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Effect of *FecX^R* polymorphism in the *BMP15* gene on natural or eCG-induced ovulation rate and litter size in Rasa aragonesa ewes and implications for on-farm application¹

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ABSTRACT

A new mutation in the *bone morphogenetic protein 15 (BMP15)* gene (*FecX^R* allele) causing increased prolificacy in heterozygous (R+) and sterility in homozygous ewes has been recently described in Rasa aragonesa, a low-prolificacy Mediterranean breed. The current study determined, firstly, the effect of this polymorphism on natural and eCG-induced ovulation rate (OR) and the effect of eCG dose on reproductive performance; and secondly, its effect on prolificacy and its interaction with progestagen + eCG treatment on farms, which have not been reported to date. The *FecX^R* allele increased OR by 0.44 and 0.63 ovulations in young (n = 91) and adult (n = 84) R+ ewes, respectively (both, $P < 0.01$), increments lower than reported in prolific breeds carrying other mutations in *BMP15*. When the standard dose of eCG used on farms (480 IU) was applied to R+ ewes (n = 36), an extremely high OR (3.95) was recorded, which was accompanied by a high partial failure of multiple ovulations (**PFMO**). On the contrary, OR using 240 IU in R+ ewes (2.90; n = 35) was similar to 480 IU in wildtype (++) ewes (2.82; n = 48; both $P < 0.01$ when compared with 480 IU in R+ ewes). No differences were found in the birth weight of the offspring between R+ and ++ eCG-stimulated ewes within the same litter size (**LS**). To validate the genealogy identification on farms, PCR genotyping was carried out in 1,667 ewes from 4 elite flocks, resulting in a negligible misclassification of R+ ewes, which demonstrated that identification by genealogy is a reliable tool to identify *FecX^R* ewes within the breeding program. In recorded farms, the natural LS of ++ ewes (1.34, n = 599,160 lambing records) was increased due to the *FecX^R* allele by 0.35 lambs ($P < 0.0001$, n = 6,593 lambing records). A similar increase (0.30) was observed when comparing ++ and R+ ewes treated with 480 IU of eCG ($P < 0.0001$, n = 62,055 and n = 866; respectively). When applying 480 IU of eCG to R+ ewes, the increase in prolificacy was only due

to increased percentages of triplets ($P < 0.001$) and quadruplets ($P < 0.0001$), but not of twin births. In conclusion, the favorable reproductive performance of R+ ewes, with 0.63 extra ovulations and 0.35 extra lambs per lambing ewe, is responsible for the increased interest in the use of this polymorphism. Nevertheless, care must be taken in the application of eCG to R+ ewes, with the current results showing that the standard dose increases prolificacy by only increasing triple and higher-order births.

Key Words: bone morphogenetic protein 15, breeding program, ovulation rate, pregnant mare serum gonadotrophin, prolificacy, sheep

INTRODUCTION

Rasa aragonesa is an autochthonous Mediterranean breed of sheep from the northeast of Spain, with about 500,000 animals recorded, mainly reared in extensive or semi-extensive farming systems and oriented to meat production. A selection program to increase prolificacy has been carried out in this breed since 1994, as the number of lambs born per ewe plays a key role in the efficiency and viability of these farms (Pardos et al., 2008). In Rasa aragonesa, phenotypic prolificacy is 1.37 (lambs/birth; 16th Catalogue of the selection program 2009; unpublished). In 2007, some descendents of the tested rams presented an uneven increase in prolificacy unexplained by polygenic heredity, leading to the discovery of a new naturally occurring polymorphism in *BMP15*, a fecundity gene with a major effect on ovulation rate (**OR**) in sheep. The polymorphism (*FecX^R* allele) consists of a deletion of 17 bp in the coding region of *BMP15*, located on the X chromosome, causing increased prolificacy in heterozygous (**R+**) and sterility in homozygous ewes (Martinez-Royo et al., 2008). As the *FecX^R* allele allows increased prolificacy while maintaining breed morphology, it is being used on farms. In fact, in 2008 the estimated population of R+ ewes was approximately 1,500, showing a clear trend towards increased numbers. In spite of its

economic interest, the increase in OR, which leads to this increase in litter size, and the interactions with progestagen + eCG treatment (widely used to induce out-of-season breeding), still remain to be determined in this breed.

Therefore, this study was performed to determine the effect of this new polymorphism on natural OR in young and adult Rasa aragonesa ewes, as well as OR and reproductive performance of adult ewes treated with fluorogestone acetate and 2 different doses of eCG. In addition, we wished to determine the increase in prolificacy due to the presence of the *FecX^R* allele and its type of birth distribution in eCG-treated or untreated ewes on farms.

MATERIAL AND METHODS

All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367-91) for the use and care of animals in research.

*Experiment 1: Natural Ovulation Rate of Young and Adult *FecX^R* Heterozygous Ewes*

This experiment was carried out from November to December (the natural breeding season) in the facilities of CITA, a Research Center located in Zaragoza (Spain). Ovulation rate was recorded in 91 heterozygous *FecX^R* carriers (R+) and 20 wild-type (++) young ewes, aged 311 ± 11 d (mean \pm SD), and in 84 R+ and 19 ++ adult ewes, aged 683 ± 37 d (mean \pm SD), previously identified by PCR genotyping as described by Martínez-Royo et al. (2009). Within each age group, R+ and ++ groups were similar in BW and BCS. Animals were kept at a constant feeding level, and treated with 30-mg fluorogestone acetate (FGA) sponges (Sincropart 30 mg, CEVA Salud Animal S.A., Barcelona, Spain) for 14 d to synchronize estrus. No eCG was used at withdrawal and no males were used for heat detection. Ovulation rate was recorded by laparoscopy during 3 consecutive cycles for each animal, always performed by the same observer, starting 6 d after

sponge withdrawal and repeating 17 and 34 d later. Ewes were fasted for 24 h. Tranquilization was carried out using acepromazine and local anaesthesia was given by a subcutaneous injection of lidocaine in the place of insertion of the trocars as described by Cognié et al. (2007). Ovulation rate of each animal was the mean of the observations recorded in the ovulating cycles. To calculate the distribution of ovulations, a total of 257 and 292 ovulation records in young and adult ewes, respectively, were considered.

Experiment 2: Ovulation Rate, Partial Failure of Multiple Ovulations, and Offspring Birth

Weight of Adult $FecX^R$ Heterozygous Ewes Treated with 2 Different Doses of eCG

This experiment was also carried out in the facilities of CITA. A total of 71 R+ and 48 ++ ewes were treated for 14 d with vaginal sponges containing 30 mg FGA (Sincropart 30 mg, CEVA Salud Animal S.A.). At sponge withdrawal, ++ ewes received 480 IU eCG i.m. (**++480**; Sincropart PMSG 6,000 UI, CEVA Salud Animal S.A.), the standard dose used for AI in Rasa aragonesa ewes on farms, whereas R+ received either 240 IU (n = 35; **R+240**) or 480 IU (n = 36; **R+480**). Cervical insemination was carried out 44.5 ± 1 h after sponge withdrawal with semen of proven fertility diluted in skim milk and refrigerated at 15 °C. Each ewe received 400×10^6 spermatozoa.

Ovulation rate was recorded once in 75 ewes (R+240: n = 21; R+480: n = 21; ++480: n = 33 ewes) 8 d after sponge withdrawal, following the procedures described in Exp. 1.

One week before lambing, ewes were placed in individual pens. At lambing, offspring were immediately identified and weighed, using a digital balance with a precision of 0.1 kg. Partial failure of multiple ovulations (**PFMO**) was calculated for each of the 3 groups, as the difference between OR and litter size (**LS**) of lambing ewes with ≥ 2 ovulations.

Experiment 3. Prolificacy of $FecX^R$ Heterozygous Ewes Identified by Genealogy, with and without eCG Stimulation, in Flocks Enrolled in the Selection Program

The Use of PCR to Validate Genotype by Genealogy. To assess the suitability of the identification method of *FecX^R* genotype by genealogy within the selection program, genotype assignment by genealogy was compared to PCR genotyping. For this purpose, only adult ewes from 4 elite flocks (n = 1,667 ewes) were genotyped for the presence of *FecX^R* allele (++, R+, RR). Blood samples were collected by jugular puncture using 5-mL vacuum tubes with EDTA. Genotypes for *BMP15* were determined by PCR using primers flanking the *FecX^R* polymorphism, as described previously by Martinez-Royo et al. (2009). Amplification by PCR was carried out under standard conditions, with PCR products separated by standard electrophoresis in a 3.5% TBE (0.045-M Tris-borate, 0.00-M EDTA) agarose gel. Amplification of the *FecX^R* and *FecX⁺* alleles by PCR produced fragments of 101 and 118 bp, respectively. On farms belonging to the selection program from 1985, all maternal ancestry is known and from 1994 paternal ancestry is known when derived from AI or controlled mating. Since the discovery of *FecX^R* polymorphism in 2007, all rams were genotyped for *FecX^R* allele presence (Martinez-Royo et al., 2009), and since 2008, all hemizygous sires have been under the control of the insemination center. For genotype identification by genealogy, daughters of *FecX^R*-genotyped hemizygous rams were assigned as R+, whereas daughters of wild-type or unknown rams were considered as ++ ewes due to the presumably low frequency of the *FecX^R* allele in the population. Daughters of known R+ ewes were not taken into account. Prolificacy estimates of R+ and ++ genotypes identified by PCR on these farms, treated or untreated with FGA + eCG, were compared to prolificacy estimates when genotype assignment was made only by genealogic information, to validate further estimates of prolificacy of both genotypes assigned by genealogy in flocks.

Prolificacy of *FecX^R* Heterozygous Ewes, with and without eCG Stimulation, in Flocks Enrolled in the Selection Program. Prolificacy of *FecX^R* heterozygous adult ewes, treated or untreated with

eCG, was investigated in all the flocks that comprise the selection program. Genotype assignment by genealogy was made as described above. As the first lambing of a known heterozygous $FecX^R$ ewe was recorded in 1998, only data from this year onwards were analyzed. A total of 668,674 lambing records, collected from 1998 to 2008 from these Rasa aragonesa controlled flocks were analyzed to calculate litter size for heterozygous $FecX^R$ and non-carrier ewes. Within each genotype, lambing records were divided into 2 groups, depending on whether mating was carried out without hormonal treatments (++: n = 599,160; R+: n = 6,593) or after the application of FGA sponges and 480 IU of eCG (++: n = 62,055; R+: n = 866).

Statistical Analysis

Differences among lots in ovulation rate, prolificacy, PFMO, and variables expressed as percentages were analysed by generalised linear models for categorical variables using the CATMOD procedure of SAS (SAS, 2004). Differences among lots in lamb birth weight were assessed by one-way ANOVA, using the GLM procedure of SAS and the Bonferroni-adjusted least significant difference (LSD) test. As significant differences were found among genotypes in prolificacy, a full factorial fixed model, including genotype and prolificacy was built to test for differences in lamb birth weight due only to genotype. The level of significance was set at $P \leq 0.05$, whereas P -values > 0.05 but ≤ 0.10 were considered trends.

RESULTS

Natural Ovulation Rate of Young and Adult $FecX^R$ Heterozygous Ewes (Exp. 1)

The mean OR and the distribution of the number of ovulations in young and adult R+ and ++ ewes are shown in Table 1. The percentage of animals ovulating in at least 1 of the 3 cycles was close to 100%, in both young and adult ewes, with no differences among genotypes ($P \geq 0.91$). In young ewes, OR was 0.44 points greater in R+ than in ++ ewes, mainly due to an increase in twin

ovulations (+50.5%; $P < 0.01$), as triple ovulations were not increased (+1.9%; $P = 0.78$). In adult ewes, OR was +0.63 points greater in R+ than in ++ ewes, because of the increase in double (+36.4%, $P < 0.01$), as well as in triple plus quadruple ovulations (+12.8%; $P < 0.05$).

Ovulation Rate, Partial Failure of Multiple Ovulations and Offspring Birth Weight of Adult $FecX^R$ Heterozygous Ewes Treated with 2 Different Doses of eCG (Exp. 2)

Ovulation rate and PFMO of R+ ewes treated with 480 or 240 IU of eCG are shown in Table 2. Ovulation rate after eCG stimulation was greater in the R+480 ewes, presenting 1.13 and 1.05 extra ovulations when compared with ++480 and R+240 ewes, respectively ($P < 0.01$ for both). In R+480 ewes, the predominant type of ovulation was quadruple or higher-order ovulation (57.1%), greater than in the ++480 (+31.4%; $P < 0.01$) and R+240 (+33.3%; $P < 0.05$) groups. No differences in OR were found between R+240 and ++480 groups ($P = 0.80$), despite that triple ovulation, the predominant type in the R+240 group (47.6%), was greater in the R+240 than in the ++480 group (+27.6%; $P < 0.01$). The different ranges in OR in both groups (1 to 4 in R+240 vs. 1 to 10 in ++480 groups; Table 2) could explain this difference. In this way, ewes ovulating more than 4 ova did increase the mean OR of the ++480 group up to a value similar to that of R+240 group, despite its lower percentage of triple ovulations ($P < 0.01$). In ++480 ewes, the predominant type of ovulation was double ovulation (42.9%), greater than in the R+480 group (+33.4%; $P < 0.01$).

Partial failure of multiple ovulations was 1.85 in R+480, greater than in the R+240 and ++480 groups (1.15 and 0.77, respectively; $P < 0.05$ for both). No differences in PFMO were found between the R+240 and ++480 groups ($P = 0.29$).

Birth weights of lambs born from adult ewes are shown in Table 3. In the R+480 group, birth weight was lower when compared with ++480 (-0.57 kg; $P < 0.05$) or R+240 group (-0.42 kg;

$P = 0.08$). Nevertheless, when lambs of the same type of birth were compared, no differences between groups were observed ($P \geq 0.10$).

Prolificacy of $FecX^R$ Heterozygous Ewes Identified by Genealogy, with and without eCG Stimulation, in Flocks Enrolled in the Selection Program (Exp. 3)

The Use of PCR to Validate Genotype Assignment by Genealogy.

When comparing the genotype results assigned by genealogy with those determined by PCR genotyping, 97.8% (83.2% ++ and 14.6% R+) of ewes were correctly classified by genealogy (Table 4). The percentage of R+ ewes in these farms, determined by PCR, was 16.3% (272), from which 10.3% (28) were misclassified as ++ ewes based on progeny data. Likewise, 0.57% of ++ ewes (8 out of 1,395) were erroneously assigned as R+ by genealogy. Similar percentages ($P = 0.86$) were obtained when expressed in terms of lambing records, with 97.9% (85.6% ++ and 12.3% R+) of lambing records coming from correctly classified ewes.

To assess the effect of genealogy-induced misclassification on prolificacy data, the mean litter size of R+ and ++ ewes was calculated taking into account only genealogy assignment or based on PCR genotyping. Very close mean litter size estimates of $FecX^R$ carrier and wild-type ewes, with or without eCG treatment, were obtained ($P \geq 0.64$). In fact, only prolificacy of the untreated ++ group was 0.01 lambs born per lambing ewe less based on genealogy records (data not shown).

Prolificacy of $FecX^R$ Heterozygous Ewes, with and without eCG Stimulation, in Flocks Enrolled in the Selection Program.

Prolificacy and litter size distribution of $FecX^R$ heterozygous Rasa aragonesa adult ewes, treated or untreated with eCG, are shown in Table 5. On farms, prolificacy recorded in untreated R+

ewes was greater than in ++ ewes ($P < 0.0001$), resulting in 0.35 extra lambs per lambing ewe. This greater prolificacy was mainly due to an increase in twin births (+15.9%; $P < 0.0001$), in addition to triplet or higher-order births (+9.2%; $P < 0.0001$).

When treated with FGA + 480 IU eCG, prolificacy was also greater in R+ than in ++ ewes, resulting in 0.30 extra lambs per lambing ewe ($P < 0.0001$), due to the increase in twin (+6.0%; $P < 0.001$) and in triplet and higher-order births (+10.4%; triplets and quadruplets: $P < 0.0001$; quintuplets: $P < 0.01$).

Heterozygous ewes for $FecX^R$ allele and ++ ewes displayed different increases in prolificacy after eCG stimulation (+0.16 and +0.21 extra lambs born, respectively; $P < 0.0001$ for both).

DISCUSSION

The polymorphism described in the *BMP15* gene of the Rasa aragonesa sheep breed ($FecX^R$; Martinez-Royo et al., 2008) produces an increase in ovulation rate of +0.44 and +0.63 in young and adult ewes, respectively. This increase is less than those found for similar mutations in the *BMP15* gene in other sheep breeds. The Galway mutation ($FecX^G$) in F700-Belclare and Cambridge breeds increases ovulation rate by +0.62 in ewe lambs and +0.72 in adults (Hanrahan et al., 2004). Conversely, Belclare ($FecX^B$) mutation in the F700-Belclare breed, and Inverdale ($FecX^I$) and Hanna ($FecX^H$) mutations in Romney, all increase ovulation rate by 1.0 (Galloway et al., 2000; Hanrahan et al., 2004; Davis, 2005). The $FecX^L$ mutation produces about 1.5 extra ovulations, likely due to an additive effect of the phenotypic background of the Lacaune breed (Bodin et al., 2007). Other polymorphisms in *GDF9* and *BMP15* genes have been described recently in the Moghani and Ghezel breeds (Barzegari et al., 2009), as well as in the Barbarine breed (Vacca et al., 2010), though their effects still are not well known. In both young and adult Rasa aragonesa ewes, this increase in ovulation rate is mainly due to an increase in double ovulations, making the use of the $FecX^R$ polymorphism suitable for application in commercial farms. This is the first mutation in the *BMP15*

gene described in a Mediterranean breed, whose natural ovulation rate and prolificacy are between 15 to 40% lower than that of other breeds in which mutations in *BMP15* have been found. This lower natural ovulation rate could explain the lesser increase in OR and prolificacy produced by the *FecX^R* mutation in this breed. This fact makes the introgression of this mutation into other Mediterranean breeds with similarly low OR and which are exploited under similar conditions of interest, such as some breeds from the North of Africa, or from other countries where sheep are reared under semi-extensive conditions.

One of the main objectives of this study was to assess the response of R+ ewes to eCG for later application on farms, because hormonal treatments are widely used in systems such as 3 lambings/2 yr, to induce out-of-season reproduction in breeds oriented to meat production. Although it has been demonstrated that prolific sheep are more sensitive to PMSG (Bindon et al., 1986), it appears that there are no works dealing with the response of *BMP15*-mutated sheep to this hormone. In mutated *Fec* gene ewes, many authors have described increased FSH sensitivity and precocious LH receptor expression in antral follicles from these ewes (Fabre et al., 2006), while others have demonstrated only an earlier acquisition of LH responsiveness by granulosa cells, but not an increase in FSH sensitivity (McNatty et al., 2009). In our study, the ovulation rate of untreated R+ ewes was 0.63 ova greater than untreated ++ ewes. This advantage increased to 1.13 ova when ewes received the standard dose of 480 IU eCG. This difference in the response to eCG could not be explained by the present work. Possible explanations could be either a greater in vivo sensitivity to gonadotrophins, or a greater population of gonadotrophin-dependent follicles, as it was demonstrated in sheep that PMSG-induced ovulation rate is significantly correlated with the number of healthy follicles from 0.8 to 2.0 mm in diameter (Driancourt, 1987). When 480 IU eCG was used, this extremely high ovulation rate was also accompanied by a high PFMO. On the contrary, when the dose was halved (240 IU), a more suitable ovulation rate and a lower PFMO were achieved. In fact, OR and PFMO of R+ ewes stimulated with 240 IU were very close to those

recorded in the wild-type ewes stimulated with 480 IU of eCG. While this is the first report of *BMP15*-mutant animal response to eCG stimulation, our results demonstrate a similar response to that reported with the Booroola genotype, a mutation in the *type 1B receptor of BMP (BMPR-1B)*, where the ovulation rate response to doses ≤ 750 IU of eCG was significantly different between heterozygous carrier and homozygous non-carrier ewes (Kelly et al., 1983; Gootwine et al., 1993). We found similar results in nulliparous Rasa aragonesa ewes at the beginning of the anestrus season (February; Lahoz et al., 2009), in agreement with previous studies showing that prepubertal ewe lambs carrying the Booroola mutation presented increased sensitivity to PMSG when compared with non carrier ewe lambs (Bindon et al., 1986). In conclusion, our results confirm that *FecX^R* heterozygous ewes present a greater response to eCG. Therefore, it may be beneficial to halve the standard dose used on farms in this breed to avoid increased PFMO. However, artificial insemination trials on farms would be necessary before recommending halving of the eCG dose in R+ ewes.

No differences were observed in birth weight among lambs of the same birth type between heterozygous or wild-type ewes. Hence, the lower weights observed in lambs born from R+ ewes were only due to their greater prolificacy. Differences between genotypes in birth weight is of concern as lower birth weights contribute to lower perinatal survival (Hinch et al., 1985; Owens et al., 1985). It appears that no published data concerning birth weight of lambs from ewes carrying any mutation in *BMP15* are available. Several studies concerning the effects of the Booroola mutation (*FecB*) on the weight of lambs at birth have been performed, with discrepant results (Fogarty, 2009); in this way, in Garole (*FecB* fixed) \times Malpura crossbred sheep, the birth weight of BB and B+ lambs was less than that of non-carriers (Kumar et al., 2008). In Booroola-Assaf crosses, BB ewe lambs were significantly lighter at birth than ++, but also than B+ ewe lambs. Moreover, a lower birth weight was observed in ewe lambs born to BB compared to B+ or ++ dams (Gootwine et al., 2006). Conversely, there was no significant effect of maternal genotype on birth

weight in the Mérinos d'Arles breed (Abella et al., 2005). In these 3 works, litter size differences among genotypes were taken into account when analyzing the effect of the genotype on the weight at birth. Hence, the effect of the Booroola mutation on birth weight, corrected for litter size, may depend on the breed where it is introgressed. It remains to be seen whether similar results will be discovered with the *BMP15* mutations where homozygous carrier ewes are sterile.

In the present work, PCR validation was used with 2 main objectives: to validate prolificacy data recorded in flocks, and to validate the suitability of identification of *FecX^R* heterozygous ewes on farms by genealogy assignation. We have shown that misclassification induced by genealogy assignation was rare. Genotyping demonstrated that 3.3% of ewes classified as R+ by genealogy were in fact ++, and 2.0% of ewes classified as ++ were confirmed as R+ after genotyping. On these farms, as only a small percentage of ewes are inseminated while the remainder (the vast majority) is naturally mated to rams at the same time, once an error of parental assignation occurs, the probability of assigning lambs from inseminated ewes to naturally-mated ewes is very great, while the probability of assigning lambs from naturally-mated ewes to inseminated ewes is negligible. Under these circumstances, the 3.3% of lambs misclassified as R+ would arise from paternity error after inseminations with R sires, while this 2% unidentified R+ ewes would mainly reflect the R+ ewes born to R rams present on farms, or to hidden R+ ewes, as the daughters of known R+ ewes have been discarded in this work. Regardless, misclassification due to incorrect genotype assignation by genealogy did not affect mean prolificacy estimates in a population with 16.3% of *FecX^R* heterozygous ewes. When considering all of the flocks, where the frequency of *FecX^R* ewes may be close to 2.96% (data not shown), the error induced by misclassification would presumably be even less. Thus, the prolificacy estimates of eCG-treated or untreated ewes of both genotypes reported here, obtained on farms in which genotypes were assigned by genealogy, can be considered reliable. Furthermore, in a recent study carried out in this breed, no significant differences in prolificacy estimates were found between non-carrier ewes born to genotyped + rams

and ewes assumed as non-carriers born to unknown rams (-0.031 lambs/lambing ewe), which indicates that the percentage of unknown R+ ewes in flocks is minimal (Jurado et al., 2008). This fact could be explained by the low percentage of R rams serving in flocks (< 7%) detected by PCR genotyping in 2007 (Martínez-Royo et al., 2009). The frequency of R rams serving on farms is low because farmers keep sires from AI as replacement animals. Because *FecX^R* is located in the X chromosome, these rams do not receive the mutated allele, unless the mother was heterozygous for *FecX^R*. As stated above, the percentage of unknown R+ ewes is negligible. Thus, assignment of *FecX^R* heterozygotes through genealogy on farms has proven to be reliable, as demonstrated by similar results found following PCR identification. This technique is simpler and less expensive for on-farm application, and allows the spread of the polymorphism in a controlled way due to the fact that *FecX^R* polymorphism is X-linked, and therefore, it could be spread from hemizygous rams to their daughters. In conclusion, identification of heterozygous ewes on farms by genealogy assignment may provide an effective and simple method of identification, once hemizygous rams and heterozygous ewes have been previously identified.

Prolificacy recorded on farms in heterozygous *FecX^R* ewes, treated or not with eCG, reflects the increase in ovulation rate observed at our experimental center. In untreated ewes, the presence of the *FecX^R* allele increases prolificacy by 0.35 extra lambs per lambing ewe, similar to the previous estimation (+0.32) reported by Jurado et al. (2008) in this breed. These results are slightly less than those described for similar polymorphisms in *BMP15*. One copy of the Inverdale (*FecX^I*) or Hanna (*FecX^H*) allele increases litter size by about 0.6 lambs per lambing ewe in the Romney breed (Davis, 2005), and Galway (*FecX^G*) allele increases prolificacy by 0.55 in the Small Tailed Han breed (Chu et al., 2007). A similar difference in prolificacy (0.30) was observed between *FecX^R* and wild-type ewes under eCG stimulation. The stimulation with eCG produced 0.21 extra lambs born in wild-type ewes, compared to only 0.16 in carrier ewes. Concerning the distribution of the type of lambing, R+ ewes receiving eCG treatment did not demonstrate an increase in the

percentage of twin births. The observed increment in prolificacy was only due to increased percentages of triple and higher-order births. Our results confirm those of Bodin and Elsen (1989) showing that a common pattern of distribution occurs independently of breed. According to this pattern, the percentage of twin births increases with prolificacy reaching a threshold, and from this point forward starts decreasing. The increased percentages of triplet and higher-order births under eCG treatment observed on farms reinforces the results obtained at our experimental center, and should be taken into account. Triple and higher-order births are problematic because of their lower lamb survival rates. In a work carried out in New Zealand it was reported that lamb survival in intensive conditions was 0.90 for single, 0.85 for twins, 0.65 for triplets, and 0.55 for quadruplets (Amer et al., 1999). In Australia, the poorer lamb survival from Booroola × Merino compared to control Merino ewes was attributed to low survival of triple and higher-order births, as survival rates were similar for singles and twins. Industry exploitation of the advantages of the *FecB* carrier ewes is dependent upon reducing lamb losses, especially among higher-order births (Fogarty et al., 2009). Rasa aragonesa is an autochthonous sheep breed oriented to meat production. In normal conditions, the breed displays a low prolificacy (1.34) accompanied by < 1.5% triplet births as shown in this study. Therefore, to avoid increased levels of lamb mortality in *FecX^R* heterozygous ewes, either eCG dose should be reduced, or their management conditions should be improved (e.g., by increasing maternal nutrition during pregnancy, or implementing supplemental supply of milk to the lambs).

A recent study carried out in the Rasa aragonesa breed highlighted the importance of the number of lambs born per ewe on the efficiency and viability of these sheep farms (Pardos et al., 2008). In this way, it has been demonstrated that Rasa aragonesa farms with > 5% of *FecX^R* ewes present better reproductive and economic results, selling 0.34 extra lambs per ewe per year when compared with farms using breeding programs without *FecX^R* ewes (Pardos et al., 2010).

Commercial use of the Inverdale mutation (*FecX^I*) in New Zealand was also shown to be highly

beneficial (Amer et al., 1998). The observed trend to increase the proportion of carrier ewes in the population of Rasa aragonesa ewes demonstrates the interest of farmers in the use of this allele. In fact, in 2009, 120 out of 203 flocks of Rasa aragonesa ewes enrolled in the breeding program had $FecX^R$ ewes, with varying percentages ranging from 0.1 to 24.8% (unpublished data). This variation in percentages of R+ ewes within flocks could be explained by an unintentional dissemination of the mutation when selecting dams and sires based upon their breeding value, before the discovery of this polymorphism. In 2009, the percentage of $FecX^R$ heterozygous ewes in relation to the total population of Rasa aragonesa ewes was 2.20%, reaching 2.96% when only considering flocks with both genotypes present. This percentage is expected to rise considerably, as shown by the fact that in 2009, 6,218 out of 10,776 (58%), and in 2010, 7,002 out of 10,833 (65%) total AI performed in flocks enrolled in the selection program, were performed with semen from $FecX^R$ hemizygous rams (unpublished data).

As has been described by Notter (2008), the optimal level of fecundity in most situations is well below the maximum attainable level, and can be targeted by combining selection within breeds using an expanding array of single-gene mutations affecting ovulation rate and litter size. Our results suggest that it is possible to quickly improve prolificacy on farms by correctly using the $FecX^R$ allele, with a minor use of hormonal treatments. In the case of mutations in the *BMP15* gene, carrier animals must be maintained in a crossbreeding system to avoid the appearance of sterile ewes, which makes the use of these genes slightly more complex when compared with other major genes that do not produce sterility in homozygous ewes. On the other hand, as these mutations are linked to the X chromosome, the control of carrier ewes coming from a carrier male makes possible their identification and control by genealogy, as has been demonstrated in this work. The use of this marker-assisted selection using *BMP15* genotypic information allows the accurate identification of valuable young animals, which is of great economic interest for farmers. However, traditional polygenic selection also remains necessary while the possible existence of associated genes,

undesirable characteristics, or inbreeding problems are being studied, as well as to preserve genetic variability.

In conclusion, in the present study the effect of the new *FecX^R* allele in the ovine *BMP15* gene was quantified for the first time. Heterozygous *FecX^R* ewes present 0.63 extra ovulations and 0.35 additional lambs per lambing adult ewe, increments lower than those previously reported in more prolific breeds carrying other polymorphisms in *BMP15* gene. These reproductive advantages are behind the increasing interest in the use of this polymorphism. Nevertheless, care must be taken in the application of eCG to ewes carrying this polymorphism, with the current results showing that a standard dose increases their prolificacy by only increasing triple and higher-order births.

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TABLES

483

484 **Table 1.** Natural ovulation rate in young (311 ± 11 d) and adult (683 ± 37 d) Rasa aragonesa
 485 ewes heterozygous for *FecX^R* allele or homozygous for the wild-type allele (LSMeans \pm SEM,
 486 range, and distribution of the number of ovulations, %). The means of 3 cycles are presented.

Item	Genotype x Age			
	R+ young ¹	++ young ²	R+ adult ³	++ adult ⁴
No. of ewes	91	20	84	19
Ewes ovulating, %	91.2 ^a	95.0 ^a	98.8 ^a	100.0 ^a
OR ⁵	1.60 \pm 0.04 ^a	1.16 \pm 0.11 ^b	1.99 \pm 0.04 ^a	1.36 \pm 0.09 ^b
OR range	1-3	1-2	1-4	1-3
Type of ovulation, %				
Single	38.9 ^a	91.3 ^b	15.5 ^a	64.8 ^b
Double	59.2 ^a	8.7 ^b	69.7 ^a	33.3 ^b
\geq Triple	1.9 ^a	0.0 ^a	14.7 ^c	1.9 ^d

487

488 In a row, within each age group, values without a common superscript differ at: ^{a, b} $P < 0.01$;
 489 ^{c, d} $P < 0.05$.

490 ¹*FecX^R* heterozygous young ewes.

491 ²Wild-type young ewes.

492 ³*FecX^R* heterozygous adult ewes.

493 ⁴Wild-type adult ewes.

494 ⁵OR = ovulation rate. Corrected means for a weight of 42.5 kg in young and 47.4 kg in adult
 495 ewes.

496 **Table 2.** Ovulation rate (means \pm SEM, range, and distribution, %) and partial failure of
 497 multiple ovulations (means \pm SEM), in adult ewes, heterozygous for *FecX^R* allele or
 498 homozygous for the wild-type allele, treated with fluorogestone acetate¹ and 480 or 240 IU of
 499 eCG².

Item	Genotype x Treatment		
	R+ 480 ³	R+ 240 ⁴	++ 480 ⁵
No. of ewes	21	21	33
OR ⁶	3.95 \pm 0.30 ^a	2.90 \pm 0.18 ^b	2.82 \pm 0.29 ^b
OR range	2-7	1-4	1-10
Type of ovulation, %			
Single	0.0 ^c	4.8 ^{cd}	12.1 ^d
Double	9.5 ^b	23.8 ^{ab}	42.4 ^a
Triple	33.3 ^{ab}	47.6 ^a	21.2 ^b
\geq Quadruple	57.1 ^{ac}	23.8 ^d	24.2 ^b
PFMO ⁷	1.85 \pm 0.41 (13) ^d	1.15 \pm 0.25 (13) ^c	0.77 \pm 0.28 (13) ^c

500

501 Within a row, values differ at: ^{a,b} $P < 0.01$; ^{c,d} $P < 0.05$.

502 ¹Sincropart 30 mg, CEVA Salud Animal S.A., Barcelona, Spain.

503 ²Sincropart PMSG 6,000 UI, CEVA Salud Animal S.A., Barcelona, Spain.

504 ³*FecX^R* heterozygous adult ewes treated with 480 IU of eCG.

505 ⁴*FecX^R* heterozygous adult ewes treated with 240 IU of eCG.

506 ⁵Wild-type adult ewes treated with 480 IU of eCG.

507 ⁶OR = ovulation rate.

508 ⁷PFMO = partial failure of multiple ovulations: Difference between OR and litter size of
 509 lambing ewes with ≥ 2 ovulations (number of ewes is shown in parentheses).

510 **Table 3.** Birth weight (kg) of lambs born from adult ewes, heterozygous for *FecX^R* allele or
 511 homozygous for the wild-type allele, treated with FGA¹ and 480 or 240 IU of eCG²
 512 (LSMeans \pm SEM. Number of lambs is shown in parentheses)

Type of birth	Genotype \times Treatment		
	R+ 480 ³	R+ 240 ⁴	++ 480 ⁵
Overall	3.09 \pm 0.17 (53) ^{bd}	3.51 \pm 0.19 (35) ^c	3.66 \pm 0.14 (33) ^a
Singletons	4.27 \pm 0.29 (3) ^a	4.21 \pm 0.16 (10) ^a	4.33 \pm 0.20 (6) ^a
Twins	3.26 \pm 0.15 (22) ^a	3.01 \pm 0.18 (16) ^a	3.40 \pm 0.16 (20) ^a
\geq Triplets	2.40 \pm 0.13 (28) ^a	2.54 \pm 0.12 (9) ^a	2.90 \pm 0.23 (7) ^a

513

514 Within a row, Least Square Means without a common superscripts differ at: ^{a,b} $P < 0.05$; ^{c,d} P
 515 < 0.1 .

516 ¹Sincropart 30 mg, CEVA Salud Animal S.A., Barcelona, Spain.

517 ²Sincropart PMSG 6,000 UI, CEVA Salud Animal S.A., Barcelona, Spain.

518 ³*FecX^R* heterozygous adult ewes treated with 480 IU of eCG.

519 ⁴*FecX^R* heterozygous adult ewes treated with 240 IU of eCG.

520 ⁵Wild-type adult ewes treated with 480 IU of eCG.

521 **Table 4.** Classification table comparing the identification of the *FecX^R* heterozygous ewes¹
 522 and wild-type ewes² by genealogy³ or by PCR genotyping

Genotype		Ewes ⁴ , %	Lambing records ⁴ , %
Genealogy	PCR		
++	++	83.2 (1,387)	85.6 (9,364)
R+	R+	14.6 (244)	12.3 (1,352)
++	R+	1.7 (28)	1.5 (163)
R+	++	0.5 (8)	0.6 (66)

523

524 ¹R+: *FecX^R* heterozygous ewes.

525 ²++: Wild-type ewes.

526 ³Daughters of *FecX^R* genotyped hemizygous rams were assigned as R+ and daughters of wild-
 527 type or unknown rams were considered as ++.

528 ⁴Whithin each row, percentages of the total of ewes (1,667) or lambing records (10,945) and
 529 number of ewes or lambing records for each row (in parentheses).

530 **Table 5.** Mean prolificacy and litter size distribution (%) of adult Rasa aragonesa ewes
 531 heterozygous for *FecX^R* or homozygous for the wild-type allele, assigned by genealogy¹,
 532 untreated or treated with FGA² and 480 IU of eCG³. Data recorded in flocks involved in the
 533 selection program during 12 yr (1998 to 2010)

Item	Genotype × Treatment			
	++ 0 ⁴	R+ 0 ⁵	++ 480 ⁶	R+ 480 ⁷
Lambing records	599,160	6,593	62,055	866
Prolificacy	1.34 ^a	1.69 ^b	1.55 ^c	1.85 ^d
LS distribution, %				
Singletons	67.7 ^a	42.5 ^b	52.2 ^c	35.7 ^d
Twins	31.0 ^a	46.9 ^b	41.1 ^c	47.1 ^{b,d}
Triplets	1.3 ^a	9.7 ^c	6.0 ^b	14.1 ^d
Quadruplets	0.054 ^a	0.77 ^b	0.63 ^b	2.8 ^c
Quintuplets	0.0042 ^b	0.091 ^{c,d}	0.066 ^c	0.35 ^d

534

535 ^{a-d}Within a row, values without a common superscript differ at:

536 Prolificacy, Singletons, Quadruplets: $P < 0.0001$.

537 Twins, Triplets : $P < 0.0001$; except ^{c,d}: $P < 0.001$.

538 Quintuplets: $P < 0.0001$; except ^{c,d}: $P < 0.01$.

539 ¹Daughters of *FecX^R* genotyped hemizygous rams were assigned as R+ and daughters of wild-
 540 type or unknown rams were considered as ++.

541 ²Sincropart 30 mg, CEVA Salud Animal S.A., Barcelona, Spain.

542 ³Sincropart PMSG 6,000 UI, CEVA Salud Animal S.A., Barcelona, Spain.

543 ⁴Wild-type ewes untreated with eCG.

544 ⁵*FecX^R* heterozygous ewes untreated with eCG.

545 ⁶Wild-type ewes treated with 480 IU of eCG.

546 ⁷*FecX^R* heterozygous ewes treated with 480 IU of eCG.