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1 **Seroconversion against SU5 derived synthetic peptides in sheep experimentally infected**
2 **with different SRLV genotypes.**

3

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15 **Abstract**

16 Synthetic peptides were generated, corresponding to SU5 domain of envelope glycoprotein of
17 Italian SRLV isolates It-561 and It-Pi1, belonging respectively to MVV- and CAEV-like
18 genotypes. The peptides, encompassing an N-terminal variable and a C-terminal conserved
19 antibody-binding site, were used in an ELISA assay to analyse the sera of two groups of sheep
20 experimentally infected with these isolates. The kinetics and specificity of the humoral response
21 to the homologous and heterologous antigen and the affinity maturation of the sera were
22 evaluated. Seroconversion occurred between week 3 and 8. The response to SU5 antigen was
23 mostly type-specific. The few broadly reacting sera may reflect the production of antibodies

24 directed to the SU5 constant antibody-binding site. All sera underwent with time avidity
25 maturation, resulting in the appearance of high affinity antibodies. This study suggests constant
26 monitoring of the circulating viral variants to develop a panel of diagnostic peptides
27 representative of local genotypes.

28

29 **Keywords**

30 Small Ruminant Lentiviruses, envelope glycoprotein, SU5 peptides, type-specific
31 seroconversion.

32 **Introduction**

33 Maedi Visna Virus (MVV) and Caprine Arthritis Encephalitis Virus (CAEV) are non oncogenic
34 lentiviruses of the Retroviridae family, presently referred to as Small Ruminant Lentiviruses
35 (SRLVs) (Zanoni, 1998). A recent phylogenetic classification of these viruses defines two main
36 groups, A and B, including respectively MVV-like and CAEV-like genotypes (Shah et al., 2004).
37 SRLV infections occur almost worldwide and cause relevant economic losses. Therapy and
38 effective vaccine are not yet available (Gonzalez et al., 2005; Petursson et al., 2005;
39 Torsteindottir et al., 2007; Niesalla et al., 2008; Reina et al., 2008), therefore eradication and
40 prevention of the infection largely depend on early, efficient and correct identification of infected
41 animals (de Andrés et al., 2005). This is routinely done by serological analysis, the ELISA
42 assay being the most sensitive and suitable technique both for large scale screening and
43 individual examination. Several ELISA protocols have been developed so far, mostly based on
44 antigens from a single viral strain. The specificity of these assays is generally high, but the
45 sensitivity shows extensive variability (de Andrés et al., 2005) due to antigenic heterogeneity of

46 SRLVs. Recent analysis of two Italian SRLV isolates, MVV-like It-561 and CAEV-like It-Pi1,
47 demonstrated that gag-encoded capsid (CA) and matrix (MA) proteins carry type-specific
48 epitopes and most sheep and goat sera reacted to these antigens in a type-specific manner,
49 irrespective of the species of origin (Grego et al., 2002 ; Grego et al., 2005). Furthermore,
50 homologous CA-MA fusion protein was able to detect seroconversion at an earlier stage
51 compared to the heterologous protein (Lacerenza et al., 2006). The present study extends the
52 analysis of the humoral response induced by It-561 and It-Pi1 to SU5, one of the major epitopes
53 of the envelope (Env) protein (Bertoni et al., 2000; Valas et al., 2000). Two SU5 synthetic
54 peptides were generated, corresponding to It-561 and It-Pi1 sequences. The peptides were
55 used as antigens to assay sera from two groups of sheep experimentally infected with these
56 viruses, in order to investigate the kinetics and specificity of the humoral response to the
57 homologous and heterologous antigen.

58 **Materials and Methods**

59 *Viral strains and experimental infection*

60 SRLV strains It-561 (genotype A) and It-Pi1 (genotype B) (Grego et al., 2002) were used in the
61 present study. Infection of sheep with these strains has been described by Lacerenza et al.
62 (2006). Detection of viral sequences in blood of experimental sheep was done by *LTR* PCR
63 (Extramiana et al., 2002) and *PoI* PCR (Grego et al., 2002).

64 *Cloning, sequencing and sequence analysis of env region coding for SU5*

65 An *env* gene fragment encompassing the 75bp "SU5 total" domain (Mordasini et al., 2006)
66 coding sequence (nt 7800-7874) was amplified from DNA of foetal ovine lung fibroblasts
67 infected respectively with It-561 and It-Pi1 viral stocks. Primer sequences (Bertoni et al., 2000)
68 were as follows: 563F: GAYATGRYRGARCAYATGAC (nt.7272-7291); 567F:
69 GGIACIAAIACWAATTGGAC (nt. 7482-7501); 564R:GCYAYATGCTGIACCATGGCATA (nt.

70 8089- 8067). The nucleotide positions refer to the CAEV sequence published by Saltarelli et al.
71 (1990; Genbank accession number M33677). Amplified fragments were cloned and sequenced.
72 Genetic distances were calculated with MEGA 3 (Kumar et al., 2001) and used to construct a
73 neighbor-joining tree with the Tamura-Nei two-parameter distance option (Tamura et al., 1993).

74 *Peptide ELISA*

75 Peptides corresponding to the 25aa “SU5 total” domain of both viruses, were resuspended in
76 0.1M carbonate buffer pH 9.6 at 5 µg/mL. ELISA plates were coated overnight at 37°C
77 (50µL/well), incubated for 1 hour at 37°C with sera diluted 1/20 and for 1 hour at room
78 temperature with a 1:8000 dilution of peroxidase-labelled anti-sheep/goat IgG monoclonal
79 antibody (Sigma). Optical density was measured at 405 nm wavelength after 40 min incubation
80 with ABTS (Sigma).

81 *Cut-off*

82 A panel of sera collected before experimental infection as well as sequential sera from two
83 mock-infected sheep, were repeatedly assayed: no significant interplate variation of the
84 absorbance was observed. The cut-off was calculated as mean absorbance of negative sera +
85 3x Standard Deviation. Samples with absorbance higher than 0.3 were considered positive with
86 a 99% confidence level. A serum collected from a sheep belonging to a long term seronegative
87 flock was loaded on each plate.

88 *Antibody avidity measurements*

89 The avidity index values of SU5-specific antibodies were measured by testing the stability of the
90 antigen-antibody complexes following a wash in 8 M urea (Mordasini et al., 2006). Antibodies
91 with avidity indexes <30% were considered to be of low affinity; those with values between 30%
92 and 50% of intermediate avidity and those with values >50% of high avidity.

93 Results

94 *Cloning and sequencing of SU5-encoding env region*

95 The *env* sequences encompassing respectively the SU5 domain of It-561 and It-Pi1 strains
96 (Genbank accession numbers: It-561: EU702487; It-Pi1: EU709743) were aligned with MVV
97 and CAEV prototypic *env* sequences of worldwide origin. It-561 clusters with representatives of
98 group A genotypes and It-Pi1 with B genotypes (fig.1 a), in agreement with the results of a
99 phylogenetic analysis of *gag* and *pol* sequences (Grego et al., 2002; 2005). The SU5 aminoacid
100 sequences of It-561 and It-Pi1 share homology in the N-terminal region, which is well
101 conserved among MVV and CAEV strains, and diverge in the C-terminal, more variable portion
102 (fig.1 b).

103 *Peptide ELISA*

104 Peptide ELISA assays were performed on a panel of sera obtained over a period of up to 92
105 weeks p.i. from three groups of experimentally infected sheep: group A, sheep 1 to 5, infected
106 with It-561; group B, sheep 6 to 9, infected with It-Pi1 (fig.2 a, b) and group C, four mock-
107 infected sheep. Group C sheep remained seronegative throughout the study (data not shown).
108 Group A sheep 1 to 4 seroconverted to the homologous antigen between week 4 and 8 and
109 remained positive throughout the study. Sheep 1 to 3 were completely negative to the
110 heterologous It-Pi1 peptide, while sheep 4 raised a weak humoral response to It-Pi1, from week
111 77 onwards. Sheep 5 did not seroconvert and was the only one negative to both *LTR* and *Pol*
112 PCR assays performed throughout the study to confirm the presence of virus in the infected
113 animals (data not shown). Group B sheep seroconverted to the homologous antigen between
114 week 3 and 8; all sheep remained positive until the end of the experiment, with high absorbance
115 values. Only sheep 9 responded to the heterologous antigen from week 8 onwards. The

116 absorbance, which was about two- to threefold lower than for the homologous antigen, reached
117 a peak at week 22 and gradually decreased thereafter, to values just above the cut-off.

118

119 *Kinetics of antibody avidity maturation*

120 The avidity maturation of sera was assayed at week 8 p.i., corresponding to the first antibody
121 peak, at week 35 and at week 78. The results obtained for group A sheep are depicted in fig.3
122 a. Sheep 2, 3 and 4 underwent a marked increase of antibody avidity over the period analyzed.
123 All avidity indexes of sheep 1 were low; therefore additional time points were analyzed.
124 Maturation to high avidity was detected at week 56, followed by a gradual decrease to low
125 avidity by the end of the experiment (data not shown. Group B sheep underwent a marked
126 avidity maturation, reaching the highest values at week 35 p.i (fig.3b).

127 **Discussion**

128 Most ovine isolates collected in Italy belong to the CAEV-like group (Grego E. et al., 2002) and
129 coinfection of goats with both MVV- and CAEV-like strains in an Italian flock has recently been
130 reported (Pisoni et al., 2007). In the last years serological assays based on Gag antigens
131 derived from Italian isolates belonging to both phylogenetic groups were developed and applied
132 in field conditions: the response obtained was largely type-specific, indicating the opportunity to
133 use antigens representative of both genotypes to improve the sensitivity of the diagnostic
134 assays (Grego et al., 2002; 2005). Furthermore, when sequential sera collected from sheep
135 experimentally infected with either It-561 or with It-Pi1, were analyzed by ELISA with both
136 homologous and heterologous CA-MA fusion proteins, the homologous antigen detected
137 infected animals up to months before the heterologous antigen (Lacerenza et al., 2006). In this

138 study the same panel of sequential sera was analyzed with an Env ELISA based on It-561 and
139 It-Pi1 SU5 peptides. Five epitopes have been identified in the SU subunit of Env (Bertoni et al.,
140 2000; Valas et al., 2000). SU5 in particular, a 25 aminoacid sequence located at the C terminus
141 of SU, is immunodominant and partly type-specific: it carries at least three antibody-binding
142 sites, one in the N-terminal conserved region, one within the C-terminal most variable domain,
143 and the third encompassing the junction between the variable and conserved regions
144 (Mordasini et al., 2006). In this study, two *env* gene fragments encoding respectively It-561 and
145 It-Pi1 SU5, were sequenced. Alignment of these fragments with sequences of MVV and CAEV
146 prototypic strains of worldwide origin showed that It-561 clusters with the MVV strains and It-Pi1
147 is phylogenetically related to classical CAEV-like prototypes. The SU5 N-terminal aminoacid
148 sequences of the two strains share extensive homology; the C-terminal region is more
149 divergent, confirming the results of Mordasini et al. (2006). Seroconversion kinetics of group A
150 and B sheep were similar, with antibodies being mostly type-specific. The few broadly reacting
151 sera may reflect the production of antibodies directed to the constant antibody-binding site. The
152 antibody levels varied between animals and for the same animal during the time course of the
153 experiment. In group A, the first antibody peak was followed by a slight decrease of reactivity
154 and then by a rise to three to fourfold higher values. One sheep of this group did not
155 seroconvert. This result was most likely caused by a technical failure during the experimental
156 infection, since the sheep was repeatedly negative to both *LTR* and *pol* PCR assays which
157 were performed on blood samples of the experimental animals to confirm the presence of viral
158 genomes. Group B sheep developed immediately an antibody response well over the cut-off. All
159 sera underwent with time avidity maturation, resulting in the appearance of high affinity
160 antibodies, although with different kinetics. It-Pi1 sera, which developed higher antibody levels
161 from the beginning, underwent high avidity maturation at an earlier time point than It-561 sera.

162 Sheep 1 showed a peculiar pattern of avidity maturation, with indexes decreasing from week
163 56 onwards, to return to low values by the end of the experiment, in spite of a persisting high
164 antibody level. The reason for sheep 1 behaviour is unclear; the genetic variability of SU5 could
165 possibly explain the disappearance of the high affinity antibody-producing clone(s).
166 Alternatively, this sheep may present some failure in the mechanisms that underly the ability to
167 maintain the production of high affinity antibodies. A failure of T helper function may be
168 responsible for the loss of high affinity antibodies as well as the associated decay of the
169 antibody titer, that have been reported in individuals who later developed AIDS (Chargelegue et
170 al., 1993; 1995; Thomas et al., 1996). SRLV infection, however, does not involve the
171 lymphocyte compartment, and sheep 1 antibody titer does not decrease, therefore a different
172 mechanism may be responsible. A comparison of the results obtained in this study and in
173 Lacerenza et al. (2006) shows that both CA-MA fusion antigens and SU5 peptides detect
174 infection at early stages, from week three onwards. Furthermore, while It-561 drives type-
175 specific antibody response towards both Gag and Env antigens, It-Pi1 seems to drive
176 production of type-specific antibody only against SU5. In fact, all group B sheep reacted against
177 the heterologous Gag subunits as well (Lacerenza et al., 2006). Since the humoral response to
178 SU5 is mainly directed towards the variable antibody-binding site, a constant monitoring of the
179 circulating viral variants will be necessary to develop a panel of SU5 peptides representative of
180 most local genotypes.

181 **Acknowledgements**

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183 **Conflict of interest statement**

184 *All authors deny any financial and personal relationships with other people or organizations that*
185 *could inappropriately influence this work.*

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265 Fig.1:

266 a: Phylogenetic analysis of It-561 and It-Pi1 *env* fragments encompassing SU5 coding region.

267 The neighbor-joining tree was constructed using MEGA. Bootstrap values are based on 5000

268 repetitions. The accession numbers refer to the following strains: AF338227 (MVV K1514,

269 Iceland); S51392 (MVV EV1, Great Britain); M31646 (SA-OMVV, South Africa); EF154467

270 (SRLV 12-5, Italy); U64439 (OLV 85/34, USA); EF154474 (SRLV 12-20, Italy); AY900630

271 (CAEV Gansu, China); M33677 (CAEV-Co, USA), AF322109 (CAEV 1GA, Norway); NC001482

272 (FIV). b: Alignment of SU5 aminoacid sequences of It-561 and It-Pi1 with MVV and CAEV

273 strains.

274

275 Fig. 2:

276 Kinetics of humoral responses of experimentally infected sheep to homologous (white bars) and

277 heterologous (black bars) antigen. The cut-off was set at 0.3 OD. *: not available. a: It-561

278 infected sheep. b: It-Pi1 infected sheep.

279

280 Fig. 3:

281 Avidity maturation of anti SU5 antibody taken at 8, 35, 78 weeks p.i. a: sheep infected with It-

282 561; b: sheep infected with It-Pi1. Serum antibodies with avidity index values lower than 30%

283 are designated low avidity antibody, those with avidity index values between 30 and 50% are

284 intermediate-avidity antibodies, and those with values higher than 50% are considered high

285 avidity antibodies.

286

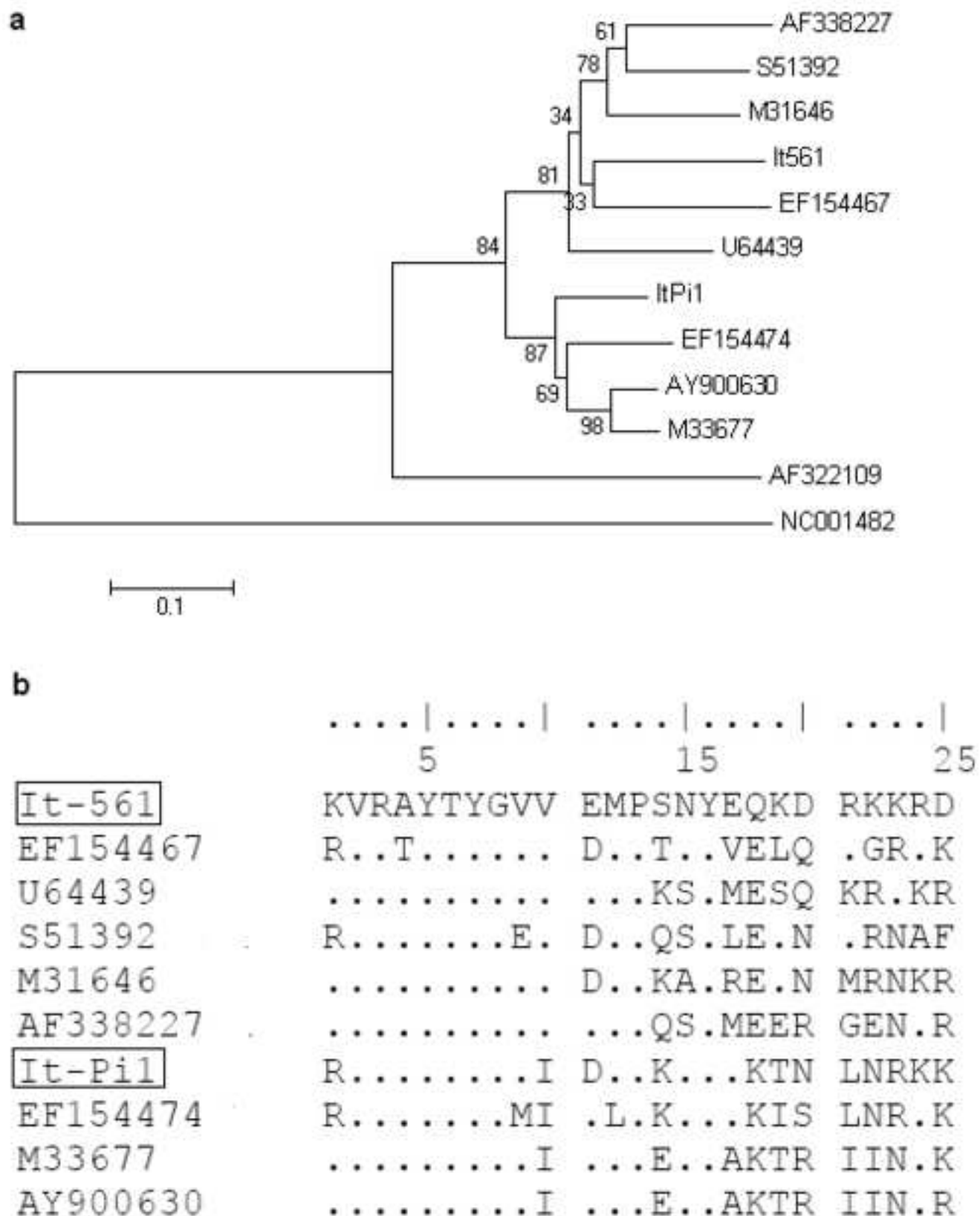


Fig. 1

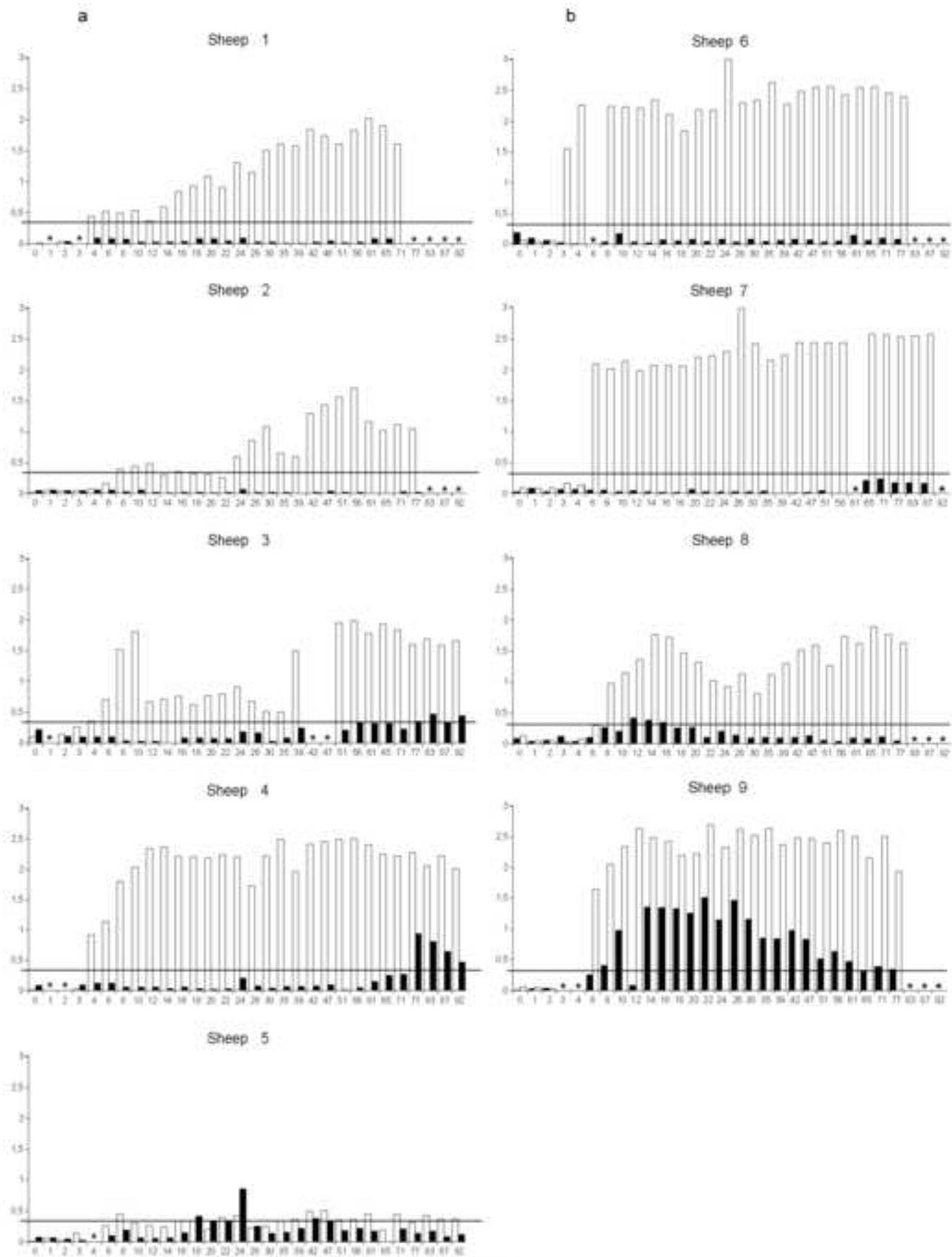


Fig 2

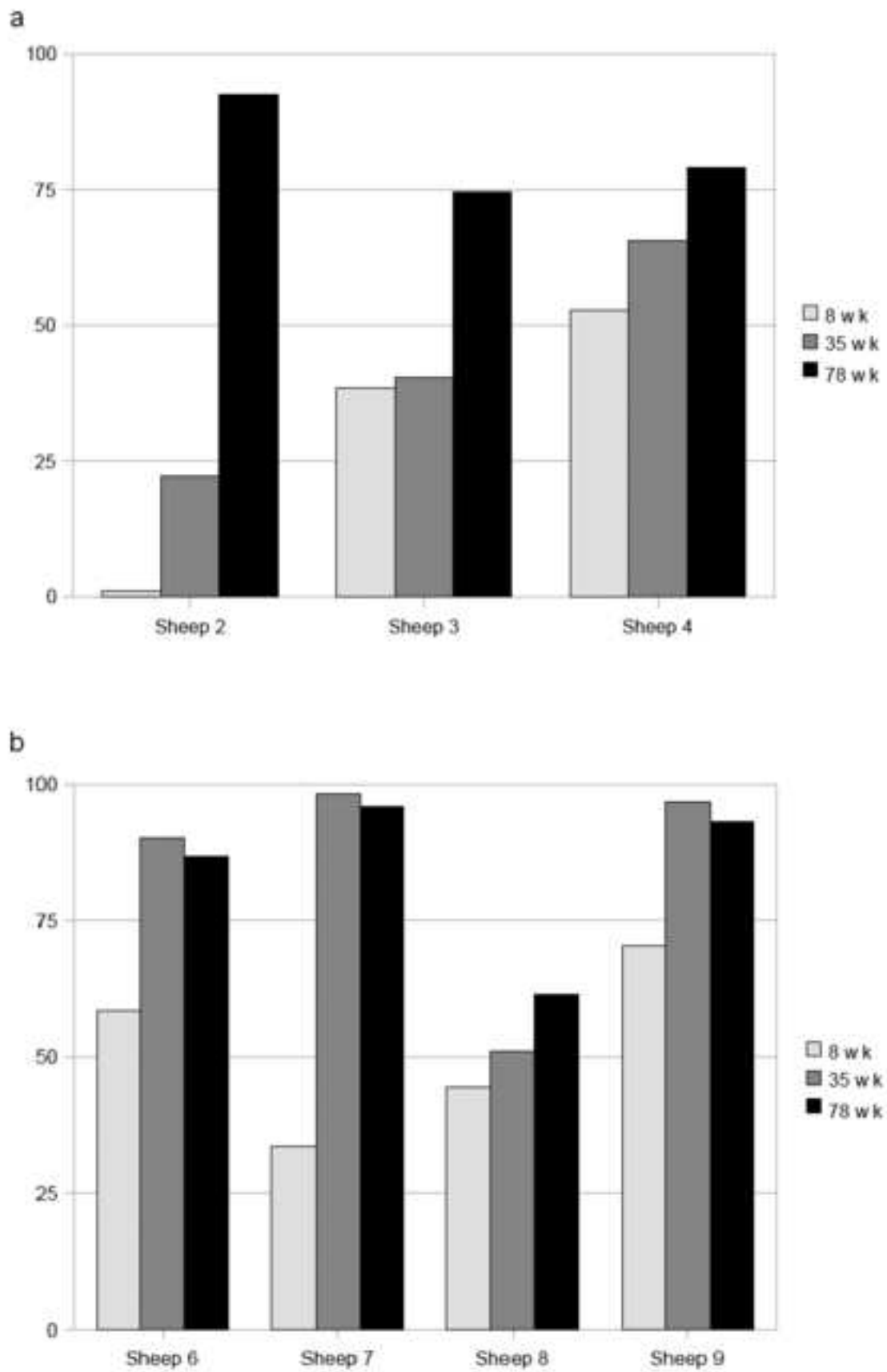


Fig. 3