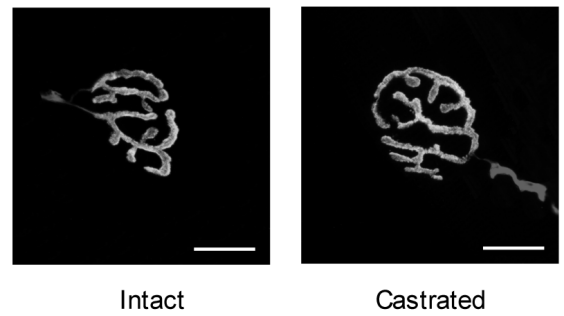
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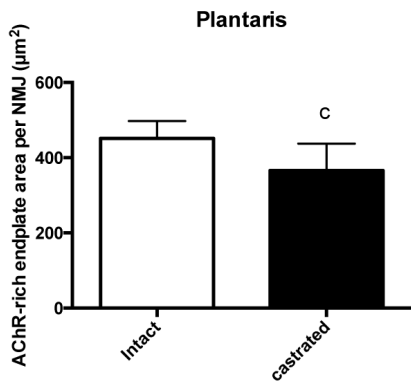
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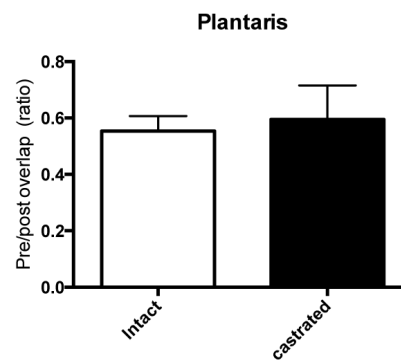
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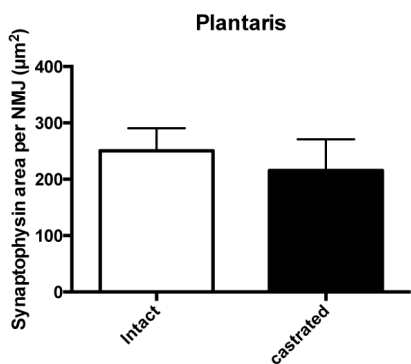
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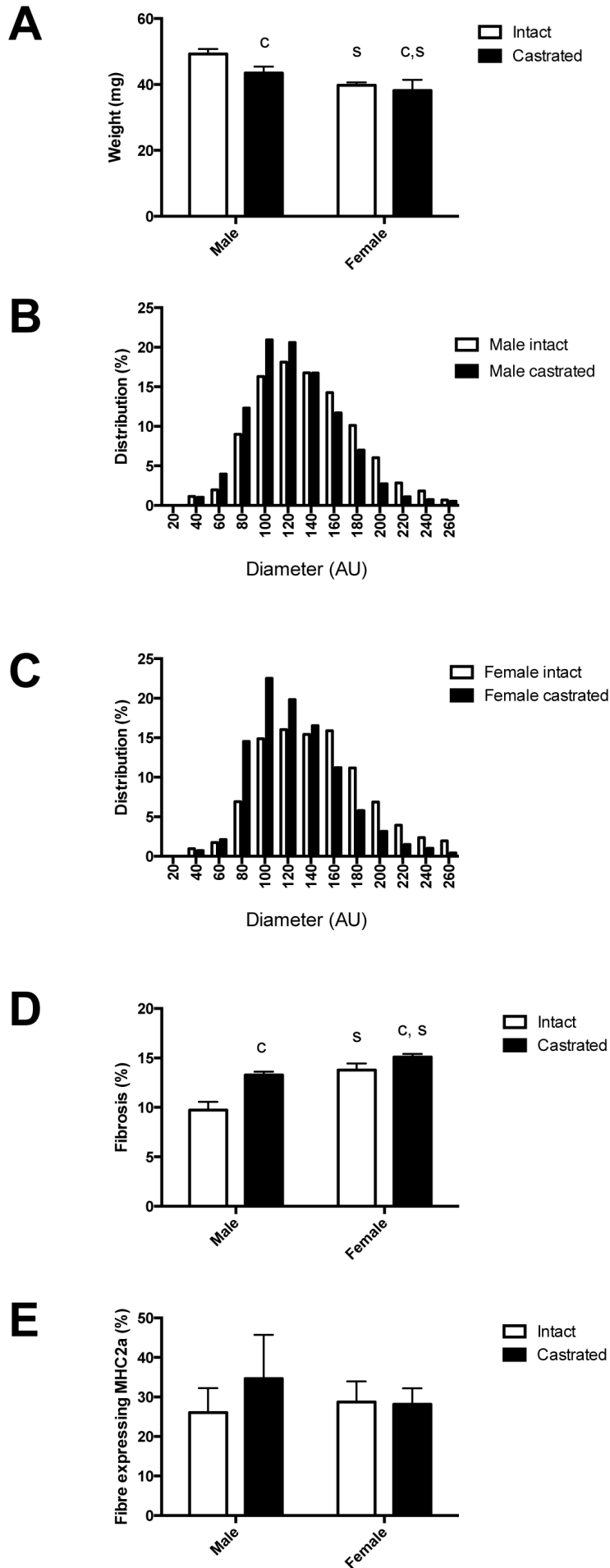
## F



## G



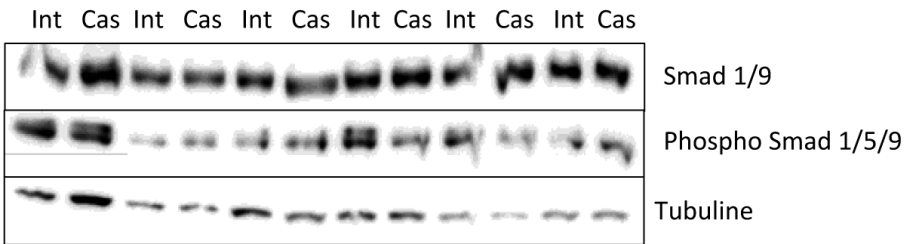
# Figure 4



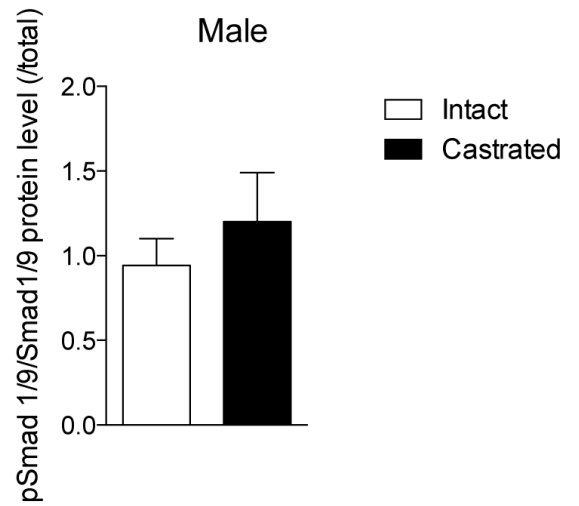


# Figure 5

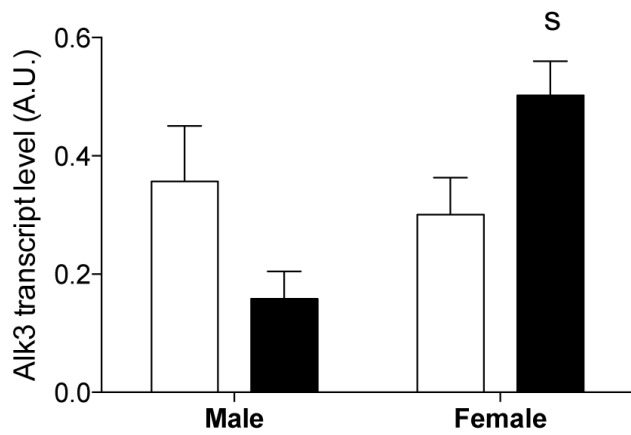
## A



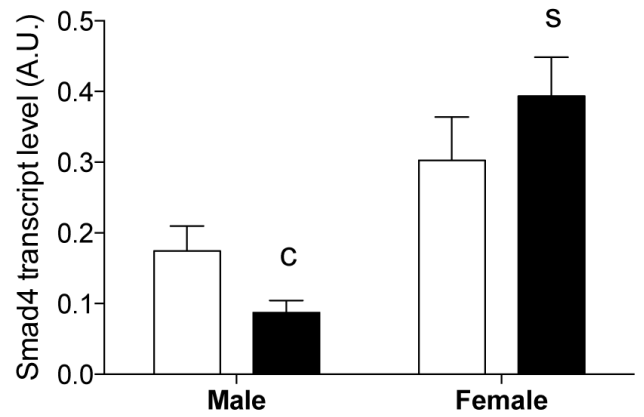
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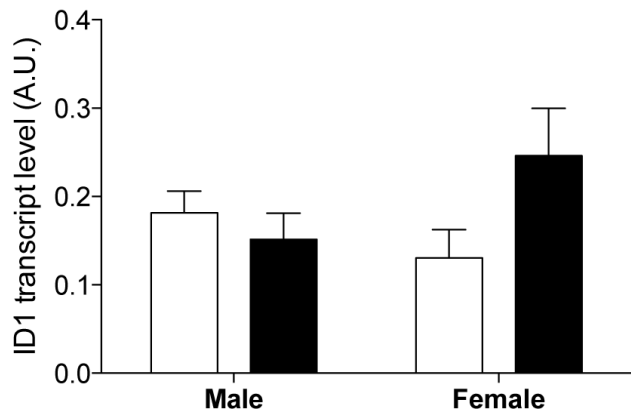
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## D

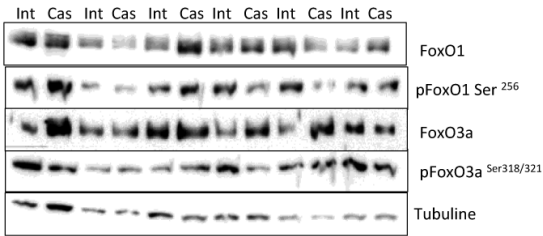


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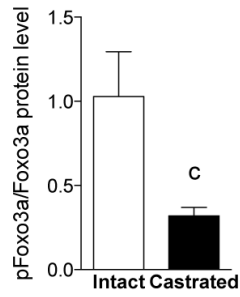


# Figure 6

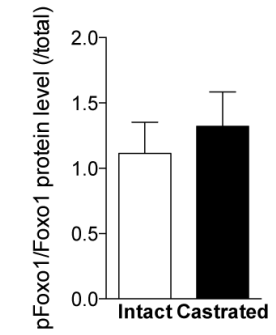
## A



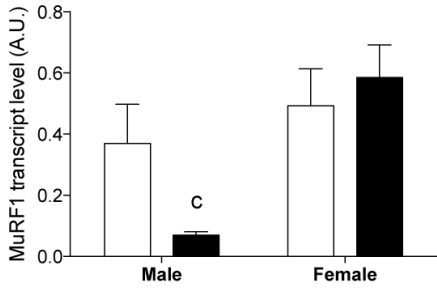
## B



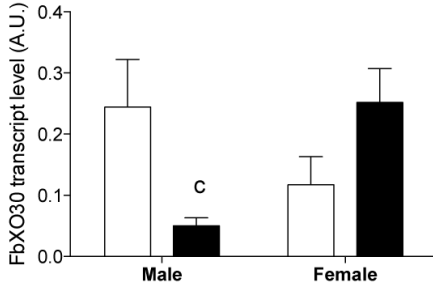
## C



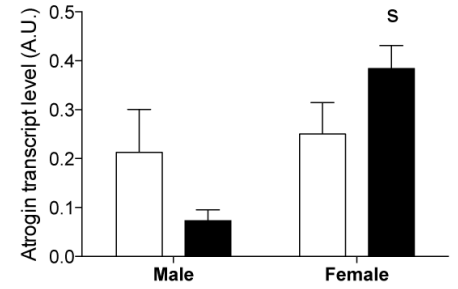
## D



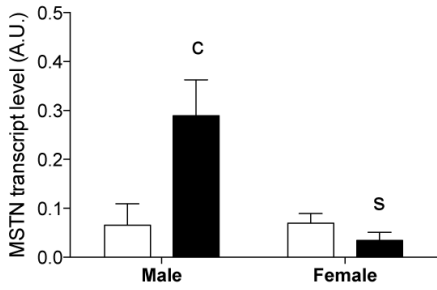
## E



## F



## G



## H

