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Genetic diversity of *GH1* and *LEP* genes in Argentine llama (*Lama glama*) populations

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ABSTRACT

Compared to other domestic species, little is known about variability of genes related to energetic metabolism and growth in camelids. Here, we have analyzed leptin (*LEP*) and growth hormone (*GH1*) genes and characterized their variability in three local llama (*Lama glama*) populations from the Argentine Northwest. Eleven novel SNPs and one indel were identified in the Lep gene. In total, eight haplotypes were found for *LEP* and seven for *GH1*. Although geographical origin clustering was not observed, SNP and haplotype frequencies varied significantly among populations. Based on variation of both loci, we detected significant genetic differentiation measured through Fst values.

Network analysis of *LEP* gene supported the well-documented history of hybridization in camelids previously identified by mtDNA analysis.

1. Introduction

South American camelids, are currently represented by two domestic species, alpaca (*Vicugna pacos*) and llama (*Lama glama*) and two wild, guanaco (*Lama guanicoe*) and vicuna (*Vicugna vicugna*). The currently accepted hypothesis, based on morphological and molecular evidence is that the llama descends from the guanaco and the alpaca from the vicuna (Wheeler, 1995; Stanley et al., 1994; Kadwell et al., 2001; Marin et al., 2007). Results from analyses of satellite DNA (Vidal-Rioja et al., 1994), mitochondrial sequences and microsatellite data (Stanley et al., 1994; Kadwell et al., 2001) have also shown the occurrence of hybridization between domestic species, which explains the long debate and variety of conclusions about their ancestry (see Wheeler, 1995).

Of the two domestic species, only llamas are bred commercially to some extent in Argentina, with the purpose of meat and fiber production. At present, the province with the highest abundance is Jujuy with a 67% of the total animals in the country, followed by Catamarca (16%) and Salta (12%). Traditionally confined to the Andean highlands, in the last decades breeding llamas in Argentina has spread to the plains. As usually extra-Andean llama herds begin with a foundation stock of animals from the Jujuy or Catamarca localities, these populations are important genetic resources to be studied.

Classically, selectively neutral molecular markers such as microsatellites or mitochondrial DNA have been the markers of choice to assess genetic variability and to determine genetic relationships among populations. Nonetheless, as livestock diversity is the result of processes of mutation, genetic drift, and natural and human selection (Long, 2008) neutral molecular markers provide limited insight into adaptive evolution. For this reason, single nucleotide polymorphisms (SNPs) have emerged as

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attractive markers. Besides occurring throughout the genome, they are relatively easy to identify and to genotype. Additionally the identification of SNPs in nuclear genes allows for detection of haplotypes which may be associated with introgression and phenotypic selection that has taken place during the domestication process (Ludwig et al., 2009; Wiener and Wilkinson, 2011; Carneiro et al., 2011).

Over the past years, SNPs were also widely used in candidate gene studies to investigate whether a genetic variant could be associated with development, diseases or phenotypic appearance (McCormack et al., 2009; Haase et al., 2009; Becker et al., 2010). However, in species whose genome sequence is not available yet, the main limitation to study putatively non neutral variation and to perform genotype-phenotype association studies, is the lack of knowledge about candidate genes variability and the absence of molecular markers.

Growth Hormone (*GH1*) and Leptin (*LEP*) genes are both involved in key metabolic pathways that regulate productive traits. GH1 plays an important role in stimulating postnatal growth and lactation (Etherton and Bauman, 1998) while *LEP* is involved in diverse processes related to fertility, regulation of food intake, energy expenditure, and whole-body energy balance in animals and humans (Ahima et al., 1996; Houseknecht et al., 1998). Also, there is evidence that *LEP* may act as a metabolic signal that regulates *GH1* secretion by acting at a hypothalamic level (Carro et al., 1997). Due to the relevance of their physiological function, *GH1* and *LEP* genes are important candidate genes for traits related to productivity in domestic animals. Several polymorphisms have been described in the *LEP* gene and its promoter for humans, cows, pigs, and other species (reviewed by van der Lende et al., 2005). In cow, association between *LEP* polymorphisms and food intake (Lagonigro et al., 2003), serum LEP concentration (Liefers et al., 2003) and postpartum luteal activity (Liefers et al., 2005) have been reported.

Most genetic studies published in llamas deals with neutral genetic diversity. Bustamante et al. (2006), using microsatellites markers showed that llama populations from the Argentine Northwest presented high genetic variability. Similar results were obtained by Barreta et al. (2012a) for Bolivian llamas and alpacas (Barreta et al., 2012a,b). Recently, Paredes et al. (2013) studied the association between microsatellite genotypes and fiber traits in Peruvian alpaca populations and found a relationship between some alleles and the fiber diameter.

Nevertheless, reports on variability of genes related to productive traits are still lacking. Very little is known about variability in growth related genes and no sequences of the *LEP* gene have been reported for llama or other South American camelids up to now.

The structure of the llama *GH1* gene and its polymorphisms have recently been described by Daverio et al. (2012). Notwithstanding, genetic variation of *GH1* locus or other candidate genes, have not been studied at the intrapopulation level in this species.

With the goal to provide genetic data for further association studies, in this work the coding sequence of the *LEP* gene was determined and variability patterns and haplotype diversity of *GH1* and *LEP* genes in three Argentine llama populations were investigated.

2. Materials and methods

2.1. Samples

Blood samples from 79 unrelated llamas, were collected from the villages of Cusi Cusi ($n = 21$) and Cieneguillas ($n = 29$), Jujuy Province. Cusi Cusi (CUSI) is located approximately 120 km southeast from Cieneguillas (CIE) and is isolated by the Santa Catalina hills and the Rio Grande of San Juan. The samples from Catamarca province were collected from the Laguna Blanca Biosphere Reserve (LB) ($n = 29$). Additionally, DNA sequencing was carried out in alpacas ($n = 5$), and in wild species of guanaco ($n = 9$) and vicuna ($n = 7$).

2.2. Primers design and PCR amplification

Total genomic DNA was extracted from whole blood using the standard phenol/chloroform procedure (Sambrook and Russel, 2001)

The *LEP* gene sequence containing the coding region, complete intron 2 and 3′ UTR region was first determined in one llama sample and used as reference to identify variable positions. As there were no camelid *LEP* sequences available on the GenBank, primer design for exon 3 amplification was done over conserved intronic regions across species using Primer3 software (Rozen and Skaletsky, 2000). For exon 2, no region suitable for primer design was found in the adjacent intron 1. Therefore, the Fw primer was designed over first bases of the coding region. Intron 2 was amplified in two overlapped fragments and assembled with exon 2 and 3.Novel *LEP* sequence was submitted to GenBank (KC295539).

Two 600–900 bp *LEP* fragments, including the two coding exons and their adjacent intronic regions were analyzed in order to determine inter and intra population variability. Primers used are listed in Table S1 (Supplementary Material).

Amplification reactions were carried out in a 25 μl volume PCR mix containing 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.7 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 6.25 pmol of each primer and 50 ng of DNA template.

The PCR cycling profile had an initial denaturation for 5 min at 94 °C followed by 30 cycles of denaturation at 94 °C (1 min), annealing at 55–58 °C (30 s), extension at 72 °C (1 min) and a final step at 72 °C for 5 min. PCR products were checked on a 2% agarose gel stained with GelRed™ (Biotium, Hayward, Ca.), sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and analyzed in an automated ABI PRISM 3730xl (Applied Biosystems, Foster City, USA).

The same animals were also genotyped for two *GH1* gene fragments (GH promoter and GH E2-4) as described in Daverio et al. (2012).

2.3. Data analysis

The sequences of *GH1* and *LEP* genes were aligned and edited by using Geneious Software v5.6.5 (http://www.geneious.com). All putative SNPs were checked by visual inspection of each chromatogram and confirmed by resequencing or by observation in two or more individuals.

Observed (Ho) and expected (He) heterozygosities, allele frequencies and Hardy–Weinberg (H–W) equilibrium were calculated using Arlequin v3.5 software (Excoffier and Lischer, 2010). DnaSP v5 (Librado and Rozas, 2009) was used to compute the number of segregating sites (S), nucleotide diversity (π) and haplotype diversity (Hd). Tajima's D test of neutrality (Tajima, 1989) was performed for both *LEP* and *GH1* gene using DnaSP v5.

Excluding 3′UTR region of *LEP*, SNPs from the two fragments of each gene were combined into single genotypes. Then, haplotypes were inferred from unphased data by means of Arlequin software (Excoffier and Lischer, 2010) Haplotypes with phase probabilities <0.95 were excluded from further analysis.

To predict the possible impact of the amino acid substitution on the *LEP* protein structure, the FFAS03 server was used to profile-profile sequence alignments (Jaroszewski et al., 2005) and Modeller for modeling the three-dimensional protein structure (Shen and Sali, 2006). ProSaweb was used to validate the protein structure modeling and FoldX to predict free energy changes (Schymkowitz et al., 2005). Prediction of the possible impact of amino acid substitutions on protein function was performed by using SIFT software (http://sift.jcvi.org/) whereas the

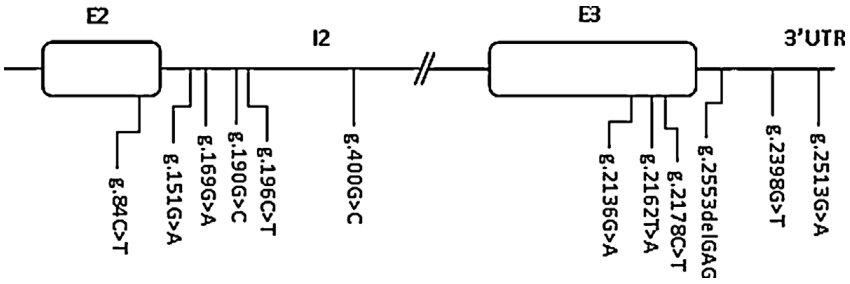

Fig. 1. Organization of the coding region and polymorphisms distribution in the LEP gene.

analysis of putative Transcription Factor Binding Sites (TFBS) within the *GH1* promoter gene was done with the NSITE program at Softberry site (http://linux1.softberry.com/berry.phtml).

To determine evolutionary relationships among haplotypes, a median-joining network was constructed using program Network 4.6.1.0 (Bandelt et al., 1999). Alpaca, vicuna and guanaco sequences were also included to infer ancestral alleles and to detect putatively introgressed alleles.

Analysis of molecular variance (AMOVA) was performed using Arlequin v3.5 considering three different hierarchical levels: within individuals, among individuals within populations, and among populations. Pairwise Fst between populations and overall *F* statistics were also estimated using Arlequin v3.5.

3. Results and discussion

3.1. GH1 and LEP gene variability

Novel *LEP* sequence obtained, encoding the mature llama *LEP* protein plus 6 aminoacid of the signal peptide, was submitted to GenBank (KC295539).

Analysis of *LEP* exon 2 and exon 3 and adjacent intronic regions across all investigated individuals (146 chromosomes) yielded 12 polymorphisms including 11 SNPs and a 3 bp indel. This polymorphism level corresponds to 1 segregating site every 124 bp and to a nucleotide diversity $\pi = 0.00329$. Organization of *LEP* coding region and polymorphisms distribution is shown in Fig. 1.

For *GH1* gene, a total of 7 SNPs were found in the 156 chromosomes analyzed, previously reported by Daverio et al. (2012). *GH1* overall nucleotide diversity, $\pi = 0.00213$, was lower than in the *LEP* gene. However, compared to other species, both values were in the same order of those reported for the same genes by other authors (Konfortov et al., 1999; Ryynanen and Primmer, 2004; Nie et al., 2005).

Localization, type and frequencies of polymorphisms found in both genes are summarized in Table 1.

LEP gene presented four SNPs in the coding region, one in exon 2 and three in exon 3. Within those in exon 3 there was a non-synonymous SNP leading to p.Gln138Leu substitution.

Through a molecular modeling approach we evaluated the effect of the amino acid replacement on the *LEP* structure. According to the llama protein model obtained, position 138 is exposed to the solvent and thus, unlikely to affect the folding. This result is also supported by the low differences in free energy of the mutation (ddG = 0.05 kcal/mol), indicating that is probably energetically neutral. We also studied the functional effect of p.Gln138Leu amino acid replacement. Results from SIFT software show that this substitution is predicted to be 'tolerated'. Nonetheless, there is experimental evidence that substitutions at 138 residue could have some functional effects. This position is usually occupied by a Gln or Arg residue in other mammals and it is located in the helix D of the protein within a region involved in *LEP*-leptin receptor interaction (Peelman et al., 2004). The replacement of a polar amino acid Gln by a hydrophobic residue Leu in llamas, occurs within a stretch of residues in the center of the binding site I. Mutations at this site in ovine *LEP* proteins have shown decreased binding affinity and enhanced ability to induce cell proliferation (Reicher et al., 2011) Thus, it is possible that Gln138 and Leu138 represent functionally different *LEP* variants.

GH1 polymorphisms were all located in non-coding regions. However, two of them, g.46G >A and g.237A >G could have some functional importance since they are situated in the promoter region of the gene. We searched for putative Transcription Factor Binding Sites (TFBS) overlapping the SNPs identified and found that SNPg.237A>G is within a predicted binding site for thyroid hormone receptor (T3R). Additionally, that SNP is located in the proximity of the TATA box (Daverio et al., 2012), which plays an important role in determining the efficiency of the promoter (Maniou et al., 2004). Therefore their

Table 1
LEP and *GH1* polymorphisms and alleles frequencies.

Gene	Region	Polymorphisms[a]	Minor allele frequencies[b]
LEP	Exon2	g.84C>**T**	0.28
LEP	Intron 2	g.151G>**A**	0.02
LEP	Intron 2	g.169G>**A**	0.09
LEP	Intron 2	g.190G>**C**	0.42
LEP	Intron 2	g.196**C**>T	0.09
LEP	Intron 2	g.400**G**>C	0.03
LEP	Exon3	g.2136G>**A**	0.12
LEP	Exon3	g.2162**T**>A	0.37
LEP	Exon 3	g.2178C>T	0.40
LEP	3′ UTR	g.2253–2255del3	ND[c]
LEP	3′ UTR	g.2398G>T	ND[c]
LEP	3′ UTR	g.2513G>A	ND[c]
GH1	Promoter	g.46G>**A**	0.15
GH1	Promoter	g.237A>**G**	0.28
GH1	Exon 1	g.310**A**>G	0.11
GH1	Intron B	g.847T>**C**	0.11
GH1	Intron B	g.884**C**>G	0.30
GH1	Intron B	g.927G>**T**	0.01
GH1	Intron B	g.941**T**>C	0.30

[a] SNPs are identified with corresponding numbering to GenBank accessions HM921333 and KC295539 for *GH1* and *LEP* respectively.
[b] The minor allele frequencies corresponds to the bolded allele.
[c] ND: non determined.

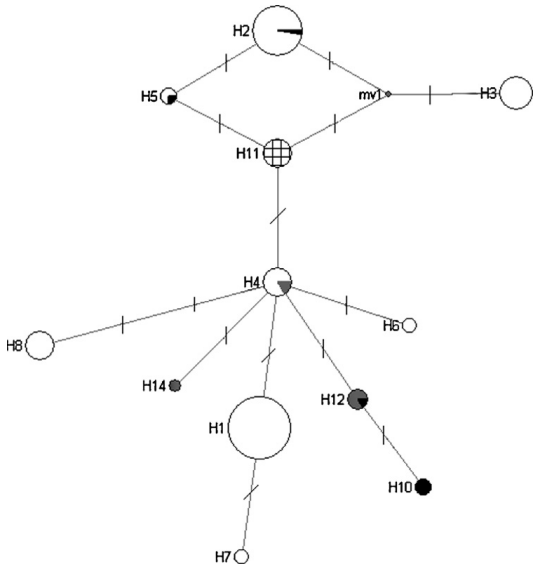

Fig. 2. Median-joining network of LEP gene, representing the evolutionary relationships among haplotypes found in both domestic and wild camelids. Size of the circles is proportional to the frequency of each haplotype. Lamas are denoted by white. Alpacas are denoted by black while the vicuñas and guanacos are denoted by gray and cross-line, respectively. Vertical lines represent the number of mutational steps.

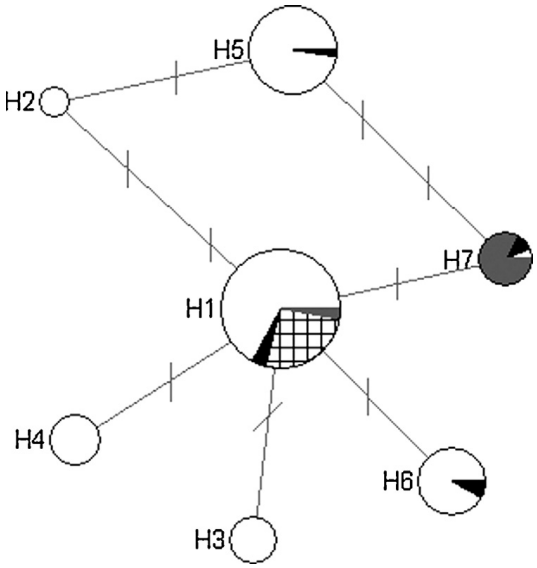

Fig. 3. Median-joining network of GH1 gene. Color code is the same as Fig. 2.

functional importance could not be discarded. For SNP g.46G >A, we did not find TFBSs for known factors regulating *GH1* promoter activity.

3.2. Relationships among haplotypes

A median joining network also including alpaca, guanaco and vicuna samples was constructed to study evolutionary relationships among camelids haplotypes.

Ancestral nodes of the *LEP* gene network (Fig. 2) corresponding to guanaco and vicuna haplotypes were identified as H11 and H4, respectively, and defined two clusters separated by only one mutational step. Haplotype network did not show the classical star-like appearance, presenting young allele frequencies considerably higher than the ancestral ones. It is interesting to note that none of the llama samples presented the ancestral guanaco haplotype H11. Haplotype sharing between vicuna and llama and also between alpaca and llama, suggest that introgression has played a role in shaping genetic diversity in local llama populations.

The *GH1* median joining network did not show a clear cluster structure. It showed reticulation involving ancestral nodes and one haplotype, H1, which was shared by the four camelid species (Fig. 3).

Table 3
Population pairwise Fst.

		CIE	CUSI	LB
LEP	CIE	0.000		
	CUSI	0.147	0.000	
	LB	0.214	0.045	0.000
GH1	CIE	0.000		
	CUSI	0.071	0.000	
	LB	0.149	0.067	0.000

Table 2
Genetic diversity indices and Tajima's D values for *GH1* and *LEP* gene.

		S[a]	π[b]	Ho[c]	He[d]	H[e]	Hd[f]	D[g]	P[h]
LEP	CIE	5	0.00218	0.215	0.216	4	0.465	1.16	>0.1
	CUSI	7	0.00342	0.356	0.322	7	0.852	1.43	>0.1
	LB	7	0.00386	0.320	0.387	6	0.772	2.01	$0.1 > p > 0.05$
GH1	CIE	6	0.00152	0.226	0.225	6	0.647	0.08	>0.1
	CUSI	6	0.00198	0.357	0.292	5	0.779	0.67	>0.1
	LB	6	0.00243	0.339	0.359	7	0.759	1.64	>0.1

[a] S, segregantes sites.
[b] π, nucleotide diversity.
[c] Ho, observed heterozygosity.
[d] He, expected heterozygosity.
[e] H, number of haplotypes;
[f] Hd, haplotypic diversity;
[g] D, Tajima's D.
[h] *p*, *P*-value.

Table 4
LEP and *GH1* AMOVA and *F* statistic.

Source of variation	Gene	Sum of squares	Variance components	Percentage of variation	Gene	Sum of squares	Variance components	Percentage of variation
Among populations	*GH1*	4.951	0.04148 Va	10.29	*LEP*	5.867	0.05440	13.67
Among individuals within populations	*GH1*	25.383	−0.02309 Vb	−5.73	*LEP*	24.411	0.01022	2.57
Within individuals	*GH1*	30.000	0.38462 Vc	95.44	*LEP*	24.000	0.33333	83.76
Total		60.333	0.40301			54.278	0.39796	
Fixation indices						Fixation indices		
Fis		−0.064				Fis	0.02976	
Fst		0.103				Fst	0.13671	
Fit		0.046				Fit	0.16240	

3.3. Within population variability and genetic differentiation

Genetic diversity was evaluated at the intrapopulation level by determining the number of segregating sites, nucleotide diversity, observed and expected heterozigosity, haplotype number and haplotypic diversity by population (Table 2). No SNP showed significant deviation from H–W expectation in any of the studied populations.

Most *LEP* haplotypes were spread amongst the three populations. However, there were differences in haplotype frequencies (Table S2, Supplementary Material). The most frequent haplotype in CIE, H1, was found in LB at a lower frequency. On the other hand, the second most frequent haplotype in LB, H8, was absent in CIE and CUSI. H6 and H7 were found only in CUSI population. Notably, haplotype H2 and H3 which have the allele Leu for the p.Gln138Leu substitution reached a combined frequency of more than 40% in CUSI and LB.

Overall, CIE showed low genetic diversity measured through different parameters, compared to CUSI and LB populations. For example, only four *LEP* haplotypes were found and among them, a unique haplotype, H1 accounts for 70% of the sampled chromosomes.

In concordance with observations for *LEP*, *GH1* haplotype frequencies were different between populations. In CIE, 55% of the samples had the H1 haplotype. In LB this haplotype was found in a much lower frequency, while H5 was the major haplotype with a frequency of 46%. In CUSI, H1 and H3 were the most frequent haplotypes with a combined frequency of 59% (Table S3, Supplementary Material).

Haplotype diversity and nucleotide diversity were also appreciably lower in CIE than in CUSI and LB. For *GH1* gene, nucleotide diversity, heterozigosity and haplotype diversity were also lower for CIE than for CUSI and LB, although differences were not so marked as in *LEP* gene (Table 2).

Bustamante et al. (2006), analyzing microsatellite data, found high levels of genetic diversity in both, CIE and CUSI population (referred as Los Pioneros and Llampa Jarán in this work) Contrasting results from microsatellite and coding genes here studied could be explained by different selection pressures acting on both regions of the genome.

Genetic differentiation between populations was studied through pairwise Fst values (Table 3). For *LEP* gene, significant genetic differentiation between CIE and CUSI (Fst = 0.147) and between CIE and LB (Fst = 0.214) was observed. CUSI and LB showed less differentiation, but Fst was still significantly different from zero.

Based on *GH1* gene, pairwise comparisons showed significant differentiation between populations, although Fst values were lower than those based on *LEP* gene.

Contrary to what is expected under neutrality, a positive correlation between genetic differentiation and geographic distance was not found. For both genes analyzed, the two furthest populations (CUSI and LB) showed the lowest Fst values.

The percentage of variation among populations from the AMOVA was 13.7% for *LEP* and 10.3% for *GH1* respectively (Table 4). Even though both genes showed significant differences in haplotype frequency among the three populations (empirical *P*-values were <0.001 for both genes), these differences accounted only for a minority of the observed variance and it was explained mainly by within-individual variation.

Table 4 also shows estimates for Fst, Fis and Fit values. Inbreeding coefficient (Fis) was not significantly different from zero for either *LEP* (Fis = 0.03) or *GH1* gene (Fis = −0.06).

Departures from neutral expectations were investigated on the basis of Tajima's *D* test for each locus and each population. Positive *D* values indicate an excess of intermediate-frequency alleles and could result from balancing selection whereas negative values reflect an excess of rare polymorphisms in a population, which is consistent with positive selection (Handcock and Di Rienzo, 2008). It was observed that across the three populations and both loci, Tajima's *D* values were positive (although not statistically significant) for both analyzed genes (Table 2). However, positive Tajima's *D* values can be also indicative of admixture or a reduction in population size (Carlson et al., 2005), processes both known to have occurred in domestic camelid species after the Spanish conquest (Kadwell et al., 2001).

Despite the fact the Tajima test failed to detect selection in the data obtained, some interesting features are apparent from *LEP* locus. Analysis in a wider range of samples could provide more insights into camelid domestication process and the selective forces acting on the *LEP* gene.

To conclude, *LEP* and *GH1* loci showed considerable variation within and between populations analyzed. The results from this work are relevant for future studies aimed

to test association of particular *LEP* and *GH1* alleles with growth and reproductive parameters.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.smallrumres.2014.03.014.

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