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

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Abstract

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

Keywords

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

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

Introduction

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

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

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

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

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

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

Materials and Methods

Mice

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

Muscle contractile performance

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

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

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

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

Fibre size and type

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

Remodeling pathways: protein

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

Remodeling pathways: mRNA

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

Statistical analysis

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 ± SEM. Significance was set at $p < 0.05$.
244

Results

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

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

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

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

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

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

2-Castration decreases absolute maximal force gain in female mice

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

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

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

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

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

3- Castration reduces sexual dimorphism regarding muscle performance

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

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

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

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

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

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

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

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

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

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

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

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

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

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

As mentioned above, part of the reduction in muscle performance is related to decreased muscle weight in 3-month old castrated mice of both sexes. Therefore, we further analysed the **reduced TA muscle weight** in 3-month castrated mice of both sexes (Figure 4A), as previously shown (Figures 1E and J), and found that it was not associated with a decrease in **bone growth** in castrated mice of both sexes. Indeed, the length of the tibia was not changed by castration in both 3-month old male (17.8 ± 0.3 mm in castrated versus 18.2 ± 0.3 mm in intact mice) and female (18.0 ± 0.1 mm in castrated versus 18.3 ± 0.2 mm in intact mice) mice. Moreover, the reduced muscle weight in castrated mice was related to muscle **fibre atrophy** since histological analyses revealed a left shift in the fibre diameter distribution in both 3-month old castrated mice of both sexes (Figures 4BC). In line, there was an increase in **fibrosis** in 3-month old castrated mice (14.2±0.9 % in castrated versus 11.7±2.0 % in intact mice)($p<0.05$)(Figure 4D). We also determined whether fibre atrophy was accompanied by an increase in the **percentage of fibres expressing MHC-2a that** are fast fibres having small fibre diameter**.** We found that the percentage of fibres expressing MHC-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.

Moreover, we found that the transcript levels of *Murf1* (Figure 6D) and *FbXO30* (Figure 6E) were reduced in 3-month old castrated male mice, as compared to age-matched intact male mice ($p < 0.05$), whereas that one of *atrogin 1* was unchanged (Figure 6F). In contrast, castration did not affect the transcript levels of *Murf1, FbXO30* and *atrogin 1* in 3-month-old female mice (Figures 6D-F). Together, these results suggest that 3-month after castration E3 ubiquitin ligases (*atrogin 1*, *Murf1*, and *FbXO30*) might be less active in males and unchanged in females.

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

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

Discussion

MGRF promotes long-term muscle contractile quality

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

Our results indicate that increased fibrosis, but not neuromuscular transmission failure, can explain in part, the reduced specific maximal force and power in 6-month old castrated male mice. It is possible that the decrease in muscle quality is due to accumulation of 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

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

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

Differential effect of FGRF versus MGFR on muscle performance gain

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

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

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

Sexual dimorphism concerning muscle performance is reduced by castration

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

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

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

Conclusion

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

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contributions

DM and AF conceived the research.
VUP, JM, ML, PR and AF performed experiments and analysed data.
AS, DJO, PN and OA provided expertise.
VUP, DM and AF wrote the manuscript.
All authors edited and approved the manuscript.

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Legends of figures

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

A: Absolute maximal force in male mice. B: Specific maximal force in male mice. C: Absolute maximal power in male mice. D: Specific maximal power in male mice. E: Muscle weight in male mice. F: Absolute maximal force in female mice. G: Specific maximal force in female mice. H: Absolute maximal power in female mice. I: Specific maximal power in female mice. J: Muscle weight in female mice.

1m : 1-month old ; 1.5 : 1.5-month old ; 3m : 3-month old; 6m : 6-month old ; cas : castrated.

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

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

n=8-16/group;

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

Figure 2. Muscle performance in 3-month old castrated male mice with deficiency in muscle fibre AR (TA muscle).

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

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

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

n=6-8/group

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

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 µm. 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

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

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

Int : intact ; Cas : castrated.

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

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

n=5-7 per group.

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

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

A : Representative images of blots (male mice). B : Protein levels of phosphorylated Foxo3a (male mice). C : Protein level of phosphorylated Foxo1 (male mice). D: mRNA levels of Murf1. E : mRNA levels of FbXO30. F : mRNA levels of atrogin 1. G : mRNA levels of MSTN. H: mRNA levels of IGF-1.

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

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

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

n=5-7/group.

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

Table 1. Body weights.

	Castrated	Intact
3-month old		
Male	23.5±0.8[c]	27.8±0.1
Female	23.2±0.5	22.9±0.4
6-month-old		
Male	30.9±1.1	30.0±0.6
Female	30.4±1.5[c]	25.4±0.8

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

n=5-11/group

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

Table 2. Primers used.

Name	Sequence
18S	5'-TCGTCTTCGAAACTCCGACT-3'
	5'-CGCGGTTCTATTTTGTTGGT-3'
ID1	5'-CTCGGAGTCTGAAGTCGGGA-3'
	5'-GAACACATGCCGCCTCGG-3'
ALK3	5'-CTCTGAGAATTCTGAAGAAAGCAGC-3'
	5'-TCCTGCTGTCTCACTGGTGT-3'
Smad4	5'-GAATAGCTCCAGCCATCAGTCT-3'
	5'-GAATGCACAATCGCCGGAGG-3'
IGF	5'-AGCAGCCTTCCAACTCAATTAT-3'
	5'-GAAGACGACATGATGTGTATCTTTATC-3'
MuRF	5'-TGAGGTGCCTACTTGCTCCT-3'
	5'-GTGGACTTTTCCAGCTGCTC-3'
MSTN	5'-GCTACCACGGAAACAATCAT-3'
	5'-CAATACTCTGCCAAATACCA-3'
Atrogin	5'-TCACAGCTCACATCCCTGAG-3'
	5'-TCAGCCTCTGCATGATGTTC-3'
FbxO30s	5'-AGGGACGTTTGTGGCAGTTT-3'
	5'-ACTGAATCGCCATACCTTCTC-3'

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

	Sex	Age	Force	Power	Weight
Contribution of MGRF					
	Male	3 month	26%	58%	31%
	Male	6 month	29%	38%	0%
Contribution of FGRF					
	Female	3 month	32%	0%	23%
	Female	6 month	20%	0%	12%

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

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

Figure 1

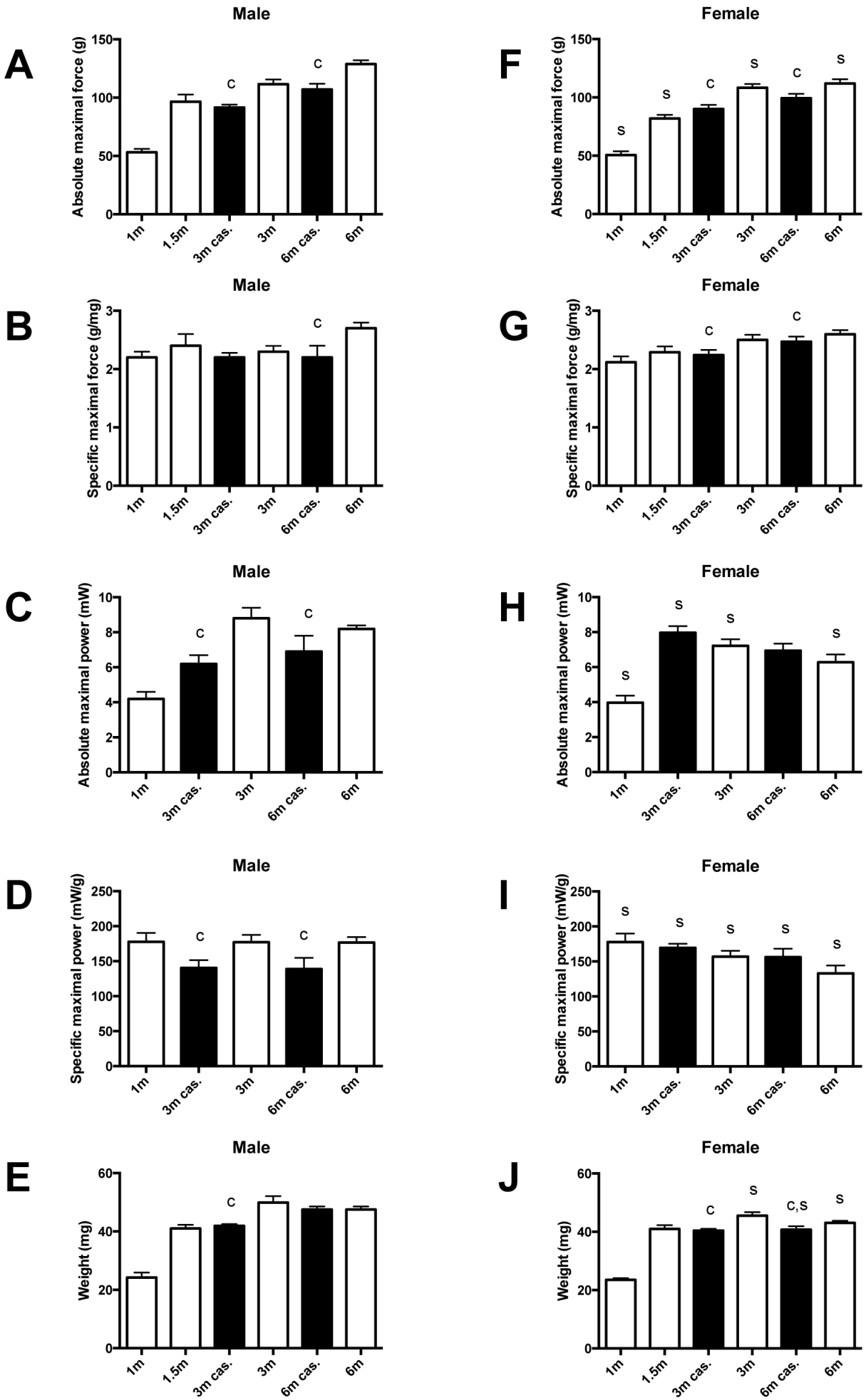

A
Male
Absolute maximal force (g)
150
100
50
0
C
C
1m
1.5m
3m cas.
3m
6m cas.
6m
F
Female
Absolute maximal force (g)
S
S
C
S
C
S
B
Male
Specific maximal force (g/mg)
3
2
1
0
C
G
Female
Specific maximal force (g/mg)
C
C
C
Male
Absolute maximal power (mW)
10
8
6
4
2
0
C
C
H
Female
Absolute maximal power (mW)
S
S
S
S
D
Male
Specific maximal power (mW/g)
250
200
150
100
50
0
C
C
I
Female
Specific maximal power (mW/g)
S
S
S
S
S
E
Male
Weight (mg)
60
40
20
0
C
J
Female
Weight (mg)
S
C
C,S
S

Figure 2

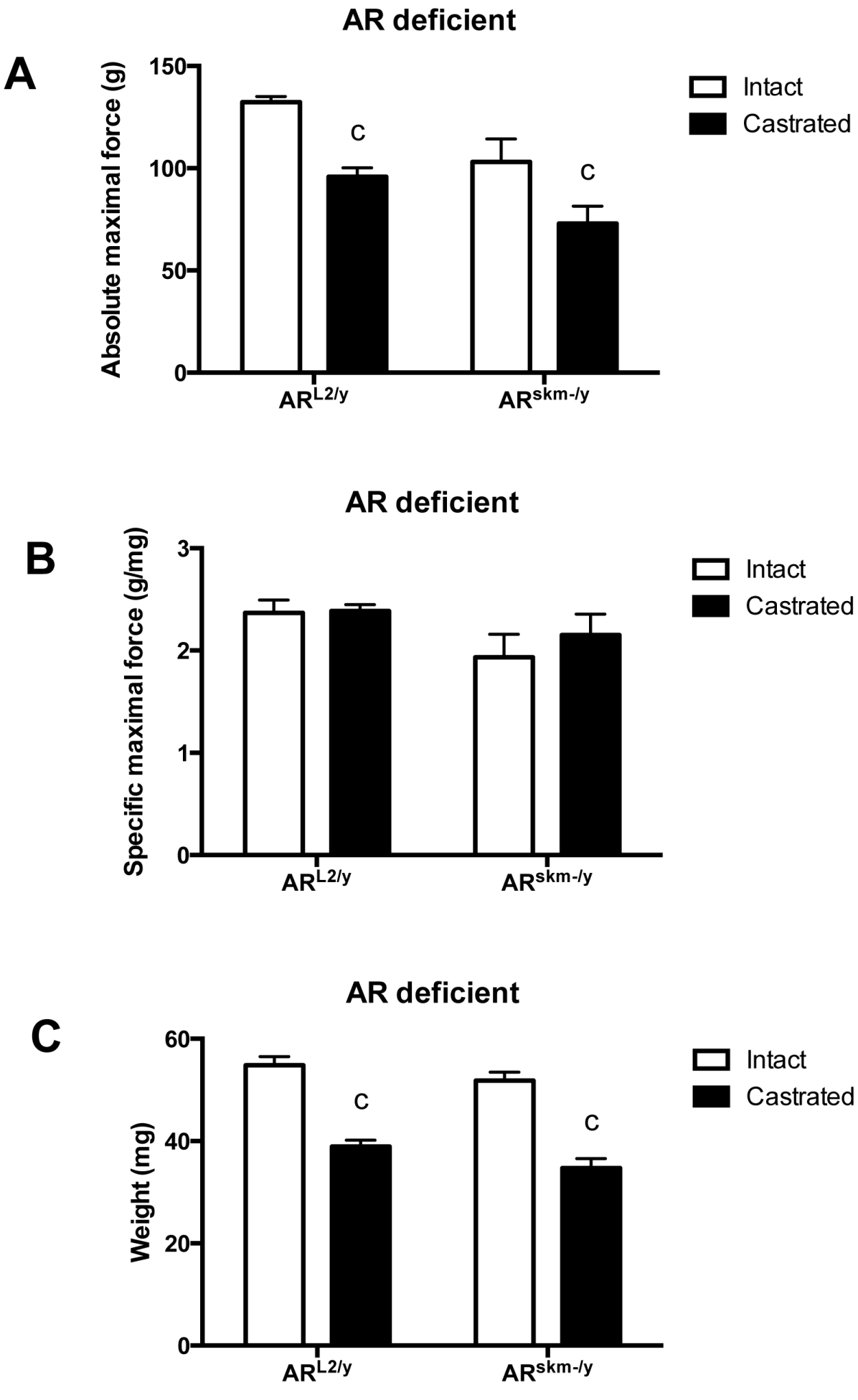

A
AR deficient
Absolute maximal force (g)
150
100
50
0
C
C
Intact
Castrated
AR^L2/y
AR^skm-/y
B
AR deficient
Specific maximal force (g/mg)
3
2
1
0
Intact
Castrated
AR^L2/y
AR^skm-/y
C
AR deficient
Weight (mg)
60
40
20
0
C
C
Intact
Castrated
AR^L2/y
AR^skm-/y

Figure 3

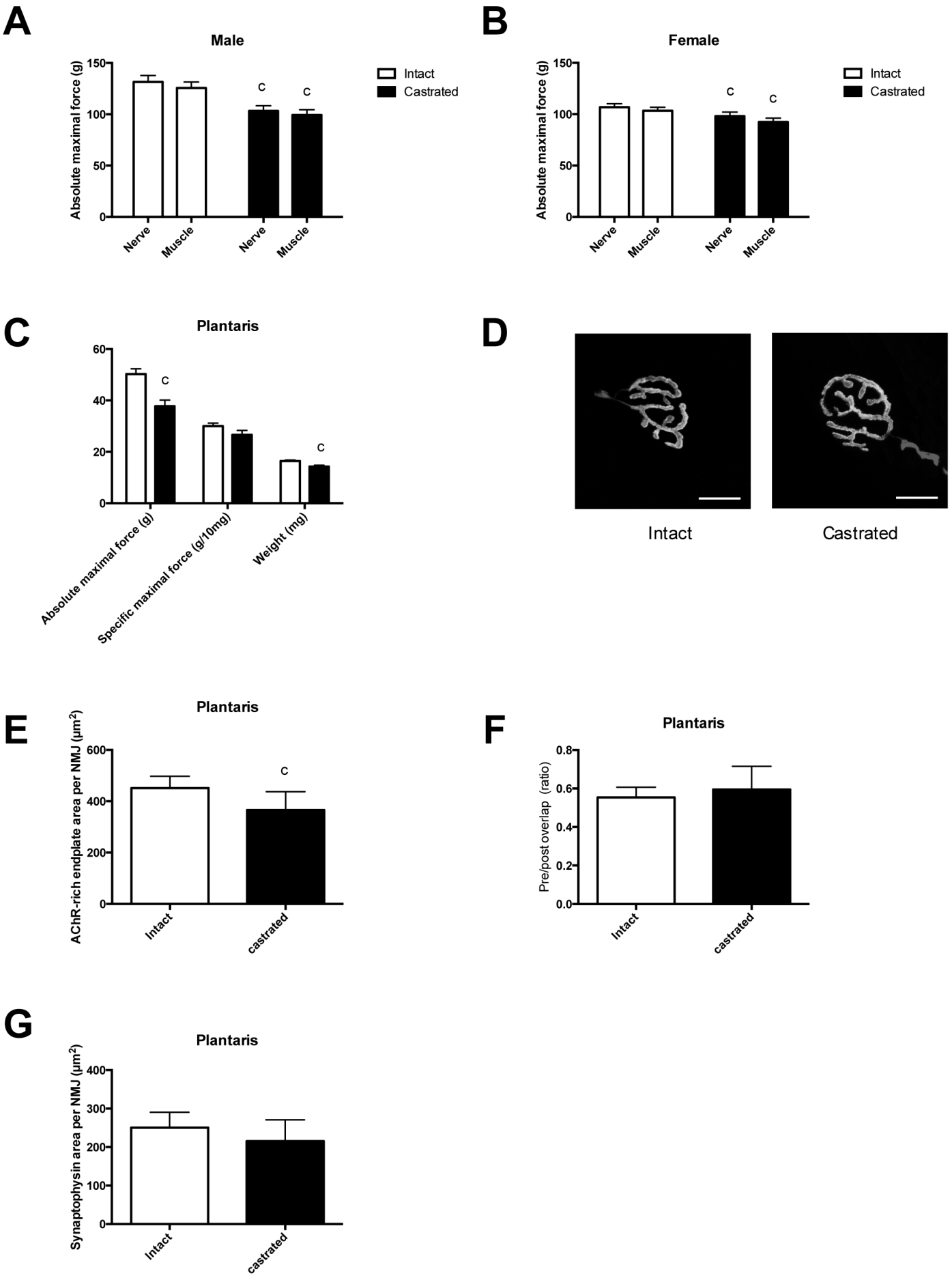

A
Male
Absolute maximal force (g)
150
100
50
0
c
c
Nerve
Muscle
Nerve
Muscle
Intact
Castrated
B
Female
Absolute maximal force (g)
150
100
50
0
c
c
Nerve
Muscle
Nerve
Muscle
Intact
Castrated
C
Plantaris
60
40
20
0
c
c
Absolute maximal force (g)
Specific maximal force (g/10mg)
Weight (mg)
D
Intact
Castrated
E
Plantaris
AChR-rich endplate area per NMJ (µm²)
600
400
200
0
c
Intact
castrated
F
Plantaris
Pre/post overlap (ratio)
0.8
0.6
0.4
0.2
0.0
Intact
castrated
G
Plantaris
Synaptophysin area per NMJ (µm²)
400
300
200
100
0
Intact
castrated

Figure 4

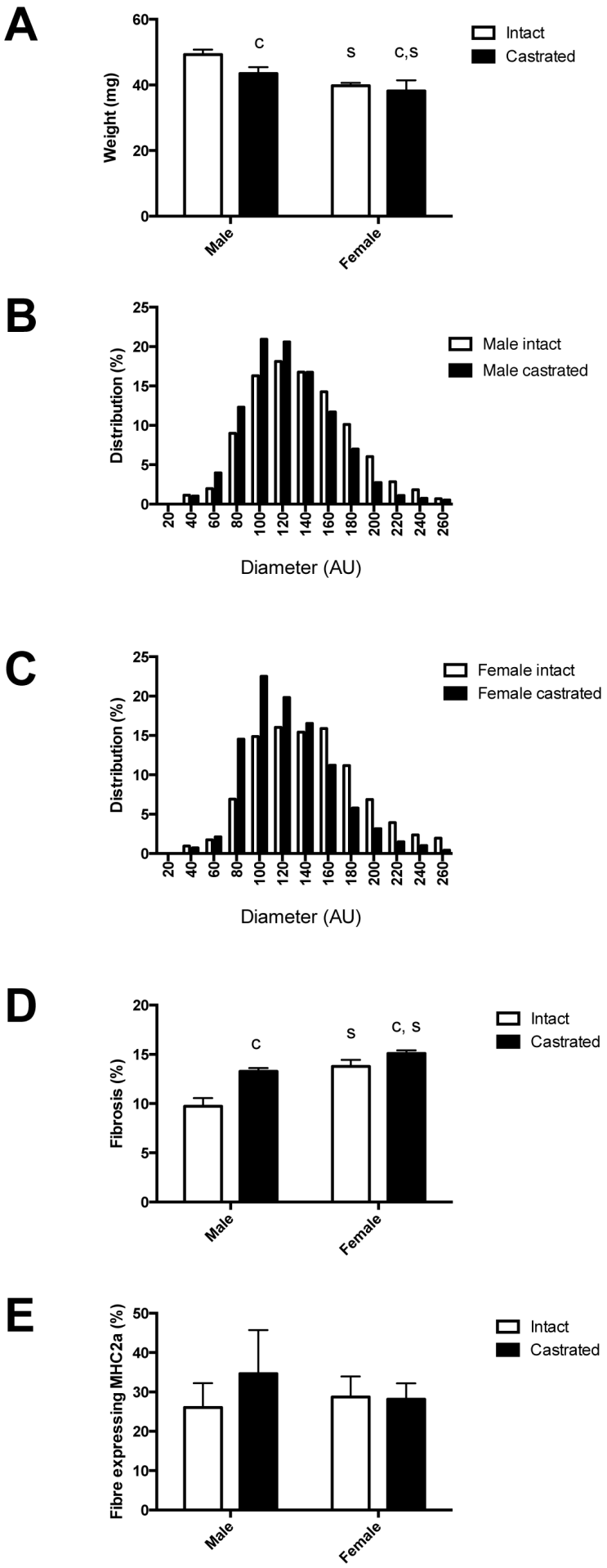

A
Weight (mg)
60
40
20
0
C
S
C,S
Intact
Castrated
Male
Female
B
Distribution (%)
25
20
15
10
5
0
20
40
60
80
100
120
140
160
180
200
220
240
260
Male intact
Male castrated
Diameter (AU)
C
Distribution (%)
Female intact
Female castrated
Diameter (AU)
D
Fibrosis (%)
C
S
C, S
Intact
Castrated
Male
Female
E
Fibre expressing MHC2a (%)
50
40
30
20
10
0
Intact
Castrated
Male
Female

Figure 5

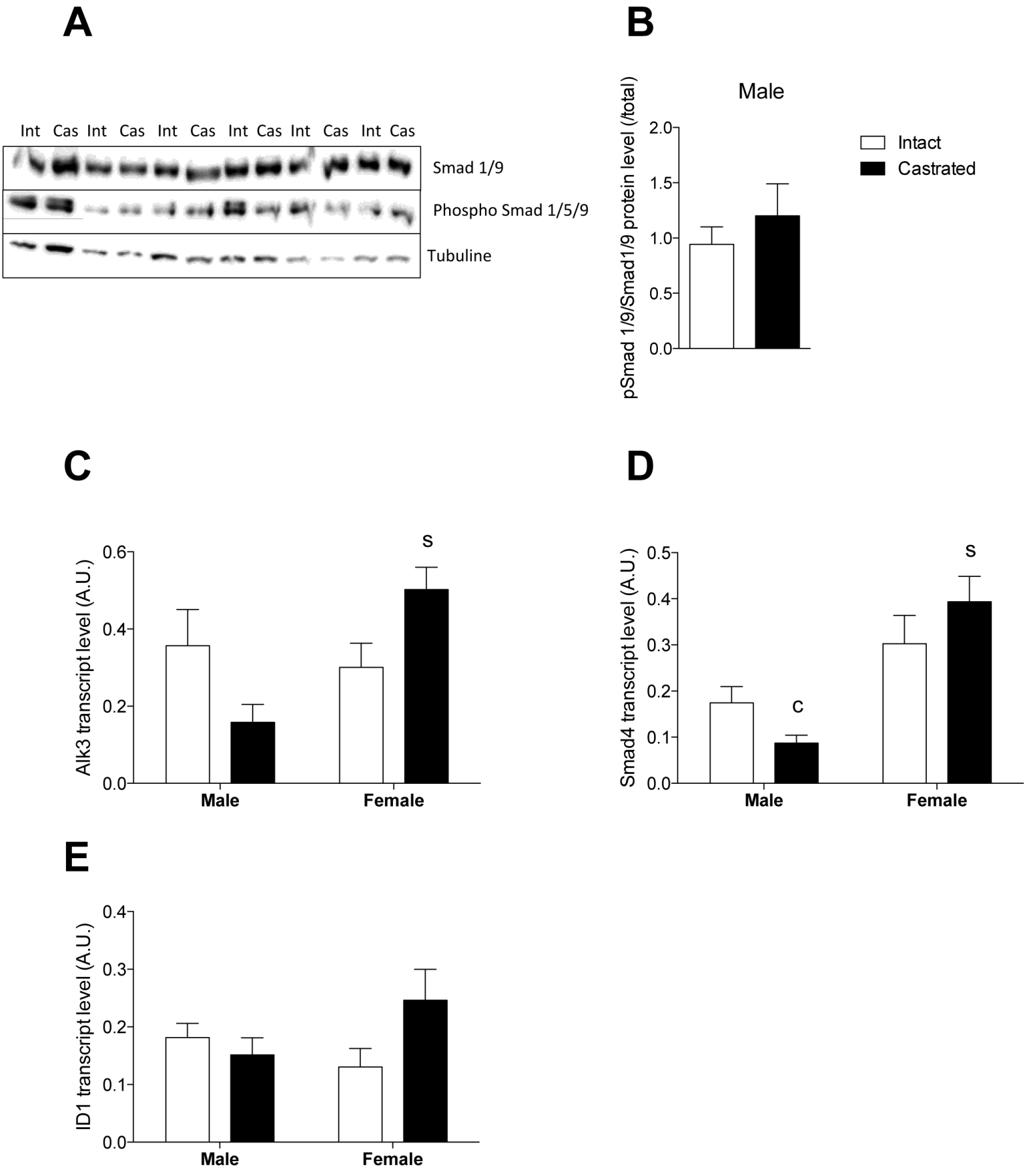
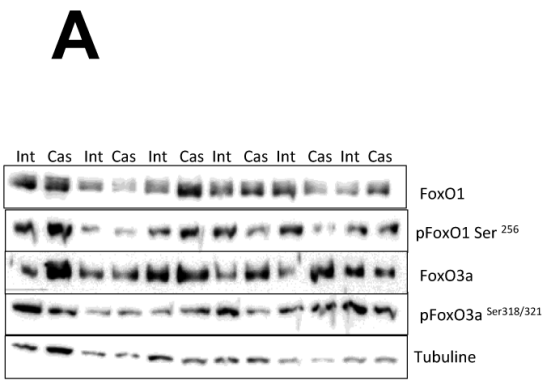
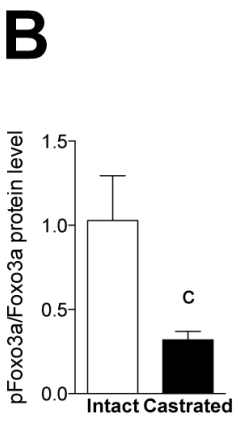
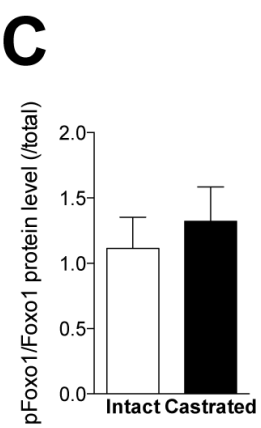
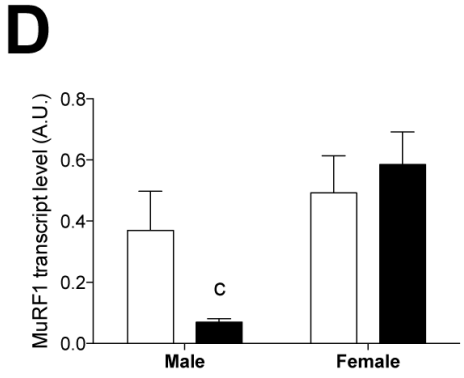

A
Int Cas Int Cas Int Cas Int Cas Int Cas Int Cas
Smad 1/9
Phospho Smad 1/5/9
Tubuline
B
Male
pSmad 1/9/Smad1/9 protein level (/total)
2.0
1.5
1.0
0.5
0.0
Intact
Castrated
C
Alk3 transcript level (A.U.)
0.6
0.4
0.2
0.0
S
Male
Female
D
Smad4 transcript level (A.U.)
0.5
0.4
0.3
0.2
0.1
0.0
C
S
Male
Female
E
ID1 transcript level (A.U.)
0.4
0.3
0.2
0.1
0.0
Male
Female

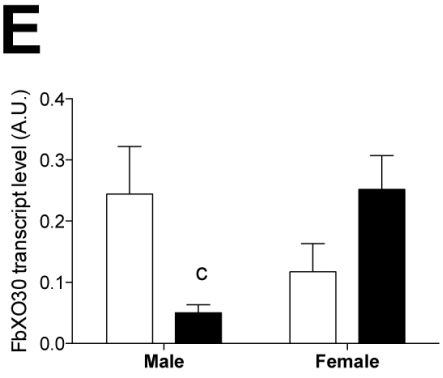
Figure 6

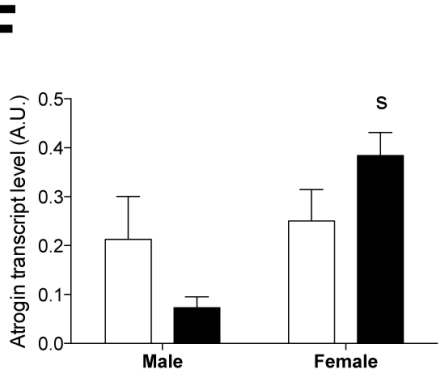

A
Int Cas Int Cas Int Cas Int Cas Int Cas Int Cas
FoxO1
pFoxO1 Ser 256
FoxO3a
pFoxO3a Ser318/321
Tubuline

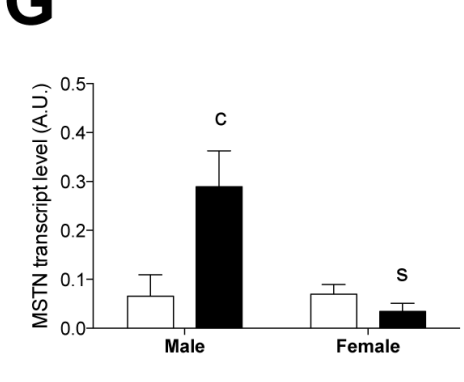

B
pFoxo3a/Foxo3a protein level
1.5
1.0
0.5
0.0
C
Intact Castrated

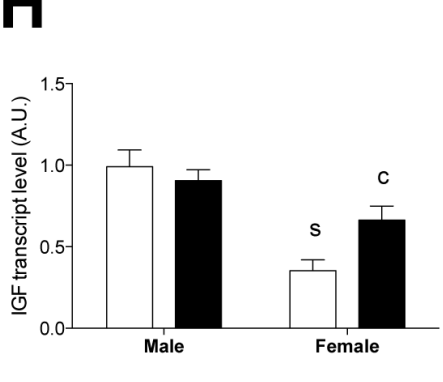

C
pFoxo1/Foxo1 protein level (/total)
2.0
1.5
1.0
0.5
0.0
Intact Castrated


D
MuRF1 transcript level (A.U.)
0.8
0.6
0.4
0.2
0.0
C
Male
Female


E
FbXO30 transcript level (A.U.)
0.4
0.3
0.2
0.1
0.0
C
Male
Female


F
Atrogin transcript level (A.U.)
0.5
0.4
0.3
0.2
0.1
0.0
S
Male
Female


G
MSTN transcript level (A.U.)
0.5
0.4
0.3
0.2
0.1
0.0
C
S
Male
Female


H
IGF transcript level (A.U.)
1.5
1.0
0.5
0.0
S
C
Male
Female