Effect of testosterone on insulin-like growth factor-I, androgen receptor, and myostatin gene expression in splenius and semitendinosus muscles in sheep

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ABSTRACT: Testosterone is known to act differentially on skeletal muscle from different regions. Two genes likely to mediate the testosterone effect are IGF-I, an important growth regulator acting in an autocrine and paracrine way, and androgen receptor (AR), as receptor density could account for differential muscle growth. Another muscle-specific gene that may play a role in differential muscle growth is myostatin, a member of the transforming growth factor-β superfamily, shown to be a negative regulator of skeletal muscle mass. The objective of this study was to quantify and compare the expression of these three genes in two different skeletal muscles in sheep. East Friesian × Dorset-sired ram lambs from Dorset ewes were used in a 2 × 4 factorial experiment. Eighteen sets of twins were assigned to four age groups corresponding to 77, 105, 133, and 161 d of age, and one individual from each set was castrated at birth. Total RNA was extracted from samples of splenius (SP) and semitendinosus muscles collected at the time of slaughter. Insulin-like growth factor-I mRNA was measured using competitive reverse-transcription PCR. Androgen receptor and myostatin mRNA were measured by ribonuclease protection assay with standard curves. Weight of SP was greater than semitendinosus in rams compared with wethers at 105, 133, and 161 d ($P = 0.05$, $P = 0.04$, and $P = 0.02$, respectively). The difference in IGF-I mRNA levels between the two muscles was greater in rams than in wethers at 133 ($P = 0.001$) and 161 d ($P = 0.014$), and the difference in AR mRNA levels was greater in rams than in wethers at 105, 133, and 161 d ($P = 0.002$, $P < 0.001$, and $P < 0.001$, respectively), with greater abundance in the SP. No difference was found in myostatin mRNA level between the two muscles in rams and wethers at any age. These results suggest that locally produced IGF-I and the regulation of AR expression are important for sexually dimorphic muscle growth patterns.

Key Words: Androgen Receptor, Gene Expression, Insulin-Like Growth Factor-I, Muscle, Myostatin, Sheep

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Introduction

Male animals are generally larger than females (Glucksmann, 1974) and have more muscle, especially in the neck and the forequarters. Different growth rates for individual muscles or muscle groups are of special interest for meat production as improving muscle growth of meat producing animals has been a major endeavor of farmers and agricultural scientists.

Sexual dimorphism in muscle growth relates to the protein anabolic effect of testicular hormones. It was shown (Arnold et al., 1997) that the splenius muscle (**SP**) in rams and in wethers implanted with testosterone was heavier and had a biphasic growth pattern compared with the single phase of growth for the same muscle in control wethers. These results confirm the hypothesis that testosterone is implicated in the increased neck muscle mass in sexually mature rams. The concentration of IGF-I was increased in response to the testosterone treatment (Arnold et al., 1996). Subsequently, the expression of IGF-I, androgen receptor (**AR**) and myostatin genes was measured in both SP and semitendinosus (**STN**) muscles in rams (Mateescu and Thonney, 2002), and it was suggested that the increased SP mass of the neck associated with the sexual maturity of rams is mediated by an increase in the mRNA of the IGF-I and AR genes, and that myostatin had no effect. Because only rams were studied, one question that our previous experiment could not address was whether the levels of mRNA observed for these three genes in SP and STN muscles was a result of testosterone.

The objective of this experiment was to analyze the role of IGF-I, AR, and myostatin genes in the differen-

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tial growth phenomena in response to testosterone. The approach was to quantify IGF-I, AR, and myostatin mRNA expression and to assess whether mRNA expression of these genes differed in SP (sexually dimorphic) and STN (not sexually dimorphic) muscles in rams and wethers of different ages.

Materials and Methods

Animals

East Friesian × Dorset-sired ram lambs from Dorset ewes were used in a 2 × 4 factorial experiment. Twenty sets of twins were assigned randomly to four age groups corresponding to 77, 105, 133, and 161 d of age. Eighteen sets of twins born between March 15 and April 2, 2002, were used in this study. Twice-weekly measurements of testosterone from 28 to 217 d of age in Dorset rams in a previous experiment (Arnold et al., 1996) showed that concentrations of testosterone began to increase at approximately 91 d of age. Therefore, the ages in our experiment were chosen to start just before puberty (77 d of age), followed by three more ages at 4-wk intervals. Within 2 d after birth, one of the twins was selected randomly and was castrated with a rubber band applied with an elastrator. Four sets of twins were assigned to each of the first two age groups, and six sets to each of the last two age groups, to ensure sufficient sets of twins were available for sampling at older ages. During the time of the experiment, one twin from each of two sets assigned to the 161-d sampling age were lost; therefore, only four sets of twins were available for this age group. Lambs were fed (10% moisture, as-fed basis) a 71% TDN, 15.6% CP diet that comprised 70% barley, 15% soy hulls added for digestible fiber, 11% soybean meal for protein, 2% vegetable oil to eliminate dust, 1.4% limestone, 0.9% sheep mineral mix, 0.5% ammonium chloride, 0.11% vitamin premix to satisfy the vitamin and mineral requirements, and 0.09% decoquinate premix (Alpharma, Fort Lee, NJ). All animals were slaughtered from June 4 to September 9, 2002, in the Department of Animal Science abattoir within a 3-d range surrounding the assigned slaughter age. The animals were weighed before slaughter, and the carcass weight was recorded after removal of the head, feet, skin, and internal organs. The STN and SP muscles were completely removed, trimmed of visible fat, and weighed. Within 15 min after exsanguination, samples from the center of both muscles were snap-frozen in liquid N, and stored at –70°C until they were subsequently assayed.

RNase Protection Assay

Preparation of the AR, myostatin, GAPDH-labeled antisense and unlabeled sense riboprobes was performed as described previously (Mateescu and Thonney, 2002). Forty micrograms of SP and STN muscle total RNA was co-precipitated with 1 ng of labeled myostatin, AR, and GAPDH riboprobes, and hybridization was performed at 42°C overnight using the protocol and the reagents supplied in the ribonuclease protection assay kit (RPA III, Ambion, Austin, TX), as described in the standard procedure. On each sample gel, known amounts (ranging from 52 to 0.25 pg) of in vitro-synthesized AR, myostatin, and GAPDH sense RNA were hybridized with an excess of labeled antisense probe (1 ng) to construct standard curves. The quantification of myostatin and AR mRNA products was performed as described previously (Mateescu and Thonney, 2002).

Competitive Reverse Transcription PCR

Construction of a heterologous competitor (**MIMIC**), and the establishment and validation of quantitative reverse-transcription PCR was performed as described previously (Mateescu and Thonney, 2002). For each of the two muscle tissues, five reverse transcription reactions were performed using 600 ng of total RNA, and fixed concentrations of the MIMIC IGF-I cRNA. Because the quantity of IGF-I mRNA in muscle decreased with age, two different dilution series of the MIMIC IGF-I cRNA were used to keep the equivalence point close to the middle (9, 3, 1, 0.33, and 0.11 attomoles for the first 18 animals, and 3, 1, 0.33, 0.11, and 0.04 attomoles for the remaining 18 animals). The quantification of PCR products was performed as described previously (Mateescu and Thonney, 2002).

Statistical Analyses

Splenius and STN Muscle Weights. The difference between the two muscle weights within each individual was analyzed to evaluate the effect of sex on the growth of the two muscles over time. Because STN was much larger than SP, a transformation to natural logarithms (**ln**) was used to bring the two muscle weights to comparable scales. A new variable (**DMW**) was created as the difference between the ln SP and ln STN muscle weights within each individual: DMW = (ln SP – ln STN). The difference between the two muscle weights was analyzed using the following fixed-effects statistical model:

$$DMW_{ijkl} = \mu + A_i + S_{ij} + P_{ki} + e_{ijkl} \quad [1]$$

where DMW_{ijkl} = difference between the ln SP and ln STN muscle weights of the lth animal, within the kth pair, of the jth sex, in the ith age class, μ = overall mean, A_i = ith age class (i = 77, 105, 133, 161d), S_{ij} = jth sex within ith age class (j = ram, wether), P_{ki} = kth pair within ith age class (k = 1, 2,...18), e_{ijkl} = error associated with $ijkl$th observation, assumed to be normally distributed, with mean = 0, and variance = σ^2_e.

The fixed effects model had 3, 4, 14, and 14 df for age, sex within age, pairs within age, and the error term, respectively. The sex within age effect was partitioned into four 1-df orthogonal contrasts comparing

the difference in ln SP and ln STN muscle weight between rams and wethers for each of the four age classes and testing for statistical significance using the residual error.

Carcass Weight. After an initial fit of Model [1] for carcass weight, the plot of the absolute residuals against the fitted values for carcass weight detected heterogeneity of residual variance. A ln transformation corrected this problem. The ln carcass weight was then analyzed using the same fixed-effects statistical Model [1]. The sex within age effect was evaluated using four 1-df orthogonal contrasts that compared the ln carcass weight between rams and wethers for each of the four age classes.

Gene Expression. The main objective was to compare the gene expression in two specific muscles. Because the two muscles were sampled from each animal, the gene expression in each muscle represented paired observations with respect to the animal. A new variable (**Diff**) was created as the difference between mRNA concentration (attomoles/μg of total RNA) in the two muscles for each gene studied: Diff = (SP – STN).

The difference in gene expression between the SP and STN muscles was analyzed using the same fixed effects statistical Model [1] as for muscle weight. The sex effect was evaluated using four 1-df orthogonal contrasts that compared the difference in gene expression in the SP and STN muscles between rams and wethers for each of the four age classes.

To test whether there was a difference between sexes in gene expression between the two muscles, a hypothesis test was performed. The null hypothesis was H_o: Diff = 0 and the alternative hypothesis was H_a: Diff > 0 for IGF-I and AR, and H_a: Diff < 0 for myostatin.

The assumptions of normality and equal variance were found to be met by checking with a normal probability plot and a plot of the absolute values of residuals against the predicted values. All tests were performed for an α level of 0.05.

Results and Discussion

Carcass Weight

A plot of carcass weight data against age for the two sexes is presented in Figure 1. Wethers were heavier than rams at 77 d of age and the difference approached significance (P = 0.07), but no sex-related differences were found at 105, 131, and 161 d of age (P = 0.69, P = 0.44, and P = 0.88, respectively). Within each twin pair, one individual is likely to be heavier than the other, especially at younger ages. Therefore, the difference in carcass weight between the two sexes found in the first age group could be attributed to chance alone. Given the anabolic effects of testosterone, it is surprising that rams were not heavier than wethers. Although not quantitatively measured, carcasses from wethers were visually fatter than rams in this experiment. This observation is in line with the report that, when animals

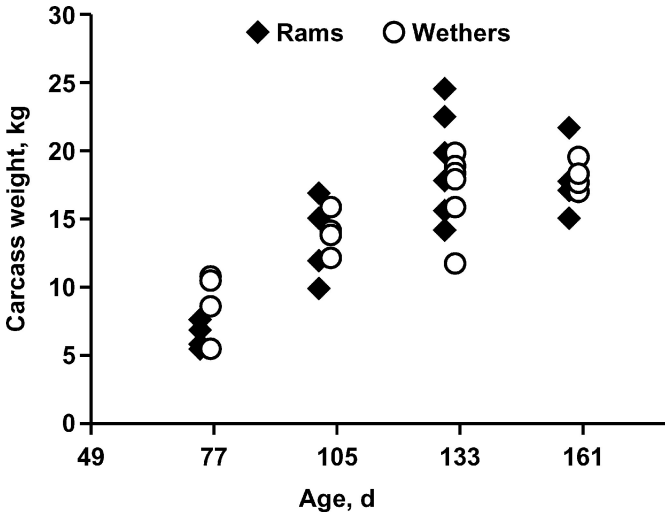

Figure 1. Carcass weights for rams and wethers with increasing age. Analysis of variance of the natural logarithms of carcass weight showed that wethers were heavier than rams at 77 d of age (P = 0.07), but there were no differences between rams and wethers at older ages. Data for rams and wethers were offset so that all data points are visible.

are allowed ad libitum access to a high-energy diet, anabolic effects of hormones sometimes observed in body composition differences are not reflected in growth rate differences (Rosemberg et al., 1989).

Semitendinosus and Splenius Muscle Weights

No significant difference (P = 0.78) was found between rams and wethers for the difference in the ln weight of the two muscles at 77 d of age, but the sex effect was significant at 105, 131, and 161 d of age (P = 0.05, P = 0.04, and P = 0.02, respectively). The heavier weight of the SP muscle in rams relative to wethers for the same age supports the hypothesis that the presence of testosterone is an important factor in the sexual dimorphism of SP muscle (Arnold et al., 1997). These differences were reflected in the raw data for SP and STN muscles classified by sex and age and by sex and carcass weight presented in Figure 2.

Gene Expression

For a general description of the data, the subclass means and standard deviations for IGF-I, AR, and myostatin mRNA concentrations are presented in Table 1.

Insulin-Like Growth Factor-I mRNA Abundance. Testosterone could exert an effect on muscle growth through the IGF-I axis. Locally produced IGF-I is an important growth regulator acting in an autocrine and paracrine manner (Weimann and Kiess, 1990), but different muscles may possess different IGF-I sensitivities (Boge et al., 1995) and/or IGF-I synthesis rates (Thissen et al., 1994; Frost et al., 1997; Pfaffl et al., 1998b) and

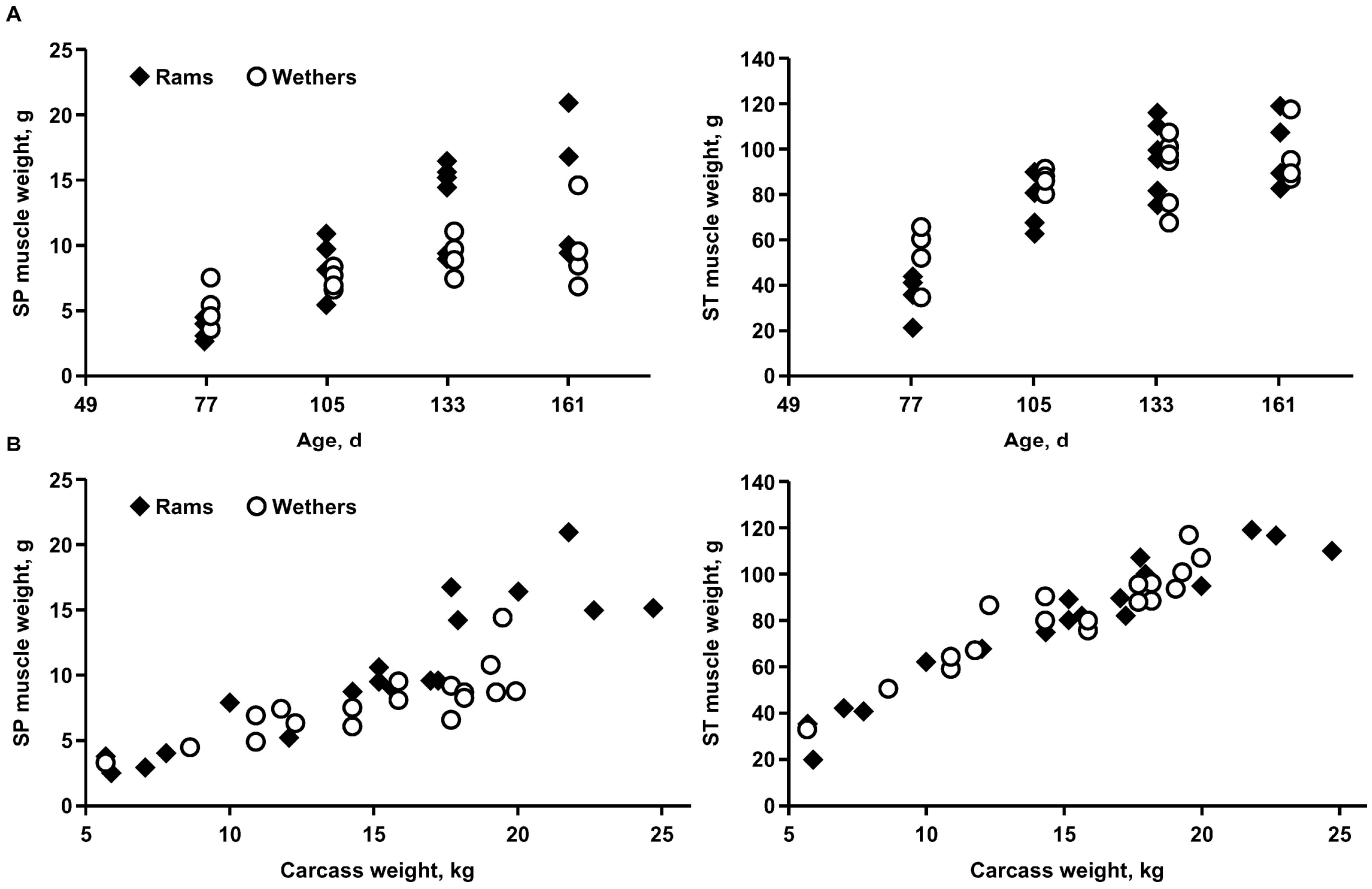

Figure 2. Splenius (SP) and semitendinosus (STN) muscle weights for rams and wethers with increasing age (A) and increasing carcass weight (B). Analysis of variance of the difference between natural logarithms SP and STN muscles weights confirmed the greater differences for rams than wethers at 105, 131, and 161 d of age ($P = 0.05$, $P = 0.04$, and $P = 0.02$, respectively). Although not reflecting the experimental design, the figures in panel B are presented to show that the effect also was present when examined from the point of view of carcass weight. Data for rams and wethers were offset so that all data points are visible.

Table 1. Mean concentrations (attomoles/μg of RNA ± SD) of IGF-I, androgen receptor (AR), and myostatin mRNA for splenius (SP) and semitendinosus (STN) muscles from rams and wethers[a]

	Age, d							
	77		105		133		161	
Item	Rams (n = 4)	Wethers (n = 4)	Rams (n = 4)	Wethers (n = 4)	Rams (n = 6)	Wethers (n = 6)	Rams (n = 4)	Wethers (n = 4)
IGF-I[b]								
SP	0.70 ± 0.12	0.68 ± 0.15	0.34 ± 0.08	0.20 ± 0.06	0.39 ± 0.06	0.26 ± 0.09	0.39 ± 0.05	0.20 ± 0.03
STN	0.59 ± 0.18	0.43 ± 0.06	0.40 ± 0.03	0.25 ± 0.08	0.23 ± 0.07	0.30 ± 0.05	0.23 ± 0.06	0.21 ± 0.05
AR[c]								
SP	4.01 ± 0.45	4.26 ± 0.56	5.40 ± 1.44	5.18 ± 1.37	4.96 ± 1.10	4.24 ± 1.27	5.77 ± 1.36	5.04 ± 1.10
STN	3.80 ± 0.32	3.95 ± 0.42	4.66 ± 1.10	4.95 ± 1.18	4.05 ± 1.03	4.19 ± 1.09	4.82 ± 1.23	4.99 ± 1.24
Myostatin								
SP	2.87 ± 0.99	2.59 ± 0.38	3.47 ± 0.55	3.28 ± 0.66	3.44 ± 0.66	3.46 ± 0.60	4.05 ± 0.31	3.79 ± 0.16
STN	3.22 ± 0.70	2.70 ± 0.49	3.63 ± 0.63	3.45 ± 0.49	3.71 ± 0.33	3.69 ± 0.55	4.02 ± 0.29	4.06 ± 0.35

[a]Differences in mRNA abundance between SP and STN of rams and wethers were analyzed. Actual subclass means and SD are presented for a more comprehensive description of the data.

[b]Difference between IGF-I mRNA abundance in SP and STN was greater in rams than wethers at 133 ($P = 0.001$) and 161 ($P = 0.014$) d of age.

[c]Difference between AR mRNA abundance in SP and STN was greater in rams than wethers at 105 ($P = 0.002$), 133 ($P = 0.001$), and 161 d ($P = 0.001$) of age.

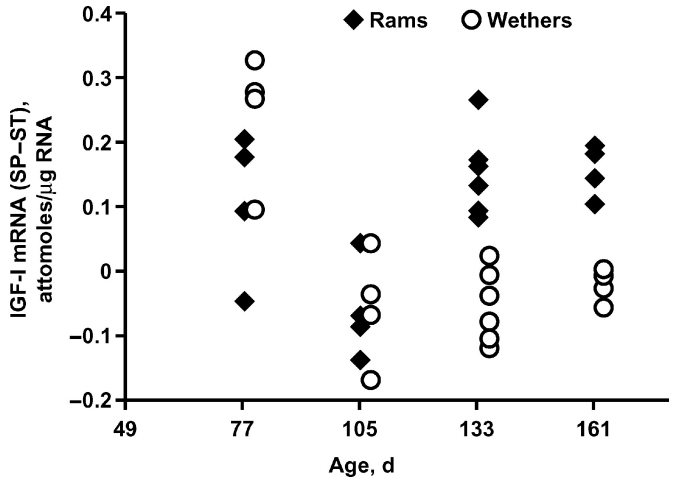

Figure 3. IGF-I mRNA differences between splenius (SP) and semitendinosus (STN) muscles in rams and wethers with increasing age. Analysis of variance showed that the difference in IGF-I mRNA concentration was greater in rams than in wethers at 133 (P = 0.001) and 161 (P = 0.014) d of age. Data for rams and wethers were offset so that all data points are visible.

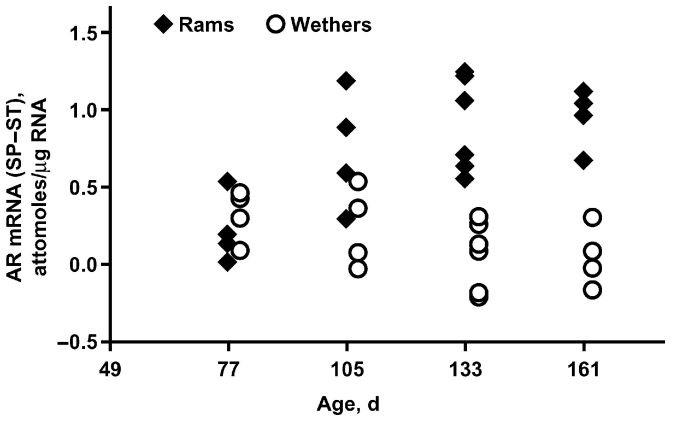

Figure 4. Androgen receptor (AR) mRNA differences between splenius (SP) and semitendinosus (STN) muscles in rams and wethers with increasing age. Analysis of variance showed that the difference in AR mRNA concentration was greater in rams than in wethers at 105 (P = 0.002), 133 (P = 0.001), and 161 (P = 0.001) d of age. Data for rams and wethers were offset so that all data points are visible.

may therefore exhibit different growth rates. Testosterone has been implicated in the increased levels of IGF-I responsible for growth rate and body composition differences among intact males, castrated males, and females (Ford and Klindt, 1989). Increased muscle growth due to testosterone is caused in part by increasing the concentration of circulating IGF-I independent of GH concentrations (Arnold et al., 1996). Steers receiving steroid hormone implants had increased circulating IGF-I concentrations compared with nonimplanted steers, and the longissimus and semimembranosus muscles of implanted steers showed increased IGF-I mRNA levels compared with nonimplanted steers (Pampusch et al., 2003; White et al., 2003).

The data for the difference in IGF-I mRNA between SP and STN muscles classified by sex and age are presented in Figure 3. The difference in IGF-I mRNA abundance between SP and STN was not greater in rams than in wethers at 77 or 105 d of age, but it was greater at 133 (P = 0.001) and 161 d of age (P = 0.014). These results support the hypothesis that the increase in SP muscle weight in rams relative to wethers is associated with an increase in locally produced IGF-I in SP muscle in response to testosterone. It is not surprising that rams and wethers did not differ at 105 d of age, which is the age when puberty is thought to occur in sheep. It is possible that some of the rams had not reached puberty at this point in time or that greater concentrations of testosterone might be necessary for an effect on IGF-I gene expression. Testosterone stimulates muscle growth by affecting the rate of protein synthesis, protein breakdown and the net gain or loss of muscle protein (Wong et al., 1993), and these actions are mediated by the AR, which acts as a nuclear transcription factor. When testosterone increases muscle protein synthesis, i.m. mRNA concentrations of IGF-I are raised and concentrations of the inhibitory IGFBP-4 are lowered (Rooyackers and Nair, 1997). The increase in IGF-I mRNA could be regulated by AR and may be required for the increase in muscle protein synthesis. The significant difference in the AR mRNA level between rams and wethers that we found at 105 d of age did not result in increased transcription of IGF-I mRNA level at the same age. We speculate that the difference in AR mRNA, even if statistically significant, was too small to cause a difference at the IGF-I mRNA level. Another possible explanation could be that there is a temporal separation between the increase at the regulatory factor (AR) level and the response in gene transcription activity for IGF-I. Additional data are needed to distinguish between these hypotheses.

Our results are consistent with a study of IGF-I gene expression in cattle (Pfaffl et al., 1998a). Expression of IGF-I mRNA levels was measured in two muscles selected because of their overproportional (SP) and underproportional (gastrocnemius) growth response to testicular steroids. In bulls, greater IGF-I mRNA concentration was found in the SP than in gastrocnemius muscle. Thus, a local difference in IGF-I expression is potentially one of the mediators of the differential growth of these muscles in intact males.

AR mRNA Abundance. The data for the difference in AR mRNA between SP and STN muscles classified by sex and age are presented in Figure 4. The difference in AR mRNA expression between SP and STN was not greater in rams than in wethers at 77 d of age, but it was greater at 105, 133, and 161 d of age (P = 0.002, $P < 0.001$, and $P < 0.001$, respectively).

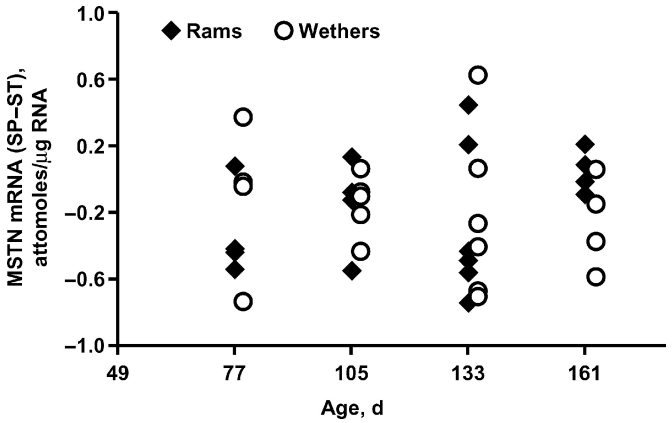

Figure 5. Myostatin (MSTN) mRNA differences between splenius (SP) and semitendinosus (STN) muscles in rams and wethers with increasing age. There was no difference between the SP and STN myostatin mRNA expression between rams and wethers at any age. Data for rams and wethers were offset so that all data points are visible.

Testosterone action is mediated by the AR, which transduces the steroid signal within cells. The response to testosterone differs among muscle groups and this differential response may be explained by the variation of AR number among skeletal muscles (Urban, 1999). Thus, sexual dimorphism can be explained partly by greater androgen sensitivities in muscles with pronounced growth under androgen stimulation (Sauerwein and Meyer, 1989). Data on the developmental regulation of AR mRNA abundance in three bovine muscles that differed in muscle fiber composition, metabolic activity, and growth pattern showed a relationship between AR mRNA concentration and differential growth (Krieg et al., 1977). A study in cattle showed that androgen receptor mRNA concentrations in muscles with different fiber type compositions and growth impetus is positively related to the individual growth patterns (Brandstetter et al., 2000). The greater AR mRNA concentration in SP muscle in rams shown in this experiment supports results of those previous studies and helps to explain the pronounced muscle growth in the neck of maturing rams (Arnold et al., 1997).

Myostatin mRNA Abundance. The ribonuclease protection assay confirmed the presence of myostatin mRNA in both skeletal muscles. The data for the difference in myostatin mRNA between SP and STN muscles classified by sex and age are presented in Figure 5.

We hypothesized that myostatin gene expression might be downregulated in the SP muscle in rams to increase the mass of SP muscle, but no significant sex effect was found in the expression of myostatin in the two muscles. The possible role of myostatin in the sexual dimorphic muscle growth of SP muscle can not be ruled out, even though there were no differences in myostatin mRNA between rams and wethers. It was recently reported that male transgenic mice that overexpress myostatin selectively in the skeletal muscle had an 18 to 24% decrease of skeletal muscle mass and a decrease of muscle fiber size (Reisz-Porszasz et al., 2003). Female transgenic mice generated the same way did not differ from wild-type controls in either BW or skeletal muscle mass, despite the fact that the expression of myostatin mRNA and protein was increased in the skeletal muscle and was not lower compared with male mice. However, it was shown that increased body and muscle mass in male compared with female mice is associated with decreased expression of processed myostatin (McMahon et al., 2003). Interestingly, this decreased expression of processed myostatin was due to regulation after translation and, presumably, after secretion because there was no difference in the mRNA or in the latency associated peptide form of myostatin. Thus, the lack of a difference in myostatin mRNA between rams and wethers in our experiment might not be reflected in the level of processed myostatin.

In conclusion, this experiment is consistent with the hypothesis that the increased SP muscle mass of the neck associated with sexual maturity of rams is mediated by an increase in IGF-I and AR gene expression. Regulation of the other components of the IGF-I system should be studied to judge the relevance of the IGF system for regulation of differential muscle growth. Also, the AR role in muscle growth regulation should be investigated further, as other genes that are regulated by this transcription factor could be associated with muscle growth regulation. There was no difference in the myostatin gene expression associated with sexually dimorphic muscle growth. It would be of considerable interest to determine whether there is a difference in the expression of processed myostatin in sexually dimorphic muscles in rams and wethers.

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