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1 **Differences in amniotic amino acids concentrations between pregnancies obtained**
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10
11 **Abstract**

12 *In vitro* embryo production (IVP) and cryopreservation are associated with a high incidence of
13 pregnancy complications and fetal abnormalities that may be linked with alterations of
14 placental development. The amniotic fluid is partly derived from the transport of water and
15 solutes across the placenta and provides the fetus with amino acids (AAs), which are the
16 building blocks for biomolecules involved in physiologic growth and development. To better
17 understand the anomalies associated with IVP pregnancies, the present study was conducted
18 to test the hypothesis that amniotic concentrations of AAs differ in pregnancies derived from
19 vitrified/thawed (V/T) IVP embryos compared to gestations obtained with natural mating (NM)
20 in sheep. Amniotic fluid was sampled in ewes that were pregnant after transfer of V/T IVP
21 embryos and that had conceived with natural mating between day 60 and 65 (V/T n=6; NM
22 n=11) and between day 80 and 85 (V/T n=5; NM n=14) of gestation via ultrasound-guided
23 amniocentesis. Concentrations of 16 AAs in the amniotic fluid were measured using high-

24 performance liquid chromatography. At day 60-65 of gestation concentrations of cystine,
25 phenylalanine and isoleucine were lower in V/T compared to NM ewes. At day 80-85 of
26 pregnancy the mean concentrations of cystine and lysine were lower in the V/T vs NM group.
27 The total AA concentration per ewe was similar between groups at day 60-65 and 80-85 and
28 decreased by 55% from day 60-65 to 80-85 of gestation in all ewes. The most abundant AA at
29 day 60-65 of gestation was alanine in both groups, whereas at day 80-85 the most abundant
30 AAs were alanine in NM and glycine in V/T ewes respectively; cystine was the less abundant
31 detectable AA in all ewes at both stages of gestation. Results demonstrate that V/T IVP
32 embryos have decreased concentrations of individual AAs in the amniotic fluid during the
33 second trimester of gestation, possibly because of an impaired placental vasculogenesis or
34 because of a reduced placental transport. These novel findings are relevant to unravel the
35 mechanisms responsible for the issues of pregnancies achieved with the transfer of IVP and
36 cryopreserved embryos.

37
38 **Keywords:** amniotic fluid; AA; *in vitro*; embryo; vitrification; ovine.

40 **1. Introduction**

41
42 Reproductive efficiency is fundamental for the economic sustainability of animal husbandry,
43 including farming of small ruminants. To control and improve animal reproduction, assisted
44 reproductive technologies (ART) have been developed, such as artificial insemination,
45 multiple ovulation, embryo transfer and *in vitro* production of embryos (IVP) [1, 2]. The latter
46 technique involves four major steps: oocyte collection (either from a live animal via ovum pick-

47 up or from ovaries recovered at slaughter), oocyte *in vitro* maturation, fertilization
48 and development of the embryo up to the blastocyst stage. The embryos produced can be
49 transferred fresh into the uterus of estrous-synchronized recipients or can be stored via
50 cryopreservation and subsequently transferred after thawing. These different steps are now
51 well established in domestic ruminant species (cattle, sheep, and goat) [3]. Nevertheless IVP
52 is still an inefficient process in ruminants because the proportion of embryos reaching the
53 transferable stage is rarely over 40% [4-6] and because the issue of abnormal pregnancies
54 involving IVP embryos has not been satisfactorily resolved [7]. These problems hold true in
55 IVP and embryo transfer in sheep, contributing heavily to limit the commercial application of
56 these techniques. According to the international embryo transfer society in 2012 890,875
57 bovine embryos – produced *in vivo* or fertilized *in vitro* – were transferred, whereas 12,458 *in*
58 *vivo* derived ovine embryos were transferred [8]. Modern ART, such as IVP, embryo
59 cryopreservation and embryo transfer, are not routinely applied in sheep breeding programs,
60 but are only used for experimental purposes [9]. Hence, more research is needed to
61 understand why, following transfer, IVP embryos perform considerably poorer than *in vivo*
62 derived embryos and have frequently been associated with fetal and neonatal abnormalities
63 [7].

64

65 The developing fetus is surrounded by the amniotic sac containing the amniotic fluid which
66 derives, in part, from (1) the transport of water and solutes across the placenta from the
67 endometrium and (2) uterine secretions [10]. The volume and composition of the amniotic
68 fluid are strictly regulated during gestation, reflecting the dynamic balance maintained through
69 exchange between maternal circulation and fetal environment [11, 12]. The fetus may actively

70 drink the amniotic fluid; it represents a reservoir of water and proteins that can influence fetal-
71 placental development and functions [10]. The amniotic fluid also contains amino acids (AAs),
72 which are the building blocks for biomolecules involved in physiologic growth and
73 development, and precursors of nitrogenous substances with varied regulatory functions [13].
74 There is now compelling evidence indicating that the disruption of placental development
75 during the initial pregnancy phases is involved in the developmental failure of embryos from
76 different species, as well as the abnormal phenotypes of pregnancies obtained by ART [7, 14,
77 15]. The present study was conducted to test the hypothesis that concentrations of AAs in the
78 amniotic fluids differ in pregnancies derived from vitrified/thawed (V/T) IVP embryos
79 compared to gestations obtained with natural mating (NM) in sheep.

80

81 **2. Materials and methods**

82

83 All animal experiments were performed in accordance with DPR 27/1/1992 (Animal Protection
84 regulations of Italy) in conformity with European Community regulation 86/609.

85

86 *2.1 Vitrified/Thawed (V/T) group*

87 *2.1.1 In vitro embryo production*

88 Ovaries of Sarda ewes were collected from a local slaughterhouse, oocytes were recovered,
89 and embryos were produced *in vitro* as previously described [16]. When not specified,
90 products were purchased from All chemicals, Sigma Chemical Co. (St. Louis, MO). Briefly,
91 adult (4-6 years old) ovine ovaries were transported to the laboratory within 1-2 h in Dulbecco
92 Phosphate Buffered Saline (PBS) with penicillin (100 µg/mL) and streptomycin (100 µg/mL).

93 Collection of cumulus-oocyte complexes (COCs) was performed in sterile Petri dishes
94 containing 20 mM Hepes-buffered TCM 199 supplemented with 0.1% (w/v) polyvinyl alcohol
95 and antibiotics (0.1 g/L penicillin and 0.1 g/L streptomycin). Only COCs showing several intact
96 cumulus cell layers and compacted cytoplasm were selected and matured *in vitro* in TCM 199
97 supplemented with 10% heat-treated estrus sheep serum, 0.1 IU/mL FSH and 0.1 IU/mL LH
98 (Pergonal, Serono, Italy) and 100 μ M cysteamine. Thirty to thirty-five COCs were cultured for
99 24 h in 5% CO₂ in air at 38.5°C in four-well petri dishes (Nunclon, Nalge Nunc International,
100 Denmark) with 600 μ L of maturation medium, layered with 300 μ L of mineral oil. *In*
101 *vitro* matured oocytes were fertilized in SOF medium with 2% heat-treated estrus sheep
102 serum for 22 h at 38.5°C and 5% CO₂, 5% O₂ and 90% N₂ atmosphere in four-well petri
103 dishes (Nunclon). Frozen-thawed spermatozoa obtained from pooled ejaculations of a single
104 ram (Sarda breed) selected by swim-up technique ($1 \cdot 10^6$ spermatozoa/mL) under mineral oil
105 were used for IVF. Presumptive zygotes were transferred separately and cultured for 7 days
106 in four well Petri dishes containing SOF + essential and non-essential amino acids at
107 oviductal concentration [17] + 0.4% bovine serum albumin (BSA) under mineral oil, in
108 maximum humidified atmosphere with 5% CO₂, 5% O₂, 90% N₂ at 39°C. The first cleavage
109 was registered between 24-26 h after the start of fertilization.

110

111 *2.1.2 Embryo vitrification and thawing*

112 Expanded blastocysts were recovered on the 7th day of culture and vitrified in Cryotop devices
113 (Kitazato Ltd., Tokyo, Japan) using the minimum essential volume method [18]. Briefly,
114 groups of 2/3 blastocysts were initially equilibrated for 1 min at room temperature in holding
115 medium (HM) consisting of 20 mM Hepes-buffered TCM-199 supplemented with 20% fetal

116 calf serum. Embryos were then incubated in HM supplemented with 7.5% ethylene glycol
117 (EG) and 7.5% dimethyl sulfoxide (DMSO) for 3 min and successively transferred into HM
118 with 16.5% EG, 16.5% DMSO and 0.25 M sucrose for 20 sec. Blastocysts were loaded on
119 Cryotop devices with a small volume of the latter solution (<2.0 μ L) and immediately plunged
120 into liquid nitrogen for storage. Embryos were thawed by placing Cryotops in HM with 1.25 M
121 sucrose for 1 min at room temperature. Recovered blastocysts were sequentially transferred
122 into HM with 0.5 M sucrose for 3 min, then into HM with 0.25 M sucrose for 3 min and in HM
123 for 1 h.

124

125 *2.1.3 Embryo transfer*

126 Eleven adult (3–5 years old) Sarda sheep were housed at the experimental facilities of the
127 Department of Veterinary Medicine at the University of Sassari (Italy) and fed a live-weight
128 maintenance ration throughout the experimental procedure. Estrous cycles were
129 synchronized using intravaginal progestagen impregnated sponges (45 mg fluorogestone
130 acetate sponges, Cronogest[®], Intervet S.r.l., Italy) for 14 days and a single administration of
131 333 IU/ewe of eCG (Folligon[®], Intervet S.r.l., Italy) on the day of the sponge removal. Twenty-
132 four hours after sponge removal a teaser ram was introduced in the flock to detect estrus.
133 Seven days after the onset of estrus, three vitrified/thawed blastocysts were surgically
134 transferred into each recipient via median laparotomy. All procedures were carried out under
135 epidural anesthesia (3.5 mg/kg, Lidocaina 2%[®], Pfizer, Italy). The recipients were secured in
136 a cradle in dorsal recumbency. The uterus was exposed throughout the laparotomic incision
137 and both ovaries were exteriorized in order to assure the presence of at least one active
138 corpus luteum. Embryos were transferred with as little as 0.1 mL medium (PBS–BSA) using a

139 plastic catheter inserted at the major curvature of the uterine horn on the same side of the
140 ovary bearing a corpus luteum. After surgery, a single dose of amoxicillin (12.5 mg/kg,
141 Longocillina^{L.A.}®, Ceva, Italy) was administered to the ewes. Pregnancy diagnosis was
142 performed 15 days after surgery by transabdominal ultrasonography (A6V®, Sonoscape Ltd.),
143 using a 9-4 MHz micro-convex probe and eleven ewes were identified as pregnant with a
144 single fetus. No twin pregnancies were identified. Amniocentesis was subsequently performed
145 on six ewes at Day 60-65 and on five ewes at Day 80-85 of gestation.

146

147 *2.2 Naturally mated group (NM)*

148 Adult (3–5 years old) Sarda ewes were housed in a commercial farm in the province of
149 Sassari (Italy) and fed a live-weight maintenance ration throughout the experiment. Estrous
150 cycles were synchronized as described above. Fertile rams of the same Sarda breed bearing
151 a marking harness were introduced in the flock for the breeding season and marked ewes
152 were identified as mated. Transabdominal ultrasonography was performed 21 days after
153 mating to identify ewes pregnant with a single fetus and was repeated on pregnant females
154 starting approximately 50 days after mating to estimate fetal age [19, 20]. Eleven ewes that
155 were 60-65 days pregnant and 14 ewes that were 80-85 days pregnant with a single fetus
156 were identified.

157

158 *2.3 Amniocentesis and amino acid analysis in the amniotic fluid*

159 To sample the amniotic fluid, on day 60-65 (V/T n = 6, NM n = 11) and on day 80-85 (V/T n =
160 5, NM n = 14) of gestation trans-abdominal ultrasound-guided amniocentesis was performed
161 (A6V[®], Sonoscape Ltd.) using a 9-4 MHz micro-convex probe. Ewes were secured in a cradle
162 in dorsal recumbency, shaved and the area between the head of the fetus and the membrane
163 of the amniotic sac was identified via ultrasonography as the site of puncture. The area was
164 accurately disinfected and the ultrasound transducer was covered with a sterile glove. A 22
165 ga sterile needle was placed on one side of the transducer and was inserted under direct
166 ultrasound visualization until the tip of the needle was at the center of the area between the
167 head of the fetus and the amniotic membrane. The needle was connected to an extension set
168 and a syringe and 10 mL of amniotic fluid were collected.

169 At term, ewes in both groups lambled single live healthy lambs whose weight was within range
170 for the Sarda breed (3-4 kg).

171

172 *2.4 High-pressure liquid chromatography*

173 Amniotic fluid samples were centrifuged at 4000 g for 10 min and the supernatant was stored
174 at -80°C for subsequent analysis by high-pressure liquid chromatography [21].

175 Concentrations of the following amino acids were measured in the amniotic fluid: alanine,
176 arginine, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine,
177 phenylalanine, proline, serine, threonine, tyrosine and valine. Amino acids concentrations
178 were determined using an Acquity ultra-performance liquid chromatography (UPLC) system
179 equipped with an Acquity UPLC Photodiode Array (PDA) eλ Detector (Waters Corporation,
180 Milford, MA, USA). Amino acids derivatization was performed using an AccQ-Tag Ultra

181 Derivatization Kit following the manufacturer's instructions (Waters Corporation, Milford, MA,
182 USA). Once derivatized, labeled AAs were separated on a 2.1 x 100 mm, 1.7 μ m AccQ-Tag
183 Ultra column and UV detected by the PDA detector set at 260 nm.

184

185 *2.5 Statistical analysis*

186 All results are expressed as mean \pm standard error of the means. Statistical analysis was
187 conducted with R-software version 3.03. The Shapiro-Wilk test of normality was performed.
188 Data were not normally distributed, hence data were \log_{10} transformed before analysis.
189 Analysis of variance was used to test whether total and individual concentrations of amino
190 acids differed between groups and day of gestation. Statistical significance was set at a
191 probability value of 0.05 or less.

192

193 **3. Results**

194

195 At day 60-65 of gestation the total amino acid concentration per ewe was similar between
196 groups (Fig 1), but the mean concentrations of cystine, phenylalanine and isoleucine were
197 lower in V/T compared to NM ewes (Fig 2a). The mean total amino acid concentration
198 decreased by 55% from day 60-65 to 80-85 of gestation in all ewes, but the decrease was
199 greater in IVP embryo transferred compared to naturally mated ewes (65% vs 52%
200 respectively; Fig 1). At day 80-85 of pregnancy no difference was detected between groups in
201 the total amino acid concentration (Fig 1), whereas the mean concentrations of cystine and
202 lysine (Fig 2b) were lower in the V/T vs the NM ewes (Fig 2b). At day 80-85 phenylalanine,
203 isoleucine, methionine, tyrosine, histidine were undetectable in four V/T ewes, hence these

204 amino acids were excluded from the analysis. The most abundant amino acid at day 60-65 of
205 gestation was alanine in both groups, whereas at day 80-85 the most abundant amino acids
206 were alanine in NM and glycine in V/T ewes respectively; cystine was the less abundant
207 detectable amino acid in all ewes at both time points (Fig 2).

208

209 **4. Discussion**

210

211 This study reports lower concentrations of individual AAs in the amniotic fluid of pregnancies
212 achieved after the transfer of cryopreserved IVP embryos compared to gestations obtained
213 with natural mating. Individual concentrations of the majority of AAs detected were lower in
214 V/T vs NM. However, a statistically significant difference was not detected in all comparisons;
215 this could be due to the limited number of samples.

216

217 The amniotic fluid is composed of water and electrolytes from both the fetus (kidneys, lungs,
218 epidermis and fetal blood vessels in the placenta and umbilical cord) and the mother
219 (decidual blood vessels via amniotic membranes) [10]. The AAs in the amniotic fluid mainly
220 derive from maternal and fetal circulation through the placenta [22, 23]; they are transported
221 across the placenta by active transport mediated by numerous different amino acid
222 transporters [24]. In the present study the lower concentration of individual AAs in the
223 amniotic fluid of V/T pregnancies could be caused by an impaired placental vascularization.
224 We speculate this because in a recent study a severe impairment vasculogenesis early in
225 gestation was observed in pregnancies obtained through IVP from oocytes of FSH-treated
226 ewes, as assessed by a reduction in the number and diameter of placental vessels and an

227 underdevelopment of the cardiovascular systems in IVP vs naturally conceived conceptuses
228 [25]. Reduced placental vascularization and expression of angiogenic factors in fetal
229 membranes were also detected early in pregnancies after transfer of embryos obtained with
230 *in vitro* fertilization [15]. In our study, the transfer of V/T embryos may have impaired placental
231 perfusion and consequently the passage of AAs from maternal circulation to fetal fluids.

232

233 Another potential cause of the lower amniotic individual AAs concentrations in V/T
234 pregnancies may be the reduction of the AAs transport systems in the placenta. Compelling
235 evidence in humans and sheep shows that in intra-uterine growth restricted pregnancies
236 placental transport of essential AAs, such as leucine, lysine and threonine, is reduced
237 because a number of placental transport systems are down-regulated [26, 27]. Similarly, a
238 reduction in the AAs transport systems may have caused the low amniotic individual AAs
239 concentrations in V/T gestations reported in the present study. Amino acid transport systems
240 are either highly specific or have a broad specificity for a group of AAs [28]. For example, one
241 transporter can transfer a certain kind of AA (e.g., system A can transfer neutral amino acids,
242 like alanine, serine, proline and glutamine) [29]. In contrast, other transport systems have
243 overlapping substrate specificity. Nevertheless, the fact that in this study the concentrations of
244 different AAs were reduced suggests a broad effect on different transport systems rather than
245 the down-regulation of a single one.

246

247 The AAs that were reduced in V/T pregnancies compared to controls were isoleucine,
248 phenylalanine, lysine and cystine. Isoleucine is a branched-chain AA, such as leucine and
249 valine; they have a positive effect on protein synthesis in skeletal muscle [30] and donate the

250 amino group for synthesis of glutamine in mammals, which plays a prominent role in fetal
251 carbon and nitrogen metabolism [31]. Phenylalanine is an aromatic AA together with tyrosine,
252 histidine, and tryptophan. They are precursors of neurotransmitters, regulating neurological
253 functions during embryonic and fetal development and alterations of these AAs are proposed
254 to exacerbate neuronal viability [32]. Interestingly all aromatic AAs analyzed were
255 undetectable at day 80-85 of gestation in the majority of V/T ewes. Cystine is one of the
256 precursors of glutathione and its supply is a rate-limiting factor in its synthesis in humans [33];
257 glutathione is vital for antioxidant reactions and is important in the regulation of immune
258 functions [34]. Lysine is widely supplemented in animal feed, especially for monogastric
259 animals like broilers, poultry and swine and as a supplement for humans, to improve the feed
260 quality by increasing the absorption of other amino acids [35]. Taken together these findings
261 suggest that pregnancies obtained from V/T embryos may have low availability of important
262 AAs and this could in turn impair conceptus development, having long term consequences on
263 the newborn.

264

265 All gestations in this study ended with the birth of a healthy lamb whose weight was within the
266 normal range for the Sarda breed. This may mean either that the decrease in individual
267 amniotic AAs levels was not severe enough to cause pregnancy complications or that
268 concentrations of AAs may have increased later in gestation. Also, we cannot exclude that
269 non-viable pregnancies from V/T may have even lower amniotic AAs concentrations. For
270 example, in a recent study amniotic AA levels were different in non-viable pregnancies
271 achieved after somatic cell nuclear transfer compared to IVP gestations, but IVF and viable
272 SCNT pregnancies had similar amniotic AA concentrations [12]. Thus, measuring amniotic

273 AAs concentrations in pregnancies with placental and fetal developmental defects that often
274 result from the transfer of IVP embryos [7] may contribute to better understand placental
275 transport of AAs in IVP pregnancies. Similarly the comparison of amniotic AAs concentrations
276 in pregnancies resulting from the transfer of fresh IVP embryos vs cryopreserved IVP
277 blastocyst merits further research to distinguish potential detrimental effects of blastocyst
278 cryopreservation. The stage of pregnancy at which amniocentesis is performed is also
279 important. We selected day 60-65 and 80-85 of gestation because amniocentesis can be
280 performed safely; placental development is completed approximately by day 60 of gestation
281 in sheep [36], but after the third month fetal movements increase the risk of fetal/membranes
282 damages.

283

284 Different sires were used to produce embryos *in vitro* and for natural mating. Since the sire
285 contributes to the development of the conceptus and of fetal membranes, we cannot exclude
286 that this may have influenced our results. Nevertheless, a sire effect would have been
287 relevant if a single female had been used for oocyte retrieval and natural mating, whereas in
288 our study, oocytes were collected from ovaries of several different females and other ewes
289 were used to obtain pregnancies with natural mating. The validity of this model is confirmed
290 by the fact that it was used in previously mentioned studies; placental vasculogenesis and
291 epigenetic regulation of gene expression during placentogenesis were compared in
292 pregnancies obtained from transfer of *in vitro* produced embryos vs natural mating using
293 Sarda breed sheep and different rams for IVP and natural mating [14, 25]. Also, only Sarda
294 sheep were used in our study and rams were all registered in the official breed herd book.
295 Weight at birth in this breed is quite constant [37], hence it is highly unlikely that the use of

296 different rams influenced fetal and placental development, and consequently amniotic AA
297 concentration.

298

299 Alanine, glycine, glutamic acid and serine were the most abundant AAs in both experimental
300 groups, in accordance with what was reported in naturally mated ewes [11] and in IVP bovine
301 pregnancies [12]. Also, the dramatic twofold decrease in amniotic AA concentration observed
302 from day 60 to 80 of gestation is similar to what was previously reported in naturally mated
303 ewes [11], but our data show a more pronounced, albeit not significant, decrease in V/T
304 pregnancies compared to controls. This intriguing finding indicates that the impairment in
305 placental transport of AAs and consequently their availability may further worsen as gestation
306 progresses.

307 In conclusion, results demonstrate that V/T IVP embryos have decreased concentrations of
308 individual AAs in the amniotic fluid during the second trimester of gestation, possibly because
309 of an impaired placental vasculogenesis or of a reduced placental transport. These findings
310 provide the basis for future studies to elucidate mechanisms responsible for the large range of
311 developmental abnormalities associated with the transfer of IVP and cryopreserved embryos.

312

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316

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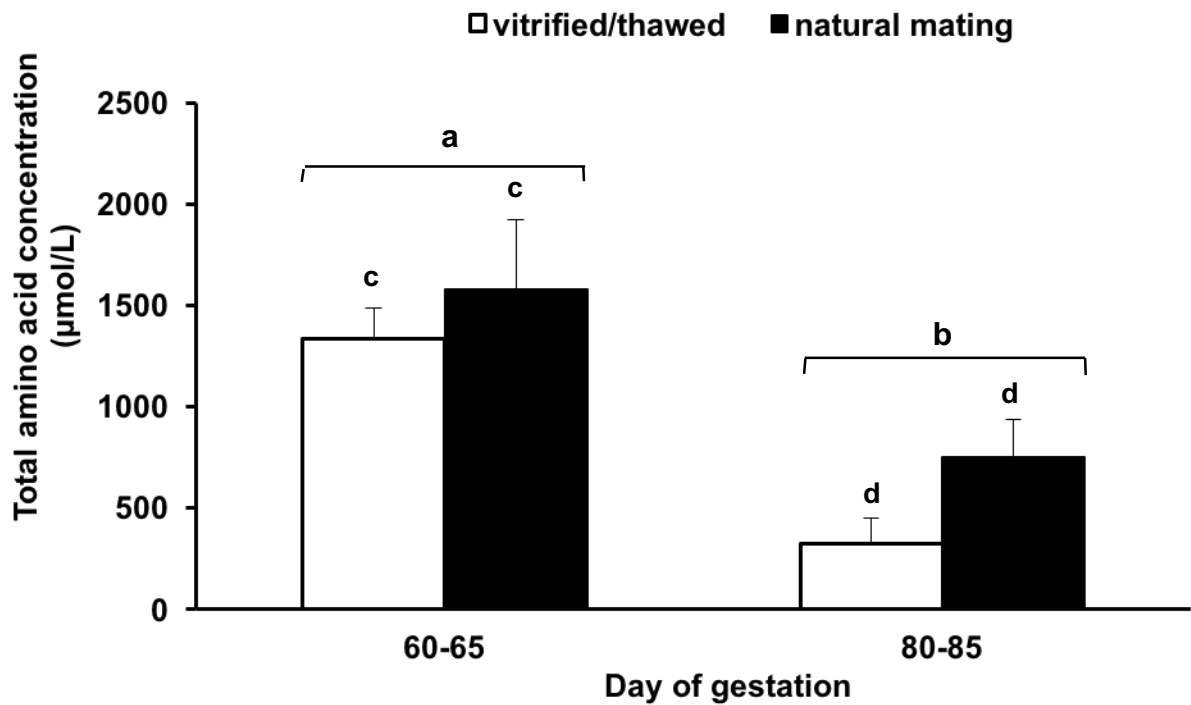
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Fig. 1. Total amino acid concentration per ewe in the amniotic fluid of ewes that were naturally mated (NM) or embryo transferred with IVP vitrified/thawed embryos (V/T) at Day 60-65 (V/T = 6; NM = 11) and Day 80-85 (V/T = 5; NM = 14) of gestation. Data are expressed as means \pm SEM. Different letters indicate statistical difference (a vs b $P < 0.001$; c vs d $P < 0.05$).

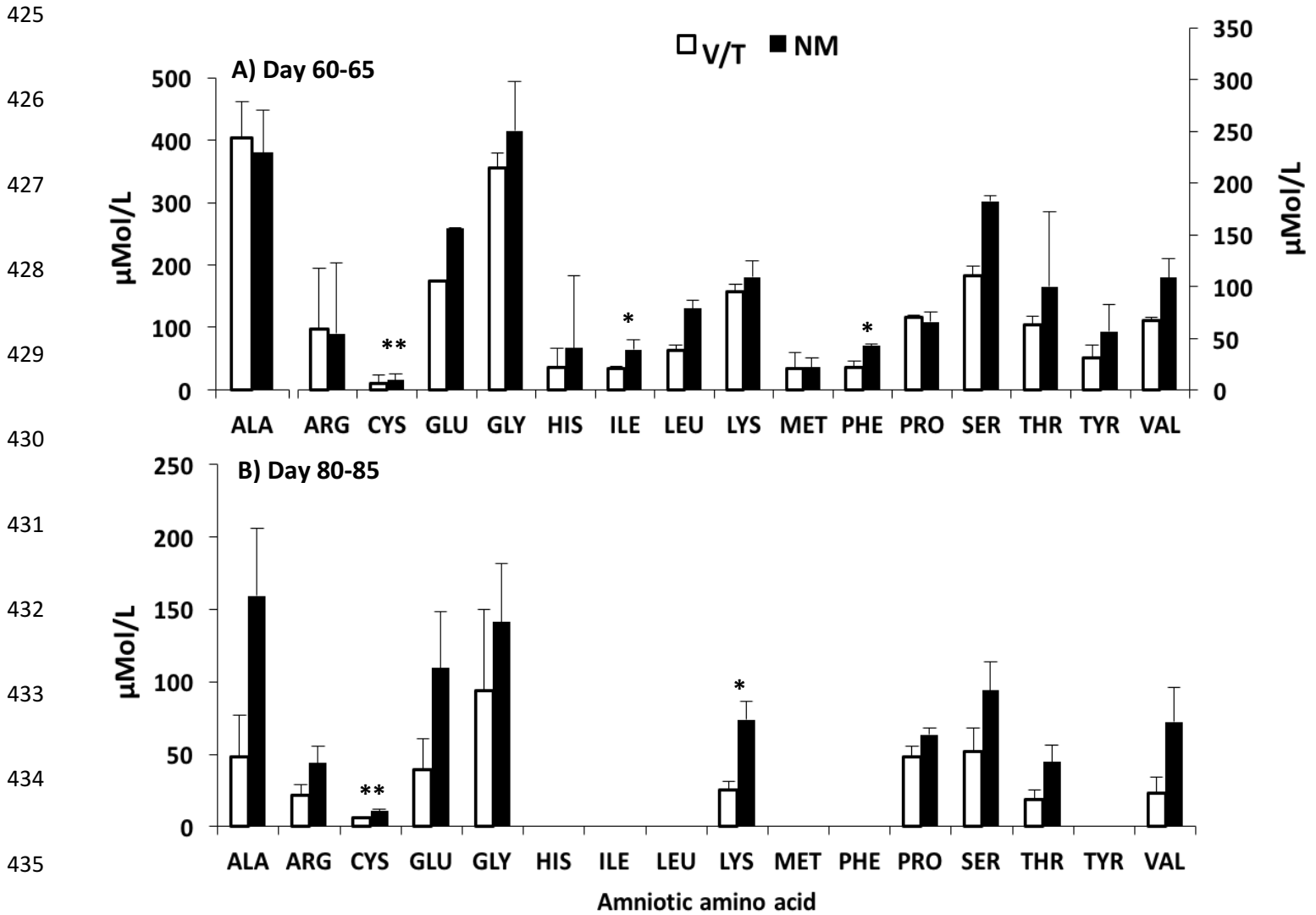


Fig. 2. Concentrations of single amino acids in the amniotic fluid in ewes that were naturally mated (NM) or embryo transferred with *in vitro* produced vitrified/thawed embryos (V/T) at day 60-65 of gestation (Panel A; V/T = 6, NM n = 11) and at day 80-85 of gestation (Panel B; V/T = 5, NM n = 14). Data are expressed as means \pm SEM. Different superscripts indicate statistical differences between groups within the same panel (* P<0.05; ** P<0.03). Abbreviations: ALA=alanine; ARG=arginine; CYS=cystine; GLU=glutamic acid; GLY=glycine; HIS=histidine; ILE=isoleucine; LEU=leucine; LYS=lysine; MET= methionine;

444 PHE=phenylalanine; PRO= proline; SER= serine; THR=threonine; TYR= tyrosine; VAL=
445 valine.