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Post-ruminal infusions of amino acids or glucose affect splanchnic, mammary and other peripheral tissue metabolisms and drive amino acid usage in dairy cows

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1 **Infusions of amino acids and glucose affect coordination of splanchnic, mammary and**
2 **other peripheral tissue amino acid metabolisms.** *By Omphalius et al.* In dairy cows, better
3 knowledge of utilization of amino acids to synthesize all proteins by different tissues is required
4 to improve the efficiency of utilization of protein, i.e., the proportion of digested proteins
5 recovered in exported proteins. Various dietary protein and energy conditions changed the
6 coordination of different tissues involved in the use of amino acids suggesting that amino acid
7 fate within each tissue should be considered to better predict the efficiency of use of amino
8 acids.

Running head

SPLANCHNIC AND MAMMARY USAGE OF AMINO ACIDS

14 **Post-ruminal infusions of amino acids or glucose affect splanchnic, mammary and other**
15 **peripheral tissue metabolisms and drive amino acid usage in dairy cows**

17 **C. Omphalius,^{*†} S. Lemosquet,^{*} D.R. Ouellet,[‡] L. Bahloul,[†] and H. Lapierre^{‡1}**

19 ^{*}PEGASE, INRA, AGROCAMPUS OUEST, 35590 Saint Gilles, France

20 [†]Adisseo France S.A.S., 10, Place du General de Gaulle, 92160 Antony, France

21 [‡]Agriculture and Agri-Food Canada, Sherbrooke, QC, Canada J1M 0C8

22 ¹Corresponding author: H el ene Lapierre, Agriculture and Agri-Food Canada, 2000 College,
23 Sherbrooke, QC, Canada J1M 0C8, Tel: (819) 780-7234, Email: helene.lapierre@canada.ca

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ABSTRACT

Effects of AA and glucose infusions on efficiency of utilization of essential AA (EAA) were studied according to a 2×2 factorial using 5 multicatherized cows in a 4×4 Latin Square plus one cow with 2-wk periods. The diet provided 87% of energy and 70% of metabolizable protein requirements and the 4 treatments were abomasal infusions of 1) water, 2) AA mixture with a casein profile (695 g/d), 3) glucose (1454 g/d) or 4) a combination of AA and glucose infusions. Milk samples were collected on the last 6 milkings. On day 14, 6 blood samples were collected from arterial, and portal, hepatic and mammary venous vessels. Splanchnic plasma flow was calculated by dilution of p-aminohippurate and mammary flow by the Fick principle using Phe+Tyr. The net flux of AA across tissues [splanchnic, i.e., portal drained viscera (PDV) + liver, and mammary gland] was calculated as the efflux minus the influx across that tissue. The efficiency of EAA was calculated as the sum of exported true proteins [milk protein yield (MPY), scurf and metabolic fecal protein (MFP)] multiplied by their respective AA profile and divided by the predicted AA supply minus AA endogenous urinary loss. In addition, catabolism was estimated for each tissue: AA supply - (portal net flux + MFP) for the PDV; -hepatic net flux for the liver; splanchnic net flux - (-mammary net flux + scurf) for the other peripheral tissues; and -mammary net flux - milk for the mammary gland. The MIXED procedure (SAS) was used with cow as random effect. There was no AA × glucose interaction on most of the measured parameters. With infusions of AA and glucose, MPY increased by 17 and 14% respectively. The decreased efficiency of EAA-N with AA infusion resulted from increased EAA-N in MPY smaller than the increased EAA-N supply and was accompanied by increased liver catabolism of His+Met+Phe (representing Group 1 AA) and increased mammary and PDV catabolisms of Group 2 AA-N (Ile, Leu, Lys and Val). In contrast, the increased efficiency of EAA-N with glucose infusion, resulting from increased EAA-N in MPY with no change in EAA-N supply, was accompanied by decreased mammary catabolism of Group 2 AA-N and

50 hepatic catabolism of His+Met+Phe. There was no mammary catabolism of His, Met and Phe
51 in all treatments, as indicated by the mammary uptake to milk output ratio close to 1 for these
52 EAA. Therefore, the mammary gland contributes significantly to variations of efficiency of
53 Group 2 AA-N through variations of AA catabolism, in response to both AA and glucose
54 supplies, whereas additional PDV catabolism was observed with increased AA supply. Partition
55 of AA usage between tissues allows to delineate their anabolic or catabolic fate across tissues
56 and better understand changes of efficiency of EAA in response to protein and energy supplies.

57
58 **Key Words:** protein, energy, net flux, catabolism, efficiency

60 INTRODUCTION

61 To face the challenge of increasing dietary N efficiency (milk N/N intake) in lactating
62 dairy cows without a detrimental effect on milk true protein yield (**MPY**), a number of options
63 have already been identified. Balancing diets for AA (Haque et al., 2012 and 2015; Lee et al.,
64 2012), increasing NE_L supply (Raggio et al., 2006b; Rius et al., 2010b) or changing the source
65 of energy (Cantalapiedra-Hijar et al., 2014b; Nichols, 2019) increased the efficiency of
66 utilization of MP, referred to as efficiency through the text. Although variations of efficiency
67 of MP under various nutritional conditions have been characterized, variations of efficiency of
68 individual EAA have not been yet thoroughly examined in response to changes of energy
69 supply, combined or not with alteration of AA supply. A meta-analysis reported a decreased
70 efficiency of EAA with increased AA supply with variations of efficiency of EAA amongst AA
71 (Doepel et al., 2004). Similarly, increasing Lys or Met supply by feeding a rumen-protected
72 form, decreased their respective efficiency of utilization for milk protein secretion (Lee et al.,
73 2015). Nevertheless, effect of NE_L or the interaction between NE_L and AA supplies on

74 efficiency of EAA need to be determined in order to better balance dairy rations for individual
75 EAA.

76 Indeed, NE_L supply may affect AA metabolism due to the energy cost of protein
77 synthesis (Lobley, 1990). It was also proposed that energy supply might affect the AA
78 utilization for gluconeogenesis (Cant et al., 1993). In fact, post-rumen infusion of glucose or
79 rumen infusion of propionate increased efficiency of Group 1 AA (i.e., His, Met, Phe and Tyr)
80 with increased mammary uptake and secretion in milk protein and no change in AA supply
81 (Lemosquet et al., 2010a). In dairy cows fed 3 levels of MP, efficiency of EAA, calculated as
82 the ratio of AA in MPY relative to net portal absorption, decreased with increased MP supply
83 (Raggio et al., 2004). For Met, Phe and Tyr, the decreased efficiency was linked to increased
84 hepatic removal whereas for Group 2 AA (i.e., Ile, Leu, Lys and Val), it was related to an
85 increased excess of mammary uptake relative to MPY, indicating an increased mammary use
86 of these AA not linked to their direct incorporation into MPY. Moreover, abomasal glucose
87 infusion (Huhtanen et al., 2002; Nichols et al., 2016) decreased branched-chain AA (BCAA)
88 plasma concentration with no effect on their mammary uptake, suggesting increased utilization
89 of the BCAA by other peripheral tissues such as muscle or splanchnic tissues, or both.

90 These observations indicate a different partition of AA utilization between tissues and
91 an alteration between anabolic or catabolic fate within tissue in response to changes in protein
92 or energy supplies. This raises the following questions: how altering supplies of AA or energy
93 (through glucose infusion), or both, affects the coordination of AA utilization by splanchnic,
94 mammary, and other peripheral tissues for anabolic or catabolic purposes and how these
95 variations affect whole body efficiency of EAA? We hypothesized that the decreased efficiency
96 of EAA observed with increased AA supply and increased efficiency of EAA with glucose
97 supply could be explained through different mechanisms, affecting differently the partition of
98 groups of AA between tissues and the partition between anabolism and catabolism within tissue.

99 To our knowledge, there is no studies yet reporting the effect of variations of levels of protein
100 and energy supplies on splanchnic and mammary net fluxes of AA and relating these measured
101 fluxes to predicted AA supply in lactating dairy cows. Consequently, this knowledge on the
102 utilization (export or catabolism) of AA by various tissues under different nutritional conditions
103 will help to determine factors affecting the efficiency of EAA and therefore improve prediction
104 of individual efficiency of EAA. This information could be used in feeding systems to either
105 improve, for a given diet, the prediction of MPY or to identify which AA are potentially in short
106 supply.

108 MATERIALS AND METHODS

110 *Cows and Surgery*

111 The trial was carried out at the Sherbrooke Research and Development Center of
112 Agriculture and Agri-Food Canada (QC, Canada). Five Holstein cows in second lactation had
113 been fitted before calving with a rumen cannula (Galindo et al., 2011). Approximately 8 wk
114 after calving, the cows were surgically implanted with catheters: 1 in the abomasum (Doepel et
115 al., 2006), 1 in a mesenteric artery, 2 in mesenteric veins, 1 in the portal vein and 1 in an hepatic
116 vein (Huntington et al., 1989). During the surgery, the right carotid artery was also raised to a
117 subcutaneous position to provide access to arterial blood if necessary.

118 At the initiation of the study, the cows averaged 158 ± 13 DIM and 655 ± 53 kg BW.
119 Before and throughout the study, cows were housed in individual tie stalls and had access to
120 fresh water. The experimental protocol was approved by the Institutional Animal Care
121 Committee of the Sherbrooke Research and Development Center and animals were treated
122 according to the Canadian Council on Animal Care guidelines (1993).

123

124 *Experimental Design, Treatments, Feeding and Milking*

125 Throughout the study, cows received the same experimental diet (Table 1). Based on
126 the cows' performance the week prior to the initiation of the project (30.8 kg milk/d at 3.2%
127 CP and 3.7% fat and an average DMI of 18.0 kg/d), the experimental diet was designed to
128 provide 87% of NE_L and 70% of MP requirements (NRC, 2001). Two factors, infusion of AA
129 or infusion of glucose, were tested in a 2×2 factorial arrangement. The 4 treatments were
130 continuous abomasal infusion of 1) water (**Ctrl**), 2) AA mixture with a casein profile (**AA_{CN}**;
131 695 g/d, Table 2), 3) glucose (**E_{Glc}**; 1600 g/d of glucose monohydrate, yielding 1454 g/d of
132 glucose) and 4) a combination of AA mixture and glucose at the same doses (**AA_{CN} + E_{Glc}**).
133 The rates of infusion of AA or glucose were selected in order that the diet plus the infusions
134 would meet MP or NE_L requirements, respectively (NRC, 2001). The cows received the 4
135 treatments in a 4×4 Latin Square design, balanced for residual effect, plus one cow, with 2-
136 wk experimental periods. The 4 treatments were delivered (6 kg/d) into the abomasum by means
137 of peristaltic pumps. The AA solutions were diluted daily from a stock solution prepared every
138 2 or 3 d whereas the glucose solutions were prepared daily. Two cows received treatments
139 through an abomasal tube installed via the rumen cannula (Gressley et al., 2006) because the
140 abomasal catheters did not function well.

141 To minimize refusals, cows were offered 98% of ad libitum intake measured the week
142 prior to the initiation of the project. The quantity of the ration offered and orts (when present)
143 was weighed every day. To minimize post-prandial variations of metabolite concentrations and
144 trans-organ fluxes, feed was supplied every 2 h in equal portions on even hours from automated
145 feeders to cows milked twice daily at 0630 h and 1830 h. Milk production was recorded at each
146 milking.

147

148 *Sampling and Laboratory Analyses*

149 **Feed and Milk.** The concentrate was made in one batch prior to the initiation of the trial.
150 Samples of ingredients used for the concentrate were then collected. Samples of dry hay and
151 concentrate were collected every week to determine the DM concentration, by drying at 80°C
152 for 48 h and pooled by period to be analyzed for chemical composition: CP, ADF, NDF, lignin
153 as described by Martineau et al. (2007). The analyses of the pooled samples of concentrate were
154 used to confirm the values obtained for the raw material and values for individual feed
155 ingredients were used to calculate the feed value of the diet (NRC, 2001). Milk samples were
156 collected on the last 6 milkings of each period and analyzed for total N, NPN and non-casein N
157 according to Raggio et al. (2004).

158 **Blood.** On last day of each period, from 0800 to the end of the sampling period, sodium
159 p-aminohippurate (**pAH**; 100 g/L) was continuously infused using a syringe pump into one
160 mesenteric vein, at 12 g/h, to determine splanchnic plasma flow by dilution. Six sets of blood
161 samples were collected at 0900, 0940, 1020, 1145, 1225 and 1305 h from each cow
162 simultaneously from arterial, portal and hepatic venous catheters. Right after each set of
163 splanchnic sampling, a blood sample was taken from one mammary vein by venipuncture,
164 alternating right and left side at each sampling. Blood was immediately put on ice and
165 centrifuged (12 min, 1,800 × g) to yield plasma. Fresh plasma sub-samples were immediately
166 used to determine urea-N concentrations, using the diacetyl monoxime method on an automatic
167 analyzer (Technicon Autoanalyzer II, Technicon Instruments Corporation, NY) according to
168 Huntington (1984). In addition, 1 g of fresh plasma was added to 0.2 g of an internal standard
169 solution of labelled AA, as described in Doepel and Lapierre (2011). The processed samples
170 were stored at -80°C until analyzed for AA concentrations, determined by isotope dilution using
171 gas chromatography/mass spectrometry (model GC 6890-MS 5973, Agilent Technologies,
172 Wilmington, DE) as previously described by Calder et al. (1999) and Doepel and Lapierre
173 (2011). The remainder of the plasma was stored at -20°C. Concentrations of pAH on arterial,

174 portal and hepatic samples, were analyzed after deacetylation of pAH as described by Lobley
175 et al. (1995).

176

177 *Calculations and Statistical Analyses*

178 **Calculations.** The dietary EAA supply was calculated from the flow of digestible EAA
179 predicted by NRC (2001) multiplied by correction factors to take into account the incomplete
180 recovery of AA after 24-h hydrolysis of a protein (Lapierre et al., 2019; Table 3). Moreover,
181 we removed from the predicted MP supply and the corrected digestible flow of individual EAA
182 the contribution from duodenal endogenous flow, included in the predicted MP supply and AA
183 digestible flows (NRC, 2001). The contribution of the endogenous duodenal flow to MP supply
184 or AA digestible flow was estimated as: $96.1 + 7.45 \times \text{DMI}_{\text{kg/d}}$ (g CP/d; Lapierre et al., 2016)
185 multiplied by 0.8 to account for intestinal digestibility (NRC, 2001); this flow was then either
186 multiplied by the ratio of TP/CP of gut endogenous proteins of 0.73 (Lapierre et al., 2019b) or
187 multiplied by its AA composition (Table 3). Therefore, total supply of individual EAA (diet +
188 infusion, Table 4) was the sum of the predicted digestible flow corrected for incomplete
189 recovery with 24-h hydrolysis minus duodenal endogenous contribution plus the rate of AA
190 infusion when applicable; the digestibility of infused AA was assumed to be 100%. The supply
191 of EAA was expressed in their hydrated form. Because the sum of the hydrated AA supply is
192 roughly 15% higher than when expressed as protein, as each AA is losing one molecule of water
193 for each peptide bond during protein synthesis, total MP supply (Table 4) was calculated as
194 predicted MP supply (NRC, 2001) minus duodenal endogenous contribution to MP, as detailed
195 above, plus the rate of AA infusion (expressed in their anhydrous form, equivalent to 600 g/d
196 of MP). From herein to the end of the text, supply of MP or AA digestible flow will refer to
197 their respective supply corrected as defined above. The NEAA supply, not predicted by NRC
198 (2001), was calculated by difference between MP supply expressed in hydrated form of AA,

199 i.e., $MP \times 1.15$, and total EAA supply. The total NE_L supply (diet + infusion, Table 4) was
200 estimated by adding dietary NE_L supply predicted by NRC (2001) and NE_L from infusion of
201 Glc [2.75 Mcal/kg of infused glucose (Armstrong et al., 1961) = 4.0 Mcal/d]. Using INRA
202 feeding system (2018), the experimental diet supplied 84 ± 0.4 g/kg DM of protein digestible
203 in small intestine (PDI, equivalent to MP) and 1.58 ± 0.01 Mcal/kg DM of NE_L .

204 The AA composition of the milk protein (Table 3) was estimated for each cow \times period
205 from the measured proportions of CN and whey proteins relative to true protein (TP), using the
206 AA composition of each family of protein reported by Farrell et al. (2004). Adapted from
207 Lapierre et al. (2019b), the following milk protein fractions were assumed to be constant (%
208 TP): lactoferrin 0.21%, albumin 1.04%, IgG1 1.64%, IgGA 0.04%, IgG2 0.18% and IgM
209 0.33%. Total CN and whey protein fractions (% of TP) measured for each cow \times period were
210 used assuming 1) the following CN distribution (% of CN): 42.7% α_{S1} -casein, 9.2% α_{S2} -casein,
211 37.5% β -casein and 10.5% κ -casein and 2) the following distribution within the remaining whey
212 protein once the fixed contribution of lactoferrin + albumin + Ig was excluded: 72.8% α -
213 lactalbumin and 27.2% β -lactoglobulin. Blood-borne proteins (BBP) were the sum of albumin
214 and Ig and represented 3.23% of milk TP. Milk protein output was estimated using MPY
215 measured during the last 6 milkings of each period and the AA composition calculated as
216 previously described. The AA composition of milk used to calculate mammary plasma flow
217 excluded BBP to consider only proteins synthesized in the mammary gland whereas the AA
218 composition of milk used to calculate whole body efficiency included all protein fractions
219 (Table 3).

220 Mammary plasma flow was determined using the Fick principle based on Phe + Tyr as
221 internal markers (Cant et al., 1993), assuming no mammary oxidation of Phe + Tyr (Lemosquet
222 et al., 2010b). Portal and total splanchnic plasma flows were calculated as infusion rate of pAH
223 divided by average portal- or hepatic-arterial concentration difference of pAH. Arterial hepatic

224 plasma flow was calculated as splanchnic minus portal plasma flow. Hepatic influx of AA was
 225 calculated as (portal plasma flow × portal concentration) + (arterial plasma flow × arterial
 226 concentration). The net fluxes of AA metabolites across portal drained-viscera (**PDV**),
 227 splanchnic tissues and mammary gland were calculated by multiplying the average veno-
 228 arterial plasma concentration difference by their respective plasma flow. The net flux of urea
 229 was calculated similarly but using plasma water concentration [plasma concentration / (1 – DM
 230 of plasma)] and blood water flow, assuming 7% and 12% DM in plasma and blood,
 231 respectively, as previously described (Doepel et al., 2007). The hepatic net flux was calculated
 232 as splanchnic minus portal net flux. Negative values of net flux indicate net removal whereas
 233 positive values indicate net release across the studied tissue. When a net removal was observed
 234 across the liver or the mammary gland, fractional removal was calculated as – (net flux divided
 235 by total influx).

236 Whole body efficiency of MP assigned the same efficiency to all proteins exported,
 237 excluding non-protein endogenous urinary losses (**EUL**) to which an efficiency of 1 is assigned
 238 (Lapierre et al., 2019b; INRA 2018). The efficiency of MP was therefore estimated as follows:

$$239 \quad \text{efficiency of MP} = \frac{\text{MPY} + \text{MFP} + \text{Scurf}}{\text{MPsupply} - \text{EUL}}$$

240 where MP supply is as previously detailed, MPY was observed and metabolic fecal protein
 241 (**MFP**), scurf proteins and EUL were calculated as described in Lapierre et al. (2019b).

242 Individual EAA efficiencies were calculated following the same pattern:

$$243 \quad \text{efficiency of EAA} = \frac{\text{MPY} \times [\text{EAA}]_{\text{milk}} + \text{MFP} \times [\text{EAA}]_{\text{MFP}} + \text{Scurf} \times [\text{EAA}]_{\text{Scurf}}}{\text{EAA digestible flow} - \text{EUL} \times [\text{EAA}]_{\text{EUL}}}$$

244 where MPY, MFP, and scurf are as described for efficiency of MP and AA composition as
 245 detailed in Lapierre et al. (2019b) except for milk which was calculated as described above
 246 (Table 3). Both equations assume no protein accretion in conceptus or other tissues. For EUL,
 247 briefly, the EAA export is related only to endogenous urea excretion using AA composition of

248 empty body, plus 3-methyl-His excretion added for His, and creatinine and creatine excretion
 249 added for Arg. The NEAA-N in protein exported were estimated as the difference between TP
 250 $\times 1.15$ and EAA-N exported and the efficiency of NEAA-N was estimated as the ratio of NEAA-
 251 N in proteins exported and NEAA-N supply previously described minus NEAA-N in EUL.

252 Potential catabolism of MP and groups of AA by different tissues were calculated based
 253 on predicted supply, proteins or AA exported (observed or predicted) and measured net fluxes
 254 across tissues. First, the estimated digestible flow not used to support MFP and not recovered
 255 into portal circulation was considered as catabolism by PDV. Therefore,

256
$$\text{PDV catabolism} = \text{digestible flow} - (\text{portal net flux} + \text{MFP}).$$

257 Then, the hepatic net removal was considered as catabolism when values were negative.
 258 Although it is recognized that part of the hepatic removal of AA is used for the synthesis of
 259 liver export proteins (Raggio et al., 2007) and therefore not catabolized, the equivalent of this
 260 synthesis must be catabolized somewhere or returned to the liver as peptides. However very
 261 limited data are available on the degradation and use of plasma proteins; therefore, we decided
 262 to include their catabolism in hepatic catabolism.

263 Post-liver supply of AA not captured by the mammary gland or secreted into scurf
 264 proteins was considered as potential catabolism by other peripheral tissues. Therefore,

265
$$\text{other peripheral tissue catabolism} = \text{splanchnic net flux} - (-\text{mammary net flux} + \text{scurf}).$$

266 And finally, the mammary uptake not secreted into milk protein was considered as
 267 mammary catabolism. Therefore, $\text{mammary catabolism} = -\text{mammary net flux} - \text{milk AA}.$

268 **Statistical Analyses.** The average intake and milk production and composition data from
 269 the last 3 d of each period were used for the statistical analyses whereas the daily means of the
 270 6 blood samples collected on the last day were used for plasma concentrations and net flux data.
 271 One cow had vaginal infection during her third period (E_{Glc} treatment) and consequently data
 272 from this cow were deleted for this period. Therefore, $n = 5$ for Ctrl, AA_{CN} and $AA_{\text{CN}} + E_{\text{Glc}}$

273 and $n = 4$ for E_{Glc} . The data were analyzed using the MIXED procedure of SAS (v 9.4, SAS
274 Institute Inc., 2004) according to the following statistical model:

$$275 \quad Y_{ijkl} = \mu + cow_i + period_j + AA_k + Glc_l + (AA \times Glc)_{kl} + \varepsilon_{ijkl}$$

276 where μ was the mean and Y_{ijkl} was the variable dependent on the fixed effects of period_j, AA_k
277 (effect of AA infusion), Glc_l (effect of glucose infusion) and their interaction, ε_{ijkl} was the
278 residual error which associated $ijkl$ observations. Cow was treated as random effect. The results
279 are expressed as least squares means with the highest standard error of the means reported.
280 Homogeneity of residues was verified. The significance level was set to $P \leq 0.05$ and the
281 tendency to $0.05 < P \leq 0.10$. When the AA and glucose interaction (**AA × Glc**) was significant,
282 the SLICE procedures of SAS (v 9.4, SAS Institute Inc., 2004) was used to test the effect of
283 one factor within the other factor. Student's t-test were used to determine if efficiency of MP,
284 efficiency of EAA and uptake to milk output ratios were different from 1 and catabolisms
285 different from 0. In addition, we tested if the efficiencies were different between EAA,
286 including the factor "EAA" as a repeated measurement. Because the interaction treatment ×
287 EAA was highly significant ($P < 0.001$), the effect of EAA was tested within each treatment
288 and means separated using adjusted Tukey test.

289

290

RESULTS

291

292 ***DMI, Energy and Protein Supplies***

293 The DMI was not affected by treatments ($P \geq 0.28$; Table 4). Also, as planned, AA
294 infusion increased ($P < 0.01$) MP supply (diet + infusion) by 43% as well as the predicted
295 digestible flow of individual EAA. There were AA × Glc interactions on the predicted digestible
296 flows of a few EAA. However, we considered that these interactions on predicted flows were
297 statistically significant only because of the very limited variations on predicted flows:

298 predictions (NRC, 2001) were based on DMI and there were minimal alterations of DMI: we
299 therefore assumed that predicted variations of ± 1 g of AA/d were not biologically relevant.

300

301 ***Milk Production and Composition***

302 There was no AA \times Glc interaction ($P \geq 0.11$; Table 5) on any of the milk parameters
303 measured. Milk yield increased ($P = 0.04$) with AA infusion whereas glucose infusion tended
304 to increase ($P = 0.07$) it. Milk TP yield and concentration increased ($P < 0.01$) with infusions
305 of AA or glucose. Milk CP concentration increased ($P < 0.01$) with infusions of AA or glucose
306 whereas the proportion of NPN relative to CP increased ($P < 0.01$) with AA infusion but
307 decreased ($P = 0.02$) with glucose infusion. The proportion of CN relative to TP tended ($P =$
308 0.08) to decrease with AA infusion.

309

310 ***MP and EAA Whole Body Efficiencies***

311 The estimated efficiency of MP decreased ($P < 0.01$) with AA infusion and increased
312 with glucose infusion ($P < 0.01$) with similar trends for Groups of AA (Table 6). The individual
313 estimated EAA efficiencies decreased ($P < 0.01$) with AA infusion and marked differences
314 among EAA were observed: Phe and Met estimated efficiencies decreased by 42 and 32%
315 respectively whereas Val, Ile and Thr estimated efficiencies decreased by 21, 21 and 19%
316 respectively. Contrary to AA infusion effect, individual EAA efficiencies increased ($P \leq 0.03$)
317 with glucose infusion in similar proportions, from 8 to 11%. No significant interaction was
318 observed between AA and glucose infusions on estimated efficiencies. Across all treatments,
319 the efficiency of Arg was the highest, followed by His and Met whereas the efficiency of Phe
320 and Thr were the lowest. Indeed, efficiency of Arg were higher than 1 in Ctrl and E_{Glc} treatments
321 whereas the efficiency of His tended to be higher than 1 in E_{Glc} treatment. All other EAA

322 efficiencies were significantly lower than 1, except for His and Met in Ctrl treatment and for
323 Lys and Met in E_{Glc} treatment which were not different ($P > 0.10$) from 1.

324

325 *Plasma Arterial Concentrations of Individual AA and Urea*

326 There was no AA \times Glc interaction ($P > 0.10$; Table 7) on the arterial concentrations of
327 any AA. Infusions of AA increased the arterial concentrations of all EAA ($P \leq 0.02$), with the
328 exception of a tendency for Trp ($P = 0.06$) and no effect for Thr ($P = 0.17$). Increases averaged
329 from 22% (Arg) to 69% (Phe) of Ctrl values. For the NEAA, the AA infusion increased ($P <$
330 0.01) arterial concentrations of Cit, Orn and Pro, decreased ($P = 0.01$) Gly and tended ($P \leq$
331 0.09) to decrease arterial concentrations of Ala and Tyr. Overall, the plasma concentrations of
332 TAA-N, EAA-N, Group 1 AA-N (sum of His, Met, Phe, Trp and Tyr; Lapierre et al., 2012),
333 HMP-N (sum of His, Met and Phe) and Group 2 AA-N (sum of Lys, Ile, Leu and Val; Mephram,
334 1982) and BCAA-N (sum of Ile, Leu and Val) increased ($P < 0.01$) with AA infusion whereas
335 arterial concentration of NEAA-N was not affected by AA infusion ($P = 0.32$). In contrast, on
336 an individual basis, glucose infusion decreased ($P \leq 0.05$) Arg, Ile, Leu, Lys, Phe, Val and Orn
337 arterial concentrations, tended ($P \leq 0.10$) to decrease Ala and Cys arterial concentrations, but
338 increased ($P \leq 0.03$) arterial concentrations of Gly and Ser. Glucose infusion decreased ($P \leq$
339 0.04) the arterial concentrations of TAA-N, EAA-N, Group 2 AA-N and BCAA-N. The arterial
340 concentration of urea increased with AA infusion (+ 126%; $P < 0.01$) and decreased with
341 glucose infusion (- 24%; $P < 0.01$).

342

343 *Net Fluxes of Individual AA and Urea across Tissues*

344 ***Splanchnic Fluxes.*** Portal and hepatic plasma flows were not altered by AA infusion
345 but tended ($P = 0.06$; Table 8) to increase with glucose infusion. The net fluxes of individual
346 AA are detailed in Table 9. The portal net fluxes of all individual AA increased ($P \leq 0.05$) with

347 AA infusion with the exception of a tendency ($P = 0.06$) for Asn and no effect for Asp, Cit and
348 Tyr. The portal net fluxes of AA were not affected by glucose infusion, except for Gln which
349 decreased ($P < 0.01$). The portal net flux of Cit had a tendency for an AA \times Glc interaction (P
350 $= 0.08$) but differences did not reach significance ($P > 0.10$) when tested within type of infusions.
351 The portal net flux of urea decreased ($P < 0.01$) with AA infusion and was not affected by
352 glucose infusion.

353 Infusions of AA increased ($P \leq 0.03$) hepatic removals of Arg, His, Lys, Met, Phe and
354 Thr and of Ala, Cys, Gln, Gly, Pro and Ser and tended ($P = 0.08$) to increase hepatic removal
355 of Asn. Among individual EAA, hepatic removals of His, Met, Phe and Thr decreased ($P \leq$
356 0.04) and those of Arg and Lys tended ($P \leq 0.08$) to decrease with glucose infusion. The hepatic
357 removal of Ala decreased ($P < 0.01$) with glucose infusion. Hepatic release of Glu decreased
358 ($P = 0.02$) with AA infusion but increased ($P < 0.01$) with glucose infusion. Net hepatic release
359 of urea increased with AA infusion ($P < 0.01$) and decreased with glucose infusion ($P = 0.01$).

360 Among individual EAA, only the splanchnic net flux of AA from Group 2 AA increased
361 ($P \leq 0.03$) whereas those of Met and Thr tended ($P \leq 0.10$) to increase with AA infusion. The
362 splanchnic net flux of Pro was the only NEAA to increase ($P < 0.01$) with AA infusion but the
363 splanchnic net flux of Asn also tended ($P = 0.06$) to increase whereas splanchnic net flux
364 decreased ($P = 0.05$) for Glu or tended ($P = 0.09$) to decrease for Ala. The splanchnic net flux
365 of His increased ($P = 0.02$) with glucose infusion. For individual NEAA, splanchnic net fluxes
366 of Ala and Glu increased ($P \leq 0.01$) with glucose infusion. The net splanchnic removal of Cys
367 tended to decrease ($P = 0.07$) with glucose infusion. An AA \times Glc interaction was significant
368 for splanchnic net flux of Cit ($P = 0.03$): AA infusion increased it only without glucose infusion
369 ($P < 0.01$) and glucose infusion increased it only without AA infusion ($P = 0.03$). The
370 splanchnic net flux of urea increased with AA infusion ($P < 0.01$) and was not affected by
371 glucose infusion.

372 Fractional removals of individual EAA removed by the liver, except Trp, increased (P
373 ≤ 0.01) with AA infusion whereas fractional removals of His, Phe and Thr decreased ($P \leq 0.05$)
374 or tended to decrease ($P \leq 0.07$) for Arg, Lys and Met with glucose infusion (Table 10). For the
375 NEAA, fractional removals increased ($P \leq 0.05$) with AA infusion for Ala, Asp, Cys, Gln, Gly,
376 Pro and Ser. Fractional hepatic removals of His, Phe, Thr, Ala, Gln and Gly decreased ($P \leq$
377 0.03) or tended to decrease ($P \leq 0.03$) for Arg, Lys and Met with glucose infusion.

378 ***Mammary Uptake of Individual AA and AA in Milk.*** Mammary plasma flow did not
379 change with AA infusion but increased ($P < 0.01$; Table 8) with glucose infusion. The net
380 mammary uptakes of all individual EAA increased ($P \leq 0.02$; Table 9) with AA infusion, except
381 for a tendency to increase for Arg and Trp ($P \leq 0.10$). However, we observed a tendency for an
382 AA \times Glc interaction ($P = 0.06$) in His mammary uptake: the increment was significant only
383 when glucose was infused ($P < 0.01$). Among NEAA, mammary uptake of Cys increased ($P \leq$
384 0.05) with AA infusion whereas mammary uptake of Ala decreased ($P = 0.05$). Glucose
385 infusion increased ($P \leq 0.02$) mammary uptakes of Arg, Met, Phe, and Thr. As previously
386 mentioned, we had a tendency for an AA \times Glc interaction ($P = 0.06$) for His mammary uptake:
387 glucose infusion was significant only with AA infusion ($P < 0.01$). Among NEAA, Ala and Glu
388 mammary uptakes increased ($P \leq 0.01$) with glucose infusion whereas increased Ser and Tyr
389 mammary uptakes only reached tendency ($P \leq 0.09$). We observed also a tendency for an AA
390 \times Glc interaction ($P = 0.09$) for Pro mammary uptake: AA infusion increased more Pro
391 mammary uptake with glucose infusion and glucose infusion were significant only with AA
392 infusion ($P < 0.01$).

393 Mammary fractional removals of individual AA decreased ($P \leq 0.05$, Table 10) with
394 AA infusion for His, Leu, Lys, Phe, Ala, Glu and Pro, tended to decrease ($P = 0.06$) for Val
395 and increased ($P = 0.05$) for Cys. On the other hand, glucose infusion increased ($P < 0.01$)
396 fractional removals of Ile, Leu, Val, Ala and Glu, tended to increase ($P = 0.06$) fractional

397 removal of Lys but decreased ($P \leq 0.03$) those of Asp, and Gln and tended to decrease ($P =$
 398 0.09) for Cys.

399 Similar to MPY, AA or glucose infusion increased all individual AA exported into milk
 400 protein ($P < 0.01$; Table 9). The ratios of mammary uptake relative to milk protein output (U:O
 401 ratio, Table 11) of Ile, Leu, Lys, Phe and Pro increased ($P \leq 0.03$) with AA infusion whereas
 402 those of Val tended to increase ($P = 0.08$). In contrast, AA infusion decreased ($P \leq 0.04$) the
 403 U:O ratios of Ala, Glu and Tyr and tended to decrease the Gln U:O ratio ($P = 0.10$). With
 404 glucose infusion, the U:O ratios of Ile, Leu and Lys decreased ($P \leq 0.03$) whereas we observed
 405 only a tendency to decrease for Val ($P = 0.07$). The U:O ratio of Ala increased ($P < 0.01$) with
 406 glucose infusion.

407

408 ***Metabolic Fates of Groups of AA by Tissue***

409 The metabolism of EAA will be followed by groups and not as total EAA, because of
 410 the large dissimilarity between the pattern of utilization of Group 1 vs. Group 2 EAA. However,
 411 because we wanted to integrate measurements of net fluxes with predicted digestible flows, the
 412 HMP-N group, i.e., the sum of His, Met and Phe, was created to replace Group 1 AA-N because
 413 NRC (2001) does not predict Trp and Tyr digestible flows. It is acknowledged that the
 414 metabolism of NEAA also differs greatly amongst NEAA, but because there is no prediction
 415 of individual NEAA digestible flows in the NRC (2001), these were estimated as a group by
 416 difference between TAA-N and EAA-N and will therefore be considered as a single group.

417 ***Net Fluxes of Groups of AA.*** The net fluxes of groups of AA-N are reported in Table
 418 12. The portal net fluxes of HMP-N, Group 2 AA-N and NEAA-N increased ($P < 0.01$) with
 419 AA infusion, and did not change with glucose infusion. The splanchnic net flux of Group 2 AA-
 420 N increased ($P = 0.01$) and those of HMP-N tended to increase ($P = 0.08$) with AA infusion.
 421 The splanchnic net flux of HMP-N increased ($P = 0.05$) with glucose infusion. Hepatic

422 fractional removal of HMP-N increased ($P \leq 0.01$, Table 10) with AA infusion and decreased
423 ($P = 0.03$) with glucose infusion. The mammary uptake of Group 2 AA-N increased ($P < 0.05$,
424 Table 12) with AA infusion. The mammary uptake of NEAA-N increased ($P < 0.05$) with
425 glucose infusion. An AA \times Glc interaction ($P = 0.05$) for HMP-N mammary uptake was
426 observed: AA and glucose infusions increased more HMP-N mammary uptake with glucose
427 infusion and AA infusion respectively. Mammary fractional removal of HMP-N and Group 2
428 AA-N decreased ($P \leq 0.02$) with AA infusion. Glucose infusion had no effect on HMP-N
429 mammary fractional removal but increased ($P < 0.01$) fractional removal of Group 2 AA-N.
430 Fractional removal of NEAA-N was not affected by treatments (Table 10). Following MPY
431 pattern, groups of AA in MPY increased ($P < 0.05$) with AA or glucose infusion. Moreover,
432 TAA-N in MPY (329, 372, 364, 436 ± 31 mmol N/h in Ctrl, AA_{CN}, E_{Glc} and AA_{CN} + E_{Glc}
433 treatments respectively) was close to MPY. Overall, the TAA-N U:O ratio was not different
434 from unity ($P > 0.10$; Table 11). There was no effect of AA and glucose infusions on the U:O
435 ratio of Group 1 AA-N. The Group 2 AA-N and BCAA-N U:O ratios increased ($P = 0.03$) with
436 AA infusion, whereas these ratios decreased ($P \leq 0.03$) with glucose infusion. With AA infusion,
437 the NEAA-N U:O ratio decreased ($P = 0.02$) and those of TAA-N tended to decrease ($P = 0.09$)
438 whereas these ratios were not affected by glucose infusion.

439 ***Sites of catabolism of groups of AA.*** Catabolism of HMP-N was only different from 0
440 across the liver in Ctrl, AA_{CN} and AA_{CN} + E_{Glc} treatments and was not different from 0 across
441 the PDV, the other peripheral tissues and the mammary gland. In contrast, there was no
442 significant hepatic removal of Group 2 AA-N whereas catabolisms by the PDV and other
443 peripheral tissues were different from 0 with AA infusion and mammary catabolism was higher
444 than 0 for all treatments. Finally, for NEAA-N, catabolism across the PDV was higher than 0
445 for the 4 treatments. A “negative” catabolism of the NEAA-N across the mammary gland for
446 all 4 treatments and by other peripheral tissues, only significant in Ctrl treatment, indicates *de*

447 *nov*o synthesis of the NEAA by these tissues. There was hepatic removal of NEAA-N in all
448 treatments, but it was not significant when glucose was infused.

449 The catabolism by the PDV of Group 2 AA-N tended ($P = 0.07$) to increase with AA
450 infusion. The hepatic removal of HMP-N and NEAA-N increased with AA infusion suggesting
451 an increased catabolism and decreased ($P \leq 0.01$) with glucose infusion suggesting a decreased
452 catabolism. The tendency ($P = 0.06$) for a decreased hepatic release for Group 2 AA-N with
453 AA infusion must be interpreted with caution because it was not significantly different from 0,
454 except a tendency in E_{Glc} treatment. The catabolism by other peripheral tissues was not affected
455 by treatments. The mammary catabolism of Group 2 AA-N increased ($P < 0.01$) with AA
456 infusion and decreased ($P = 0.01$) with glucose infusion. The “negative” mammary catabolism
457 of NEAA-N increased ($P < 0.01$) with AA infusion, indicating increased *de novo* synthesis of
458 NEAA-N.

460 DISCUSSION

461 *Nutrient Supplies*

462 The DMI were similar between treatments; consequently, AA and glucose infusions
463 created substantial variations in MP (+ 43%) and NE_L (+ 14%) supplies, respectively, with no
464 AA \times Glc interaction. The increased digestible flow of individual EAA with AA infusion did
465 not rely on the ration formulation model because it is directly related to the infusion. There was,
466 however, large variation of relative increments between EAA explained by the CN profile
467 different from the dietary MP profile. For example Thr digestible flow increased by 41%,
468 whereas Met digestible flow increased by 72%; Phe digestible flow had the largest increment
469 (102%) due to fact that Phe replaced Tyr in the infusate.

471 *Milk Yield and Composition*

472 Infusing AA or glucose increased milk yield by 6 and 5%, respectively, although the
473 latter only reached the tendency level; there was no AA \times Glc interaction, the effects being
474 therefore additive. Also, similarly to milk yield, infusing AA or glucose increased MPY, with
475 no AA \times Glc interaction, the effects being also additive, as previously reported when the effects
476 of protein and energy supplies have been tested in a factorial arrangement (e.g. Clark et al.,
477 1977; Vanhatalo et al., 2003a; Raggio et al., 2006a). In a recent meta-analysis, Daniel et al.
478 (2016) also concluded that there was no interaction between MP and energy supplies.
479 Interaction between energy and protein supplies was, however, reported by Brun-Lafleur et al.
480 (2010) using a specific experimental design with many levels of energy and protein supplies.
481 This discrepancy indicates the need to conduct studies thoroughly designed to test the energy \times
482 protein interaction on milk yield and MPY.

483 The MPY response to AA infusion (+ 17%) was similar to what was usually reported
484 with a marginal recovery of infused AA into MPY averaging 0.23, similar to marginal protein
485 efficiency reported by Hanigan et al. (1998) and Martineau et al. (2017) of 0.21 and 0.26,
486 respectively, in response to post-ruminal CN infusions. In contrast, the MPY response to
487 glucose infusion (+ 14%) was slightly larger than the response usually reported with increased
488 energy supply. For example, Curtis et al. (2018), who also infused glucose, reported a 8%
489 increment whereas many studies reported just a tendency for increased MPY with infusions of
490 glucose or other energy sources (Rulquin et al., 2004; Raggio et al., 2006a) or no effect of
491 energy infusion (Rius et al., 2010a; Curtis et al., 2014; Nichols et al., 2016). These different
492 effects of energy infusions could be due to the supplementary NE_L provided by infusions:
493 Raggio et al. (2006a) infused 3.73 Mcal/d of NE_L from propionate, close to our glucose infusion;
494 in contrast, Curtis et al. (2014) and Nichols et al. (2016) infused only 2.5 and 2.8 Mcal/d
495 respectively. Moreover, NE_L supply of their control treatments were greater than ours (32.1 and

496 28.4 Mcal/d respectively for Curtis et al. (2014) and Nichols et al. (2016) vs. 27.4 Mcal/d in
497 our study). These differences could explain our marked MPY response to glucose infusion.

498

499 *Efficiency of Utilization of MP and EAA*

500 The decreased efficiency of MP with AA infusion (- 27%) was the consequence of an
501 increase of MPY smaller than the increase of MP supply, consistent with previous observations
502 (Doepel et al., 2004; Metcalf et al., 2008). In contrast, efficiency of MP increased with glucose
503 infusion by 10%: this increment with glucose infusion was due to an increased MPY with no
504 change in MP supply. In the current context where MP supply was largely increased with AA
505 infusion, the magnitude of the variation of efficiency of MP was less important with increased
506 energy supply than with decreased protein supply as already observed by Omphalius et al.
507 (2019). Moreover, there was no AA × Glc interaction on efficiency of MP, as previously
508 reported for efficiency of utilization of N (Broderick, 2003; Rius et al., 2010b).

509 The highest efficiency of EAA occurred in E_{Glc} treatment, suggesting very limited
510 catabolism of EAA whereas the lowest efficiencies observed in AA_{CN} treatment suggested
511 increased catabolism. Efficiencies of individual EAA varied following a pattern similar to
512 efficiency of MP but with different ranges of variation. For example, with AA infusion, Phe
513 had the largest variations of efficiency whereas Thr showed the smallest variations. These
514 different variations of efficiency of EAA were linked to the relative variations of EAA supplies,
515 due to CN profile infused relative to AA supplied from the diet. Efficiencies of some EAA were
516 not different or even higher than 1; this was the case for Arg, His, Lys and Met under Ctrl and
517 E_{GLC} treatments. An efficiency higher than 1 may be explained by either an overestimation of
518 EUL or MFP, an underestimation of the digestible flows of these AA or an unaccounted source
519 of endogenous supply. The last assumption would involve different metabolic pathways for the
520 3 AA with the highest efficiency. For Arg, it is acknowledged that there is significant de novo

521 synthesis in dairy cows, which could account for up to 30% of the digestible flow (Doepel et
522 al., 2004). For His, a possible endogenous source could be from depletion of muscular
523 intracellular pools of the dipeptides carnosine and anserine or of blood hemoglobin rich in His.
524 For Lys, protein mobilisation would need to be assumed and this route would also provide
525 endogenous supply of all AA. Although we do not have measurements to assess if protein
526 mobilisation occurred when those high efficiencies were observed, splanchnic fluxes greater
527 than mammary uptake would suggest that protein mobilisation did not occur in our study.
528 Similarly, Lee et al. (2015) also reported that, with a low protein diet (13.7% CP), His and Met
529 had the highest efficiency of utilization, although their calculation was only varying the
530 efficiency of lactation as the efficiency for maintenance was assumed to be fixed. They also
531 reported a decreased efficiency of Lys and Met when their respective supply was increased.
532 Altogether these observations suggest that His, Met and Lys were potentially in short supply,
533 especially under Ctrl and E_{GLC} treatments.

534 In line with previous findings on the effect of AA supply (Hanigan et al., 2004; Raggio
535 et al., 2006b; Nichols et al., 2016), the pattern of plasma urea concentrations was the inverse of
536 estimated efficiencies of MP and EAA-N. A large increase of urea concentration with AA
537 infusion would indicate increased AA catabolism whereas in contrast, the decreased plasma
538 urea concentrations with glucose infusion, as already observed (Raggio et al., 2006b), is
539 consistent with increased estimated efficiencies suggesting a reduction of AA catabolism.
540 However, a decreased urea concentration was not observed in Nichols et al. (2016) but the rate
541 of glucose infusion was smaller than in our experiment (1.0 kg/d vs. 1.5 kg/d in our study).

542 Variations of efficiency of MP and efficiency of EAA-N are indicators of whole body
543 efficiency of utilization of AA. We wanted to understand better the mechanisms underlying
544 their variations by studying the coordination of utilization of AA by splanchnic tissues, other

545 peripheral tissues and the mammary gland, for anabolic or catabolic purposes in response to
546 variations in protein and energy supplies.

547

548 *Splanchnic and Mammary Net Fluxes Varied among EAA*

549 The portal net fluxes of all EAA infused increased with AA infusion, as usually
550 observed with a post-rumen infusion of a mixture of free AA in cows (Larsen et al., 2015) or
551 of CN in sheep (El-Kadi et al., 2006). The apparent recovery of the infused AA reaching portal
552 circulation averaged 109, 78, 69, 66, 77, 89, 83, 86, 100 and 50% for the EAA Arg, His, Ile,
553 Leu, Lys, Met, Phe, Thr, Trp, and Val respectively and 203, 99, 22, 133, 99, 12, 129, 75 and
554 98% for the NEAA Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro and Ser, respectively. Amongst the
555 EAA, the BCAA showed the lowest recovery rate, as also observed in cows in early lactation
556 (Larsen et al., 2015). Also, in sheep, amongst the EAA, only the BCAA had a slope of portal
557 recovery relative to infusion rate of AA (CN) lower than unity (El-Kadi et al., 2006). Low portal
558 recoveries of BCAA suggest that these AA are catabolized by the PDV when supply is
559 increased. Indeed, direct measurements have shown oxidation of Leu across the PDV in
560 lactating dairy cows (Lapierre et al., 2002) and in sheep (Lobley et al., 2003). Although no
561 statistical effect could be detected, we noted an increase in PDV recovery with glucose infusion
562 (E_{Glc} and $AA_{CN} + E_{Glc}$ vs. Ctrl and AA_{CN}) by 43, 32 and 58% for Ile, Leu and Val suggesting
563 that increased glucose supply might have decreased PDV metabolism of BCAA. Across the
564 NEAA, as also reported in cows (Larsen et al., 2015) and in sheep (El-Kadi et al., 2006), Ala
565 portal net flux increment, greater than infusion rate, suggests increased transamination of
566 pyruvate with increased AA supply. Moreover, Asp and Glu low recovery rates indicate
567 intensive catabolism of these 2 AA by the PDV, in agreement with their large catabolism by
568 the PDV observed in sheep and non-ruminant species (Wolff et al., 1972; Windmueller et al.,
569 1974; Ball, 2002).

570 The net hepatic removals of His, Lys, Met, Phe and Thr increased with AA infusion as
571 usually observed with increased protein or AA supply in lactating dairy cows (Raggio et al.,
572 2004; Cantalapiedra-Hijar et al., 2014a; Larsen et al. 2015). It was initially proposed that net
573 hepatic removal of EAA would be driven by the portal net flux (Reynolds, 2006). Indeed,
574 usually, increased portal net flux of EAA leads to increased circulating plasma concentrations.
575 However, a situation where increased net PDV absorption was accompanied by decreased AA
576 concentrations, e.g. when comparing cows pre- and post-calving (Doepel et al., 2009), clearly
577 demonstrated that net hepatic removal was not related to portal net flux. Another proposed
578 option was that total hepatic AA influx would drive the hepatic removal (Hanigan, 2005;
579 Lapierre et al., 2005). However, the effect of both AA and glucose infusions on hepatic
580 fractional removal for HMP-N indicates that there was no linear relationship between net
581 hepatic removal and hepatic influx: therefore other factors than mass action must have an
582 impact on hepatic AA removal. As previously reported in dairy cows (Raggio et al., 2004;
583 Cantalapiedra-Hijar et al., 2014a), hepatic removals of BCAA were not different from 0, but
584 this observation is not yet explained.

585 Overall, the TAA-N mammary uptake to milk output ratio did not differ from 1, but the
586 mammary gland response to AA or glucose infusion differed amongst groups of AA. On the
587 one hand, the mammary uptake of most EAA increased with AA infusion through increased
588 mammary VA differences (data not shown) with no change in mammary plasma flow as
589 observed previously with increased AA supply [Raggio et al. (2006a) with CN infusion; Nichols
590 et al. (2016) with EAA infusion; Omphalius et al. (2019) with dietary changes] although
591 mammary plasma flow was reported to decrease with increased MP supply (review: Lapierre
592 et al., 2012). For His, Met and Trp, the increment was just sufficient to cover the increased
593 MPY, as shown by no effect of AA infusion on the U:O ratio. In contrast, the U:O ratio of
594 Group 2 AA-N increased with AA infusion, indicating that the mammary uptake increased at a

595 greater rate than the increased MPY response to AA infusion. On the other hand, the mammary
596 uptake responses to glucose infusion occurred differently between EAA. First, for most of the
597 Group 1 AA (especially His, Met and Phe) and Tyr, mammary uptake increased with glucose
598 infusion with unchanged VA differences coupled to an increased mammary plasma flow.
599 Second, glucose infusion did not affect mammary uptake of Group 2 AA-N resultant of the 2
600 components of uptake calculations varying in opposite directions: the VA difference decreased
601 (data not shown) whereas mammary plasma flow increased. In fact, to accompany an increased
602 MPY, an increased mammary uptake of Group 1 AA-N is obligatory if there is a stoichiometric
603 transfer of their uptake to MPY; however, for Group 2 AA-N, as their mammary uptake is in
604 excess of MPY, their uptake does not need to increase to support an increment of MPY. This
605 indicates that mammary catabolism of Group 2 AA-N is not obligatory, or at least, it can
606 decrease without a negative effect on MPY. Finally, mammary uptake increased with glucose
607 infusion for many NEAA (Ala, Glu Pro, Ser and Tyr). The increased Ala and Glu uptakes were
608 already reported by Lemosquet et al. (2010a). Nevertheless, the uptakes were not sufficient to
609 cover the milk output in protein, with Gln being the only NEAA for which the U:O ratio is
610 never lower than 1. With AA infusion, i.e., when AA supply was in excess, decreased U:O
611 ratios of Ala, Glu and Tyr indicate increased mammary *de novo* synthesis. So, overall, although
612 it is acknowledged that AA infusion increases rate of appearance of glucose (Lapierre et al.,
613 2010; Galindo et al., 2011), MPY response to AA infusion was likely not driven by potential
614 increment in glucose availability as MPY increased through different intra-mammary
615 mechanisms in response to AA or glucose infusion.

616 Hepatic and mammary fractional removals responded differently to treatments. Infusion
617 of AA infusion increased HMP-N liver fractional removal but decreased their mammary
618 fractional removal, clearly indicating that the liver was coping with increased influx by
619 increasing its removal. At the opposite, infusion of glucose had no effect on mammary

620 fractional removal of HMP-N but decreased their hepatic fractional removal. As mentioned
621 previously, this might suggest that the liver is not only responding to mass action, but might
622 also have an active role in decreasing HMP-N catabolism.

623

624 ***Coordination between Tissues for Utilization of AA in Response to Changes in AA and***
625 ***Energy Supplies.***

626 As each tissue uses AA for both anabolic and catabolic purposes, it is interesting to
627 analyze the coordinated changes between tissues in response to alteration in MP or energy
628 supplies. Indeed, the efficiency of EAA-N increased with glucose infusion and decreased with
629 AA infusion but the tissue where the inefficiency occurred varied between AA, i.e., different
630 sites of catabolism could be identified. First, catabolism by the PDV of HMP-N did not differ
631 from 0 for the 4 treatments whereas for Group 2 AA-N, we noted a significant PDV catabolism
632 for all treatments. This latter catabolism, linked to low net PDV recoveries, represented between
633 one third and half of whole body catabolism, as already observed for Leu in dairy cows
634 (Lapierre et al., 2002). The PDV catabolism tended to increase with AA infusion, similarly to
635 increased Leu oxidation by the PDV with increased MP supply (Lapierre et al., 2002). Second,
636 the hepatic removal of HMP-N decreased with glucose infusion and increased with AA infusion
637 linked to increased and decreased efficiency of HMP-N with glucose and AA infusions,
638 respectively. Liver is the major site of catabolism for HMP group and represented from 85 to
639 95% of whole body catabolism, but plays a very minor role in catabolism of Group 2 AA. Third,
640 the fact that mammary catabolism of HMP-N was not different from 0 agrees with the U:O ratio
641 equal to unity reported in previous studies, which did not change with variations of energy or
642 protein supplies (Clark et al., 1977; Raggio et al., 2006a; Omphalius et al., 2019). This indicates
643 no mammary catabolism as, on a net basis, net HMP-N uptake was transferred to MPY.
644 Consequently, for HMP-N, the variations of efficiency of utilization were the result of

645 coordinated and opposite actions of the mammary gland and the liver. For example, in response
646 to glucose infusion, increased efficiency of HMP-N resulted from increased mammary uptake
647 to support MPY and decreased hepatic catabolism. The same pattern of U:O ratio and hepatic
648 removal could be observed on 2 EAA, often limiting in dairy rations, His and Met. In contrast,
649 mammary catabolism of Group 2 AA-N was present and decreased with glucose infusion. The
650 observed decreased mammary catabolism of BCAA-N with glucose infusion is consistent with
651 the fact that BCAA could provide 3-carbon intermediates and acetyl-CoA as demonstrated by
652 Raggio et al. (2006a) who directly measured Leu oxidation or by Bequette et al. (2006) who
653 incubated bovine mammary explants to study AA catabolized through the Krebs cycle for
654 energetic purposes. In Ctrl treatment, we noted that Group 2 AA-N were mainly catabolized in
655 the mammary gland which had a catabolism different from 0. Nevertheless, with AA infusion,
656 other tissues seemed to be involved in catabolism of Group 2 AA-N given observed PDV and
657 peripheral tissue catabolisms different from 0 in these treatments. With AA infusion, mammary
658 catabolism increased but its proportion relative to total catabolism decreased from 43% to 25%
659 in Ctrl vs. AA_{CN} and AA_{CN} + E_{Glc} treatments respectively. Consequently, for this group of AA,
660 mammary catabolism and also PDV catabolism could explain the decreased efficiency of
661 utilization with AA infusion. The data presented indicate that the positive response of MPY to
662 glucose infusion was accompanied by a decreased hepatic removal of HMP-N in the same range
663 than the increased mammary uptake and MPY whereas for Group 2 AA-N, as there was no net
664 liver removal, the decreased catabolism occurred mainly within the mammary gland. However,
665 our net flux measurements do not allow us to delineate if these decreased catabolisms of EAA
666 are a response to a stimulation of MPY from extra energy supply or if the increased MPY is the
667 result of “sparing” these EAA by different tissues.

668

669

CONCLUSION

670 Studying the fate of AA clearly showed that the response of tissue usage of AA differs
671 amongst tissues and the response to changes in AA or glucose supplies is coordinated and
672 explains variations of efficiency of EAA-N. These variations are explained by different
673 anabolic and catabolic fates among groups of AA according the nutrient supplies. With AA
674 infusion, efficiency of EAA-N decreased because EAA-N in MPY increased to a lesser extent
675 than the increased EAA-N supply. The decreased efficiency of EAA-N was related to increased
676 PDV and mammary catabolisms of Group 2 AA-N and increased hepatic removal of Group 1
677 AA-N. In contrast, with glucose infusion, increased efficiency of EAA-N was explained by an
678 increased MPY, supported by a similar increased mammary uptake of HMP-N, and a decreased
679 hepatic catabolism. In parallel, mammary catabolism of Group 2 AA-N decreased, linked to the
680 increased mammary uptake of NEAA-N which supported the increment of NEAA-N in MPY.
681 This study clearly delineates the coordinated tissue utilization of AA, between anabolic or
682 catabolic purpose, in response to variations of AA or glucose supplies. This knowledge could
683 improve the prediction of efficiency of EAA and this information could be used in feeding
684 systems to either improve, for a given diet, the prediction of MPY or to identify which AA are
685 potentially in short supply.

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913 **Table 1.** Composition of the experimental diet and chemical composition of the feed
 914 ingredients

	Ingredients						Experimental diet
	Grass hay mature	Barley grain ground	Corn grain ground	Beet sugar pulp dried	Wheat middlings	Soybean meal	
Composition of the experimental diet, % DM ¹	50.6	18.3	9.4	8.5	7.4	2.5	-
Analysis, g/kg DM							
CP	87	128	92	96	195	530	129
ADF	309	56	26	244	137	32	201
NDF	594	175	102	392	421	65	408
Lignin	42	12	7	27	30	5	42

¹The diet also contained a mineral and vitamin premix (2.6% DM) and urea (0.6% DM).

915

For Peer Review

916 **Table 2.** Infusion rate of AA

AA	g/d
EAA	
Arg	25.0
His	18.6
Ile	33.7
Leu	63.0
Lys	52.9
Met	19.6
Phe	72.0
Thr	29.3
Trp	8.1
Val	38.3
NEAA	
Ala	20.8
Asp	20.8
Asn	25.7
Cys	2.7
Glu	51.6
Gln	89.5
Gly	12.6
Pro	71.7
Ser	38.6

For Peer Review

918 **Table 3.** Correction factors and AA composition of proteins used in the calculation of the individual AA supplies and efficiencies

Item	Correction factors ¹	g AA _{corrected} / kg CP ²	g AA _{corrected} / kg of true protein ²			g AA _{calculated} / kg of true protein ³	
		Duodenal endogenous	Scurf	Endogenous urinary urea	Metabolic fecal protein	Milk with BBP ⁴	Milk without BBP ⁵
Arg	1.06	46.1	96.0	82.0	59.0	37.7 ± 0.3	36.9 ± 0.3
His	1.07	29.0	17.5	30.4	35.4	29.3 ± 0.2	29.3 ± 0.2
Ile	1.12	40.9	29.6	36.9	53.9	62.4 ± 0.1	63.1 ± 0.2
Leu	1.06	76.7	69.3	82.7	91.9	104.0 ± 0.9	103.6 ± 0.9
Lys	1.07	62.3	56.4	79.0	76.2	87.5 ± 0.7	87.5 ± 0.7
Met	1.05	12.6	14.0	23.7	17.3	30.2 ± 0.1	30.6 ± 0.1
Phe	1.06	39.8	36.1	44.1	52.8	52.6 ± 0.3	52.4 ± 0.3
Thr	1.07	51.8	40.1	48.4	73.6	46.0 ± 0.2	45.3 ± 0.2
Trp	1.06	12.9	7.3	10.5	17.9	16.3 ± 0.4	15.7 ± 0.4
Val	1.10	52.9	46.6	51.5	70.1	69.8 ± 0.3	69.9 ± 0.2

¹Corrections factors used to account for incomplete recovery of AA with 24-h hydrolysis of proteins, from Lapierre et al. (2019a).

²From Lapierre et al. (2019b).

³Average of 19 milk compositions (cow × period): see text for calculation details.

⁴Including blood-borne proteins.

⁵Excluding blood-borne proteins.

920 **Table 4.** Protein, AA and energy supplies from the experimental diet and infusions

Item	Treatments ¹				SEM ³	P-values ²		
	Ctrl	AA _{CN}	E _{Glc}	AA _{CN} + E _{Glc}		AA	Glc	AA×Glc
DMI, kg/d	17.9	17.9	17.9	17.9	0.83	0.28	0.82	0.47
NE _L supply ⁴ , Mcal/d	27.4	27.4	31.4	31.3	1.11	0.23	<0.01	0.56
N intake ⁵ , g/d	365	462	367	463	17.0	<0.01	0.41	0.81
MP supply ⁶ , g/d	1,302	1,901	1,304	1,899	176.1	<0.01	0.78	0.30
EAA digestible flow ⁷ , g/d								
Arg	65	91	66	90	3.1	<0.01	0.22	0.03
His	29	48	30	48	1.4	<0.01	0.62	0.34
Ile	74	107	74	108	3.4	<0.01	0.06	0.07
Leu	118	181	118	180	5.6	<0.01	0.23	0.21
Lys	94	147	95	147	4.2	<0.01	0.55	0.01
Met	27	47	27	47	1.2	<0.01	0.55	0.15
Phe	70	142	70	142	3.4	<0.01	0.34	0.67
Thr	71	100	71	100	3.2	<0.01	0.66	0.24
Val	82	121	82	120	3.7	<0.01	<0.01	<0.01
NEAA digestible flow ⁸ , g/d	866	1,202	866	1,201	97.4	<0.0	0.66	0.70

1

¹Ctrl: water infusion; AA_{CN}: 695 g/d of AA mixture (casein profile) infusion; E_{Glc}: 1,454 g/d of glucose infusion; AA_{CN} + E_{Glc}: combination of AA mixture and glucose infusion.

²Probability values correspond to the effect of AA (without vs. with AA infusion), Glc (without vs. with glucose infusion) and AA×Glc interaction.

³SEM given for n = 4.

⁴NE_L supply from NRC (2001) + glucose infusion, see text for detail.

⁵N ingested + N infused.

⁶MP supply from NRC (2001) - MP from endogenous origin + AA infusion, see text for detail.

⁷Digestive EAA flow estimated from NRC (2001) corrected for incomplete recovery with a 24-h hydrolysis – AA from MP duodenal endogenous flow + AA infusion, expressed in hydrated form, see text for detail.

⁸Digestive NEAA flow estimated by difference between MP and EAA supplies, expressed in hydrated form, see text for detail.

921

922 **Table 5.** Effects of AA and glucose infusions on milk production and composition¹

Item	Treatments ²				SEM ⁴	P-values ³		
	Ctrl	AA _{CN}	E _{Glc}	AA _{CN} + E _{Glc}		AA	Glc	AA×Glc
Milk yield, kg/d	21.4	22.3	22.1	24.0	1.75	0.04	0.07	0.41
True protein yield, g/d	722	815	797	956	68.1	<0.01	0.01	0.27
True protein concentration, g/kg	33.9	36.7	36.3	40.0	0.87	<0.01	<0.01	0.57
Casein, % of true protein	81.5	80.3	81.1	81.1	0.87	0.08	0.48	0.11
CP concentration ⁵ , g/kg	35.2	38.5	37.6	41.6	0.87	<0.01	<0.01	0.58
NPN, % of CP	3.8	4.6	3.5	4.0	0.19	<0.01	0.02	0.50

¹Average of the last 6 milkings.

²Ctrl: water infusion; AA_{CN}: 695 g/d of AA mixture with casein profile infusion; E_{Glc}: 1,454 g/d of glucose infusion; AA_{CN} + E_{Glc}: combination of AA mixture and glucose infusion.

³Probability values correspond to the effect of AA (without vs. with AA infusion), Glc (without vs. with glucose infusion) and AA×Glc interaction.

⁴SEM given for n = 4.

⁵CP = total N milk × 6.34, as proposed by Karman and van Boekel (1986).

923

924 **Table 6.** Effects of AA and glucose infusions on the efficiency of utilization of individual
 925 EAA, MP and groups of AA

Item	Treatments ¹				SEM ³	P-values ²		
	Ctrl	AA _{CN}	E _{Glc}	AA _{CN} + E _{Glc}		AA	Glc	AA×Glc
Arg	1.42 ^{a*}	0.82 ^{a*}	1.49 ^{a*}	0.92 ^{a*}	0.035	<0.01	0.03	0.62
His	1.04 ^b	0.68 ^{b*}	1.10 ^{b†}	0.77 ^{b*}	0.046	<0.01	<0.01	0.58
Ile	0.79 ^{d,e*}	0.60 ^{e*}	0.85 ^{e,f*}	0.68 ^{c*}	0.040	<0.01	<0.01	0.52
Leu	0.84 ^{c,d*}	0.60 ^{d,e*}	0.90 ^{d,e*}	0.68 ^{c*}	0.041	<0.01	<0.01	0.61
Lys	0.88 ^{c*}	0.62 ^{c*}	0.95 ^{c,d}	0.71 ^{c*}	0.043	<0.01	<0.01	0.60
Met	0.99 ^b	0.63 ^{c,d*}	1.06 ^{b,c}	0.72 ^{c*}	0.054	<0.01	0.02	0.72
Phe	0.73 ^{e*}	0.40 ^{g*}	0.78 ^{f*}	0.45 ^{e*}	0.033	<0.01	0.02	0.97
Thr	0.73 ^{e*}	0.56 ^{f*}	0.77 ^{f*}	0.62 ^{d*}	0.032	<0.01	<0.01	0.50
Val	0.83 ^{c,d*}	0.62 ^{c*}	0.89 ^{d,e*}	0.70 ^{c*}	0.042	<0.01	<0.01	0.52
MP	0.87 [*]	0.62 [*]	0.94	0.71 [*]	0.038	<0.01	<0.01	0.59
EAA-N ⁴	0.91 [†]	0.62 [*]	0.98	0.71 [*]	0.040	<0.01	0.01	0.59
HMP-N ⁵	0.92 [†]	0.56 [*]	0.98	0.63 [*]	0.042	<0.01	0.01	0.75
Group2 AA-N ⁶	0.85 [*]	0.61 [*]	0.91 [†]	0.70 [*]	0.042	<0.01	<0.01	0.57
NEAA-N ⁷	0.75 [*]	0.56 [*]	0.80 [*]	0.64 [*]	0.034	<0.01	0.01	0.54

¹Ctrl: water infusion; AA_{CN}: 695 g/d of AA mixture with casein profile infusion; E_{Glc}: 1,454 g/d of glucose infusion; AA_{CN} + E_{Glc}: combination of AA mixture and glucose infusion.

²Probability values correspond to the effect of AA (without vs. with AA infusion), Glc (without vs. with glucose infusion) and AA×Glc interaction.

³SEM given for n = 4.

⁴EAA-N: Sum of Arg, His, Ile, Leu, Lys, Met, Phe, Thr and Val.

⁵HMP-N: Sum of His, Met, Phe, used in Table 12.

⁶Group2 AA-N: Sum of Ile, Leu, Lys and Val.

⁷NEAA-N: obtained by difference between MP and EAA-N.

a,b,c,d,e,f,g Within a column, values with a different superscript differ; only tested within individual EAA.

*Different from 1 at $P \leq 0.05$ and † at $0.05 < P \leq 0.10$.

926

927 **Table 7.** Effects of AA and glucose infusions on plasma arterial concentrations (μM)

Item	Treatments ¹				SEM ³	P-values ²		
	Ctrl	AA _{CN}	E _{Glc}	AA _{CN} + E _{Glc}		AA	Glc	AA×Glc
EAA								
Arg	79	89	63	84	6.5	0.01	0.04	0.24
His	50	65	41	68	7.1	<0.01	0.43	0.13
Ile	113	139	62	87	10.2	<0.01	<0.01	0.98
Leu	122	195	69	116	15.2	<0.01	<0.01	0.19
Lys	70	95	49	74	8.7	<0.01	<0.01	0.91
Met	20	23	18	24	2.3	0.02	0.83	0.53
Phe	46	79	42	70	2.6	<0.01	0.01	0.32
Thr	86	99	90	101	9.7	0.17	0.70	0.93
Trp	38	40	35	40	2.8	0.06	0.48	0.32
Val	186	269	116	166	19.9	<0.01	<0.01	0.20
NEAA								
Ala	207	170	174	167	11.0	0.06	0.10	0.15
Asn	33	46	48	40	5.0	0.56	0.40	0.20
Asp	37	34	26	29	8.4	0.95	0.21	0.62
Cit	87	106	65	112	11.0	<0.01	0.34	0.10
Cys	126	118	117	117	5.9	0.16	0.09	0.23
Gln	220	234	245	265	26.4	0.31	0.11	0.87
Glu	52	51	54	47	3.6	0.30	0.78	0.35
Gly	188	153	255	199	13.2	0.01	<0.01	0.43
Orn	31	59	24	47	6.8	<0.01	0.05	0.53
Pro	69	161	61	143	8.2	<0.01	0.12	0.53
Ser	77	77	90	91	5.9	0.90	0.03	0.93
Tyr	46	41	46	40	3.5	0.09	0.81	0.95
TAA-N ⁴	2,498	2,900	2,252	2,699	163.6	<0.01	0.05	0.82
EAA-N ⁵	1,180	1,546	868	1,255	105.5	<0.01	<0.01	0.86
Group1 AA-N ⁶	300	378	264	378	28.3	<0.01	0.28	0.26
HMP-N ⁷	216	297	183	298	23.5	<0.01	0.24	0.21
Group2 AA-N ⁸	561	793	344	518	60.3	<0.01	<0.01	0.38
BCAA-N ⁹	421	603	247	369	44.5	<0.01	<0.01	0.29
NEAA-N ¹⁰	1,241	1,274	1,315	1,364	78.1	0.50	0.19	0.89
Urea-N (mM)	4.0	8.3	2.6	6.7	0.30	<0.01	<0.01	0.62

¹Ctrl: water infusion; AA_{CN}: 695 g/d of AA mixture with casein profile infusion; E_{Glc}: 1,454 g/d of glucose infusion; AA_{CN} + E_{Glc}: combination of AA mixture and glucose infusion.

²Probability values correspond to the effect of AA (without vs. with AA infusion), Glc (without vs. with glucose infusion) and AA × Glc interaction.

³SEM given for n = 4.

⁴TAA-N: Sum of Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

⁵EAA-N: Sum of Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val.

⁶Group1 AA-N: Sum of His, Met, Phe and Tyr.

⁷HMP-N: Sum of His, Met, Phe, used in Table 12.

⁸Group2 AA-N: Sum of Ile, Leu, Lys and Val.

⁹BCAA-N: Sum of Ile, Leu and Val.

¹⁰NEAA-N: Sum of Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser and Tyr.

928

For Peer Review

929 **Table 8.** Effects of AA and glucose infusions on plasma flows (L/h)

Tissue	Treatments ¹				SEM ³	P-values ²		
	Ctrl	AA _{CN}	E _{Glc}	AA _{CN} + E _{Glc}		AA	Glc	AA×Glc
Portal	1,103	1,112	1,154	1,182	90.5	0.53	0.06	0.73
Splanchnic	1,192	1,163	1,233	1,257	97.1	0.93	0.06	0.41
Mammary	593	561	669	753	57.4	0.44	<0.01	0.10

¹Ctrl: water infusion; AA_{CN}: 695 g/d of AA mixture with casein profile infusion; E_{Glc}: 1,454 g/d of glucose infusion; AA_{CN} + E_{Glc}: combination of AA mixture and glucose infusion.

²Probability values correspond to the effect of AA (without vs. with AA infusion), Glc (without vs. with glucose infusion) and AA × Glc interaction.

³SEM given for n = 4.

930

For Peer Review

931 **Table 9.** Effects of AA and glucose infusions on net fluxes of portal drained-viscera (PDV),
 932 hepatic (HEP), splanchnic tissues (TSP), and mammary gland (MG) and milk output (Milk¹)
 933 of individual AA and urea-N (mmol/h)

		Treatments ²				SEM ⁴	P-values ³		
		Ctrl	AA _{CN}	E _{Glc}	AA _{CN} + E _{Glc}		AA	Glc	AA×Glc
EAA									
Arg	PDV	13.9	20.1	13.4	21.2	1.87	<0.01	0.85	0.63
	HEP	-2.0	-8.5	1.4	-6.1	1.97	<0.01	0.08	0.73
	TSP	11.9	11.7	15.1	15.1	2.36	0.94	0.16	0.96
	MG	-12.8	-14.3	-15.4	-17.7	1.67	0.10	0.02	0.73
	Milk	6.2	7.0	6.8	8.2	0.60	<0.01	0.01	0.27
His	PDV	6.0	9.8	6.0	10.1	0.61	<0.01	0.70	0.77
	HEP	-1.1	-4.5	-0.1	-2.9	0.76	<0.01	0.02	0.52
	TSP	4.9	5.4	6.0	7.3	0.80	0.13	0.02	0.43
	MG	-5.7	-6.1	-6.1	-7.5	0.46	0.01	0.01	0.06
	Milk	5.5	6.2	6.1	7.3	0.53	<0.01	0.01	0.27
Ile	PDV	18.3	24.0	17.3	27.2	2.11	<0.01	0.52	0.26
	HEP	3.2	3.4	4.1	2.6	1.81	0.64	0.95	0.50
	TSP	21.4	27.4	21.4	29.9	2.73	0.02	0.64	0.64
	MG	-18.2	-22.5	-18.8	-24.0	1.83	<0.01	0.21	0.58
	Milk	14.0	15.8	15.5	18.6	1.31	<0.01	<0.01	0.28
Leu	PDV	27.2	38.2	26.0	42.9	3.35	<0.01	0.57	0.32
	HEP	4.4	4.4	5.3	3.8	2.45	0.69	0.95	0.69
	TSP	31.7	42.6	31.2	46.7	4.12	0.01	0.66	0.57
	MG	-27.1	-33.6	-27.4	-36.0	2.43	<0.01	0.29	0.40
	Milk	22.9	26.0	25.3	30.5	2.09	<0.01	<0.01	0.28
Lys	PDV	22.9	33.7	21.0	34.4	2.40	<0.01	0.78	0.55
	HEP	-0.3	-5.6	2.8	-3.1	1.98	<0.01	0.06	0.84
	TSP	22.6	28.1	24.0	31.4	2.71	0.01	0.27	0.64
	MG	-21.3	-25.1	-20.6	-27.1	1.57	<0.01	0.49	0.18
	Milk	17.4	19.7	19.2	23.1	1.59	<0.01	<0.01	0.28
Met	PDV	7.7	12.2	6.9	12.5	0.93	<0.01	0.79	0.54
	HEP	-1.9	-5.9	-1.0	-4.3	0.81	<0.01	0.02	0.36
	TSP	5.8	6.3	5.9	8.2	0.94	0.10	0.23	0.25
	MG	-5.7	-6.1	-6.2	-7.3	0.51	0.02	0.01	0.22
	Milk	6.0	6.7	6.6	7.9	0.57	<0.01	0.01	0.27
Phe	PDV	16.1	30.5	14.6	31.4	2.01	<0.01	0.87	0.55
	HEP	-7.3	-20.6	-5.3	-17.9	1.54	<0.01	0.04	0.74
	TSP	8.8	10.0	9.5	13.5	1.72	0.12	0.18	0.38
	MG	-9.8	-11.4	-10.8	-14.3	1.02	<0.01	<0.01	0.09
	Milk	9.3	10.4	10.2	12.2	0.89	<0.01	0.01	0.27
Thr	PDV	15.9	23.7	14.3	25.0	1.77	<0.01	0.92	0.37
	HEP	-2.9	-9.4	-0.5	-6.5	1.49	<0.01	0.04	0.83

	TSP	13.0	14.4	13.9	18.4	1.72	0.09	0.14	0.33
	MG	-12.0	-14.1	-13.7	-16.3	1.22	0.01	0.02	0.69
Trp	Milk	11.1	12.5	12.2	14.7	1.03	<0.01	<0.01	0.28
	PDV	3.5	4.6	2.6	4.9	0.56	0.01	0.50	0.23
	HEP	-1.7	-2.8	-0.8	-1.9	0.88	0.24	0.31	0.98
	TSP	1.8	1.8	1.8	3.0	0.78	0.40	0.46	0.43
Val	MG	-2.7	-3.2	-2.8	-3.8	0.38	0.06	0.34	0.45
	Milk	2.2	2.5	2.5	3.0	0.19	<0.01	<0.01	0.28
	PDV	17.0	21.8	16.5	26.1	2.02	<0.01	0.26	0.16
	HEP	3.0	3.1	4.5	2.5	2.04	0.56	0.79	0.50
NEAA	TSP	20.0	25.0	20.8	28.5	2.52	0.03	0.38	0.57
	MG	-18.4	-22.7	-19.2	-24.1	1.87	<0.01	0.23	0.76
	Milk	17.4	19.6	19.2	23.0	1.66	<0.01	0.01	0.27
Ala	PDV	47.0	65.6	39.4	61.2	5.05	<0.01	0.16	0.70
	HEP	-29.5	-56.4	-8.6	-33.8	5.60	<0.01	<0.01	0.75
Asn	TSP	17.5	9.2	30.9	27.4	4.33	0.09	<0.01	0.45
	MG	-11.5	-4.2	-18.7	-16.2	3.12	0.05	<0.01	0.28
	Milk	11.4	12.9	12.6	15.2	1.03	<0.01	<0.01	0.28
	PDV	15.1	23.1	13.2	21.1	2.87	0.06	0.42	1.00
	HEP	-7.5	-11.8	-4.4	-10.1	2.40	0.08	0.25	0.79
Asp	TSP	7.0	11.2	8.0	11.0	1.20	0.06	0.74	0.70
	MG	-6.1	-6.3	-6.7	-7.6	1.97	0.70	0.55	0.87
	Milk	9.8	11.0	10.8	12.9	0.90	<0.01	<0.01	0.28
	PDV	7.3	8.3	6.5	8.4	2.03	0.42	0.86	0.78
	HEP	-4.2	-4.6	-1.6	-4.3	1.81	0.18	0.23	0.32
Cit	TSP	3.2	3.7	5.4	4.1	2.10	0.85	0.47	0.63
	MG	-5.1	-4.4	-3.2	-3.4	1.64	0.80	0.20	0.68
	Milk	7.8	8.9	8.7	10.4	0.66	<0.01	<0.01	0.29
	PDV	4.9	7.6	7.9	6.1	1.38	0.72	0.55	0.08
	HEP	-2.5	1.6	0.9	0.4	2.37	0.35	0.56	0.23
Cys	TSP	2.5	9.2	9.0	6.4	2.01	0.26	0.31	0.03
	MG	-0.6	1.0	1.0	-3.3	1.43	0.34	0.35	0.06
	PDV	0.4	1.4	0.6	1.9	0.70	0.01	0.31	0.71
	HEP	-2.3	-3.1	-1.8	-2.9	0.42	0.03	0.42	0.73
	TSP	-1.9	-1.7	-1.2	-1.0	0.56	0.56	0.07	0.95
Gln	MG	-1.4	-1.7	-1.2	-1.8	0.26	0.05	0.95	0.47
	Milk	1.8	2.1	2.0	2.4	0.12	<0.01	<0.01	0.36
	PDV	-1.4	23.3	-10.9	15.8	2.37	<0.01	<0.01	0.68
	HEP	0.6	-30.2	7.3	-16.9	5.95	<0.01	0.01	0.26
	TSP	-0.8	-6.9	-3.3	-1.1	5.08	0.46	0.53	0.13
	MG	-19.5	-20.0	-20.9	-22.3	2.10	0.57	0.26	0.81

Glu	Milk	19.6	22.1	21.6	25.9	1.90	<0.01	0.01	0.27
	PDV	3.7	4.5	2.0	4.6	1.53	0.05	0.30	0.25
	HEP	27.2	22.4	43.0	33.5	5.03	0.02	<0.01	0.38
	TSP	30.8	26.9	45.0	38.1	4.16	0.05	<0.01	0.55
	MG	-18.4	-16.0	-24.2	-22.1	1.72	0.19	0.01	0.94
Gly	Milk	25.9	29.2	28.6	34.3	2.47	<0.01	0.01	0.27
	PDV	26.5	33.5	24.1	35.2	2.40	<0.01	0.84	0.27
	HEP	-32.5	-39.5	-31.4	-45.0	2.53	<0.01	0.26	0.10
	TSP	-6.0	-6.1	-7.2	-9.8	2.45	0.35	0.11	0.38
	MG	-4.0	-3.8	-2.4	-2.3	1.93	0.95	0.41	0.96
Orn	Milk	7.5	8.4	8.2	9.9	0.71	<0.01	0.01	0.27
	PDV	6.9	8.5	4.0	9.5	1.56	0.04	0.54	0.21
	HEP	3.6	3.4	4.5	2.4	1.26	0.20	0.91	0.26
	TSP	10.5	11.9	8.1	11.9	2.18	0.24	0.57	0.58
	MG	-9.5	-11.5	-10.4	-10.8	1.65	0.37	0.93	0.53
Pro	PDV	11.9	30.0	9.9	31.3	1.89	<0.01	0.86	0.34
	HEP	-3.4	-12.4	-2.1	-10.5	1.44	<0.01	0.24	0.82
	TSP	8.5	17.6	7.8	20.8	1.79	<0.01	0.44	0.25
	MG	-6.6	-10.7	-7.3	-13.6	1.53	<0.01	0.02	0.09
	Milk	26.9	30.2	29.6	35.4	2.67	<0.01	0.01	0.27
Ser	PDV	25.0	40.0	26.7	42.5	2.57	<0.01	0.37	0.86
	HEP	-20.7	-33.8	-21.3	-33.5	2.12	<0.01	0.92	0.79
	TSP	4.4	6.3	5.4	9.1	2.56	0.21	0.39	0.68
	MG	-8.7	-10.5	-11.6	-13.9	1.90	0.19	0.06	0.86
	Milk	18.8	21.1	20.7	24.8	1.83	<0.01	0.01	0.27
Tyr	PDV	14.0	13.7	12.8	14.7	1.04	0.35	0.91	0.19
	HEP	-6.0	-6.1	-4.7	-5.6	0.98	0.44	0.19	0.56
	TSP	8.0	7.5	8.1	9.1	1.12	0.77	0.39	0.44
	MG	-9.1	-9.7	-10.0	-10.7	0.96	0.24	0.09	1.00
	Milk	9.6	10.8	10.5	12.6	0.93	<0.01	0.01	0.27
Urea-N	PDV	-309	-497	-241	-440	71.0	<0.01	0.29	0.92
	HEP	458	800	303	698	62.1	<0.01	0.01	0.49
	TSP	149	304	75	259	58.0	0.01	0.30	0.79

¹The milk output associated with mammary uptake exclude BBP, as detailed in text.

²Ctrl: water infusion; AA_{CN}: 695 g/d of AA mixture with casein profile infusion; E_{Glc}: 1.454 g/d of glucose infusion; AA_{CN} + E_{Glc}: combination of AA mixture and glucose infusion.

³Probability values correspond to the effect of AA (without vs. with AA infusion), Glc (without vs. with glucose infusion) and AA × Glc interaction.

⁴SEM given for n = 4.

934

935 **Table 10.** Effects of AA and glucose infusions on fractional removal¹ of AA by the liver and
 936 the mammary gland
 937

	Treatments ²				SEM ⁴	P-values ³		
	Ctrl	AA _{CN}	E _{Glc}	AA _{CN} + E _{Glc}		Cas	Glc	Cas × Glc
Liver								
EAA								
Arg	0.02	0.08	-0.02	0.05	0.018	<0.01	0.06	0.84
His	0.01	0.06	0.00	0.03	0.013	<0.01	0.04	0.60
Lys	0.00	0.04	-0.03	0.02	0.017	0.01	0.07	0.64
Met	0.06	0.16	0.04	0.10	0.021	<0.01	0.06	0.26
Phe	0.10	0.17	0.08	0.15	0.015	<0.01	0.05	0.90
Thr	0.03	0.08	0.01	0.04	0.014	<0.01	0.05	0.47
Trp	0.03	0.05	0.02	0.04	0.015	0.27	0.31	0.97
NEAA								
Ala	0.10	0.22	0.04	0.13	0.022	<0.01	<0.01	0.37
Asn	0.13	0.16	0.06	0.14	0.039	0.18	0.23	0.48
Asp	0.08	0.12	0.06	0.10	0.033	0.05	0.33	0.79
Cys	0.02	0.03	0.01	0.02	0.003	0.01	0.23	0.67
Gln	-0.01	0.10	-0.03	0.05	0.018	<0.01	0.01	0.21
Gly	0.13	0.19	0.09	0.16	0.014	<0.01	0.03	0.87
Pro	0.04	0.06	0.03	0.05	0.010	0.02	0.26	0.97
Ser	0.18	0.27	0.15	0.22	0.022	0.01	0.11	0.53
Tyr	0.09	0.10	0.07	0.09	0.015	0.15	0.21	0.89
HMP-N ⁵	0.04	0.10	0.02	0.07	0.002	<0.01	0.03	0.36
Mammary gland								
EAA								
Arg	0.29	0.31	0.38	0.31	0.034	0.24	0.11	0.10
His	0.21	0.19	0.27	0.17	0.039	0.05	0.58	0.21
Ile	0.30	0.32	0.45	0.40	0.038	0.45	<0.01	0.16
Leu	0.41	0.34	0.61	0.46	0.042	0.01	<0.01	0.25
Lys	0.57	0.52	0.66	0.53	0.052	<0.01	0.06	0.11
Met	0.54	0.52	0.53	0.45	0.052	0.15	0.29	0.36
Phe	0.38	0.27	0.41	0.29	0.032	<0.01	0.36	0.90
Thr	0.25	0.27	0.25	0.23	0.029	0.93	0.31	0.44
Trp	0.13	0.16	0.12	0.14	0.021	0.26	0.49	0.82
Val	0.19	0.16	0.25	0.21	0.022	0.06	<0.01	0.50
NEAA								
Ala	0.10	0.04	0.17	0.13	0.026	0.04	<0.01	0.53
Asn	0.32	0.26	0.25	0.29	0.065	0.77	0.71	0.48
Asp	0.21	0.21	0.18	0.15	0.030	0.49	0.03	0.32
Cys	0.020	0.027	0.015	0.021	0.004	0.05	0.09	0.88
Gln	0.16	0.17	0.14	0.12	0.016	0.85	0.03	0.38
Glu	0.63	0.60	0.70	0.65	0.015	0.03	<0.01	0.54

Gly	0.04	0.05	0.01	0.02	0.016	0.57	0.13	0.81
Orn	0.67	0.45	1.42	0.47	0.420	0.15	0.33	0.36
Pro	0.17	0.12	0.18	0.14	0.026	0.01	0.32	0.65
Ser	0.23	0.27	0.21	0.22	0.055	0.40	0.29	0.66
Tyr	0.36	0.45	0.36	0.38	0.054	0.22	0.45	0.47
HMP-N ⁵	0.28	0.24	0.32	0.22	0.063	0.01	0.63	0.25
Group2 AA-N ⁶	0.36	0.32	0.48	0.39	0.037	0.02	<0.01	0.32
NEAA-N ⁷	0.15	0.15	0.14	0.13	0.013	0.81	0.31	0.65

¹Fractional removal calculated as $-(\text{net flux across the tissue}/\text{total influx})$; values for AA released by the tissue or all treatments are not reported; a negative value indicates a release for that treatment.

²Ctrl: water infusion; AA_{CN}: 695 g/d of AA mixture with casein profile infusion; E_{Glc}: 1,454 g/d of glucose infusion; AA_{CN} + E_{Glc}: combination of AA mixture and glucose infusion.

³Probability values correspond to the effect of AA (without vs. with AA infusion); effect of Glc (without vs. with glucose infusion); and interaction between AA and Glc: AA × Glc.

⁴SEM given for n = 4.

⁵HMP-N = sum of His, Met and Phe.

⁶Group2 AA-N: Sum of Ile, Leu, Lys and Val.

⁷NEAA-N: Sum of Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser and Tyr.

938

939 **Table 11.** Effects of AA and glucose infusions on the ratio of mammary uptake to milk
940 output¹ of AA

Item	Treatments ²				SEM ⁴	P-values ³		
	Ctrl	AA _{CN}	E _{Glc}	AA _{CN} + E _{Glc}		AA	Glc	AA×Glc
Arg	2.09*	2.09*	2.25*	2.17*	0.178	0.78	0.40	0.77
His	1.05	0.99	1.01	1.04	0.041	0.61	0.75	0.14
Ile	1.29*	1.43*	1.20*	1.29*	0.044	0.02	0.02	0.61
Leu	1.18*	1.31*	1.08	1.18*	0.047	0.03	0.03	0.80
Lys	1.23*	1.29*	1.07	1.18*	0.041	0.03	<0.01	0.52
Met	0.95	0.92*	0.95	0.92*	0.029	0.28	1.00	0.85
Phe	1.06	1.10*	1.06	1.17*	0.035	0.03	0.27	0.32
Thr	1.09*	1.13*	1.12*	1.11*	0.033	0.63	0.88	0.58
Trp	1.23†	1.27*	1.13	1.31*	0.129	0.39	0.82	0.59
Val	1.05	1.17*	0.99	1.05	0.048	0.08	0.07	0.49
Ala	0.97	0.27*	1.45†	1.05	0.201	<0.01	<0.01	0.31
Asn	0.65	0.58†	0.62	0.61†	0.139	0.72	0.97	0.86
Asp	0.65†	0.50*	0.37*	0.33*	0.202	0.53	0.14	0.71
Cys	0.78†	0.82†	0.61*	0.73*	0.108	0.43	0.20	0.67
Gln	1.00	0.91	0.99	0.88	0.079	0.10	0.70	0.86
Glu	0.72*	0.57*	0.88*	0.65*	0.074	0.02	0.13	0.60
Gly	0.55†	0.45*	0.27*	0.25*	0.254	0.80	0.34	0.88
Pro	0.24*	0.34*	0.24*	0.38*	0.033	<0.01	0.24	0.19
Ser	0.49*	0.52*	0.58*	0.57*	0.113	0.85	0.24	0.80
Tyr	0.94	0.89*	0.96	0.85*	0.037	0.04	0.67	0.45
TAA-N ⁵	1.03	1.00	1.03	1.00	0.021	0.09	0.98	0.87
EAA-N ⁶	1.31*	1.36*	1.28*	1.31*	0.040	0.41	0.31	0.85
Group1 AA-N ⁷	0.98	0.95*	0.97†	0.98	0.019	0.32	0.97	0.23
HMP-N ⁸	1.03	1.01	1.01	1.06†	0.027	0.69	0.51	0.13
Group2 AA-N ⁹	1.20*	1.30*	1.09*	1.17*	0.035	0.03	0.01	0.87
BCAA-N ¹⁰	1.17*	1.30*	1.08*	1.17*	0.044	0.03	0.03	0.64
NEAA-N ¹¹	0.70*	0.60*	0.74*	0.65*	0.038	0.02	0.18	0.85

¹The milk output associated with mammary uptake exclude BBP, as detailed in text.

²Ctrl: water infusion; AA_{CN}: 695 g/d of AA mixture with casein profile infusion; E_{Glc}: 1.454 g/d of glucose infusion; AA_{CN} + E_{Glc}: combination of AA mixture and glucose infusion.

³Probability values correspond to the effect of AA (without vs. with AA infusion), Glc (without vs. with glucose infusion) and AA × Glc interaction.

⁴SEM given for n = 4.

⁵TAA-N: Sum of Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

⁶EAA-N: Sum of Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val.

⁷Group1 AA-N: Sum of His, Met, Phe, Trp and Tyr.

⁸HMP-N: Sum of His, Met, Phe, used in Table 12.

⁹Group2 AA-N: Sum of Ile, Leu, Lys and Val.

¹⁰BCAA-N: Sum of Ile, Leu and Val.

¹¹NEAA-N: Sum of Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser and Tyr.

*Different from 1 at $P \leq 0.05$ and † at $0.05 < P \leq 0.10$.

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942 **Table 12.** Effects of AA and glucose infusions on supply, net flux (NF) and utilization of
 943 groups of AA by portal drained-viscera (PDV), hepatic (HEP), splanchnic tissues (TSP), and
 944 mammary gland (MG) and resulting estimated catabolism (mmol N/h)

	Treatments ¹				SEM ³	<i>P</i> -values ²		
	Ctrl	AA _{CN}	E _{Glc}	AA _{CN} + E _{Glc}		AA	Glc	AA×Glc
MP								
Supply	620	905	621	904	31.3	<0.01	0.75	0.31
Endo Uri	99.7	99.7	101.3	99.7	-	-	-	-
MFP	107	107	107	107	4.9	0.28	0.82	0.47
Scurf	3.9	3.9	3.9	3.9	-	-	-	-
Milk	344	388	379	455	32.4	<0.01	<0.01	0.27
HMP-N⁴								
Supply	49	88	49	88	2.3	<0.01	0.55	0.32
Endo Uri	2	2	2	2	-	-	-	-
MFP	10	10	10	10	0.5	0.28	0.82	0.47
PDV _{catabolism} ⁵	-3	5	-1	3	5.0	0.18	0.99	0.60
PDV _{NF}	42	72	40	74	4.6	<0.01	1.00	0.61
HEP _{NF}	-12*	-40*	-7	-30*	4.2	<0.01	0.01	0.40
TSP _{NF}	29	32	33	44	4.7	0.08	0.05	0.28
Scurf	0.2	0.2	0.2	0.2	-	-	-	-
Peri _{catabolism} ⁶	-4	-4	-2	0	2.5	0.66	0.33	0.65
MG _{NF}	-33	-36	-35	-44	2.7	<0.01	<0.01	0.05
MG _{catabolism} ⁷	0	-1	-1	1	1.0	0.69	0.41	0.07
Milk	33	37	36	43	3.1	<0.01	<0.01	0.28
Group2 AA-N⁸								
Supply	144	219	144	218	6.6	<0.01	0.40	0.08
Endo Uri	4.0	4.0	4.0	4.0	-	-	-	-
MFP	26	26	26	26	1.2	0.28	0.82	0.47
PDV _{catabolism} ⁵	11	41*	17	27*	12.6	0.07	0.71	0.35
PDV _{NF}	108	152	102	165	11.6	<0.01	0.73	0.36
HEP _{NF}	10	0	19†	3	9.0	0.06	0.33	0.59
TSP _{NF}	117	152	122	168	14.2	0.01	0.43	0.64
Scurf	0.7	0.7	0.7	0.7	-	-	-	-
Peri _{catabolism} ⁶	10	22*	14	29*	12.9	0.21	0.61	0.91
MG _{NF}	-107	-129	-108	-138	9.1	<0.01	0.28	0.33
MG _{catabolism} ⁷	15*	25*	6*	16*	2.8	<0.01	0.01	0.89
Milk	92	104	101	122	8.5	<0.01	<0.01	0.27
NEAA-N⁹								
Supply ¹⁰	359	498	359	497	19.3	<0.01	0.70	0.74
Endo Uri ¹⁰	59	59	59	59	-	-	-	-
MFP ¹¹	50	50	50	50	2.4	0.28	0.82	0.47
PDV _{catabolism} ⁵	161*	155*	190*	178*	29.9	0.62	0.16	0.85
PDV _{NF}	148	293	115	269	20.7	<0.01	0.14	0.79
HEP _{NF}	-76*	-210*	-21	-155*	24.3	<0.01	<0.01	0.99
TSP _{NF}	72	82	95	114	22.0	0.45	0.17	0.82
Scurf ¹⁰	2.1	2.1	2.2	2.1	-	-	-	-
Peri _{catabolism} ⁶	-39†	-24	-35	-25	20.5	0.54	0.94	0.90
MG _{NF}	-108	-104	-124	-137	10.2	0.49	<0.01	0.22
MG _{catabolism} ⁷	-63*	-89*	-65*	-90*	12.2	<0.01	0.88	0.94
Milk ¹⁰	172	194	189	227	16.2	<0.01	<0.01	0.27

¹Ctrl: water infusion; AA_{CN}: 695 g/d of AA mixture with casein profile infusion; E_{Glc}: 1,454 g/d of glucose infusion; AA_{CN} + E_{Glc}: combination of AA mixture and glucose infusion.

²Probability values correspond to the effect of AA (without vs. with AA infusion); effect of Glc (without vs. with glucose infusion); and interaction between AA and Glc: AA × Glc.

³SEM given for n = 4.

⁴HMP-N: Sum of His, Met and Phe.

⁵PDV_{catabolism}: catabolism across PDV.

⁶Peri_{catabolism}: catabolism across peripheral tissues other than mammary gland.

⁷MG_{catabolism}: catabolism across mammary gland.

⁸Group2 AA-N: Sum of Ile, Leu, Lys and Val.

⁹NEAA-N: Sum of Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser and Tyr.

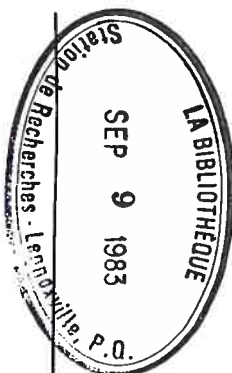
¹⁰Obtained by difference between MP and EAA-N fractions.

*Catabolisms different from 0 at $P \leq 0.05$ and † at $0.05 < P \leq 0.10$.

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ANALYSIS OF VARIANCE III: FACTORIAL EXPERIMENTS

15.1 Introduction

In this chapter, we deal with factorial experiments. Here there are a number of treatments in each of several categories and they define a treatment grid. This choice of treatment design leads to a logical partitioning of the treatment sum of squares into additive components with corresponding tests of hypotheses.

The topic of independent comparisons, introduced in Sec. 8.3, is discussed further, with special attention given to equally spaced treatments and regression. Tukey's test for nonadditivity is also given.

15.2 Factorial Experiments

A *factor* is a kind of treatment, and in factorial experiments, any factor will supply several treatments. For example, if diet is a factor in an experiment, then several diets will be used; if baking temperature is a factor, then baking will be done at several temperatures.

The concept of the factorial experiment can be illustrated by an example. Consider an experiment to evaluate the yielding abilities of several

soybean cultivars. In a single-factor approach, all variables other than cultivar are held as uniform as possible, that is, one particular level of each of the other factors is chosen. Suppose that a second factor, distance between rows, is of interest. A two-factor experiment can be planned in which the treatments consist of all combinations of cultivars and the chosen row spacings, that is, each cultivar is present at all row spacings. In a single-factor experiment, all cultivars would be planted at only one row spacing or one cultivar would be planted for all row spacings. In soils, an experiment could be designed to compare all combinations of several rates of phosphorus and potassium fertilizers. In an animal feeding experiment, factors under consideration might be amounts and kinds of protein supplement.

The term *level* refers to the several treatments within any factor. It is derived from some of the earliest factorial experiments. These dealt with soil fertility where combinations of several amounts, or levels, of different fertilizers were the treatments. Today the word has a more general meaning, implying a particular amount or state of a factor. Thus, if five cultivars of a crop are compared using three different management practices, the experiment is called a 5×3 factorial experiment with five levels of the cultivar factor and three levels of the management factor. The number of factors and levels which may be compared in a single experiment is limited only by practical considerations.

Thus, a *factorial experiment* is one in which the set of treatments consists of all possible combinations of the levels of several factors. A treatment design is implied in the word "factorial."

Factorial experiments are used in practically all fields of research. They are of great value in exploratory work where little is known concerning the optimum levels of the factors, or even which ones are important. Consider a new crop for which several promising cultivars are available but little is known regarding a suitable date and rate of planting. A three-factor experiment is indicated. If the single-factor approach is used, one date and rate of planting are selected and a cultivar experiment is conducted. However, the cultivar that does best at the chosen date and rate may not be the best for some other date and rate. Any other single factor must involve only one cultivar. At other times, the experimenter may be primarily interested in the interaction between factors, that is, she may wish to know if the differences in response to the levels of one factor are similar or different at different levels of another factor or factors. Where considerable information is available, the best approach may be to compare only a very limited number of combinations of several factors at specific levels.

Thus, we see that the scope of an experiment, or the population concerning which inferences can be made, can often be increased by the use of a factorial experiment. It is particularly important to do this where information is wanted on some factor for which recommendations are to be made over a wide range of conditions.

Main effects are seen to be computed on a per-unit basis. Equations (15.1) and (15.2) are easily extended to 2^n factorials.

Main effects are averages over a variety of conditions just as are any other treatment means. For a factorial experiment in a randomized complete block or Latin square design, the variety of conditions exists within blocks as well as among blocks; thus factor A is replicated within every block since it is present at both levels for each level of factor B . This is *hidden replication*. Averaging implies that the differences, that is, the simple effects, vary only because of chance from level to level of the other factor or factors. This is, in fact, a hypothesis that is usually subjected to a test of significance when treatments are factorially arranged; the hypothesis is that of no interaction between factors.

For the data under I and II, the simple effects for both kind and rate differ. Under III, the simple effects for A are the same, as are the simple effects for B ; here, they also equal the corresponding main effect. When simple effects for a factor differ by more than can be attributed to chance, this differential response is termed an *interaction* of the two factors. The relation is a symmetric one; that is, the interaction of A with B is the same as that of B with A . From Table 15.1 you will see that the difference between the simple effects of A equals that for B in all three cases; it would be impossible to construct a table otherwise. In our notation, the interaction of A and B is defined in

$$AB = \frac{1}{2}[(a_2b_2 - a_1b_2) - (a_2b_1 - a_1b_1)] \\ = \frac{1}{2}[(a_2b_2 + a_1b_1) - (a_1b_2 + a_2b_1)] \quad (15.3)$$

The value $\frac{1}{2}$ is used so that interaction, like the main effects, is on a per-unit basis. For the data under I,

$$AB = \frac{1}{2}(8 - 2) \quad \text{in terms of simple effects of } A \\ = \frac{1}{2}(12 - 6) \quad \text{in terms of simple effects of } B \\ = 3$$

For the data under II, we find

$$AB = \frac{1}{2}(26 - 36 - 32 + 30) = -6$$

and under III,

$$AB = \frac{1}{2}(38 - 36 - 32 + 30) = 0$$

Note that the interaction is also one-half the difference between the sums of the two diagonals of the 2×2 table, which is one-half the difference between the sums of the treatments where both A and B are present at the higher and lower levels and of the treatments where only one is present at the higher level. This is always true of the 2^2 factorial.

Interaction measures the failure of the A effect, or the response to A , to be the same for each level of B or, conversely, the failure of the B effect to be the same for each level of A . Under I, the simple effects for kind are 2 and 8 whereas the main effect is 5. Interaction may be defined as a measure of the departure of the simple effects from an additive law or model based on main effects only.

Under I, Table 15.1, the response to A or the increase from a_1 to a_2 is greater for b_2 than for b_1 , that is, there has been a change in the magnitude of the increase. Under II, the response to A is an increase in the presence of b_1 and a decrease in the presence of b_2 ; there has been a change in the direction of the increase. In terms of treatment means presented in a two-way table, sufficiently large changes in the magnitudes of the differences between treatment means in a column (or row), as one goes from column to column (or row to row), constitute an interaction. Also, changes in the rank of any treatment mean for a column (or row), as one changes columns (or rows), may constitute an interaction.

Figure 15.1 illustrates graphically what is meant by an interaction. The presence or absence of main effects tells us nothing about the presence or

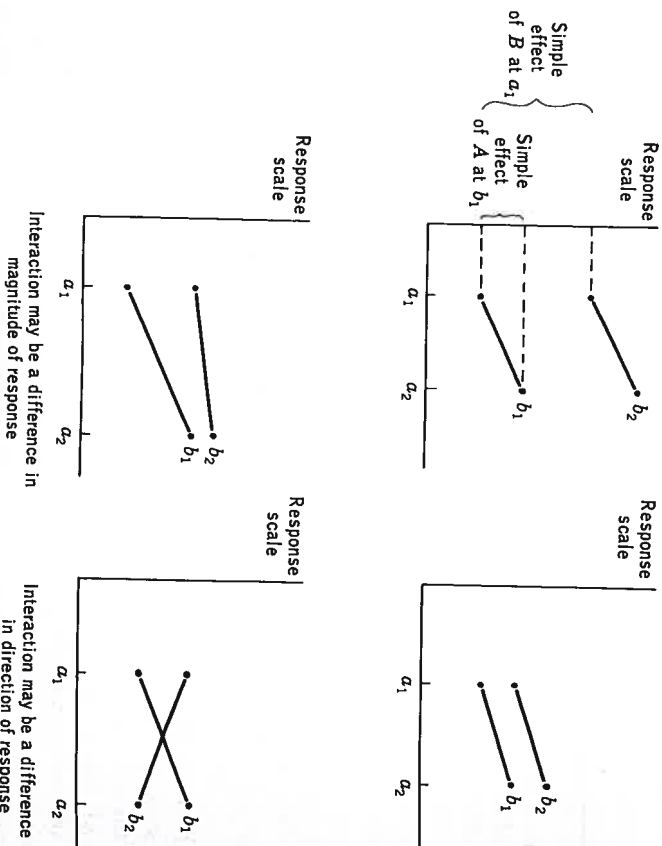


Figure 15.1 Illustration of interaction.

absence of interaction. The presence or absence of interaction tells us nothing about the presence or absence of main effects but does tell us something about the homogeneity of simple effects.

A significant interaction is one that is too large to be explained on the basis of chance and the null hypothesis of no interaction. With a significant interaction, the factors are not independent of one another; the simple effects of a factor differ and the magnitude of any simple effect depends on the level of the other factor of the interaction term. Where factors interact, a single-factor experiment will lead to disconnected and possibly misleading information.

If the interaction is nonsignificant, it is concluded that the factors under consideration act independently of each other; the simple effects of a factor are the same for all levels of the other factors, within chance variation as measured by experimental error. The average of simple effects, namely, the main effect, is appropriate and the best estimate of the common difference. The evidence is that there is no departure from a simple additive model with components due to the levels of the factors only. Where factors are independent, the factorial experiment saves considerable time and effort. This is so since the simple effects are equal to the corresponding main effects and a main effect, in a factorial experiment, is estimated as accurately as it would be if the entire experiment had been devoted to that factor.

In the analysis of a factorial experiment, it is correct to partition the treatment degrees of freedom and sum of squares into the components attributable to main effects and interactions even when the overall F -test of the differences among treatments is not significant; main effects and interaction comparisons are planned comparisons. It is easy to visualize a situation where one factor, say B , does neither good nor harm in itself nor has any effect on A and hence contributes no more to the treatment sum of squares than can be attributed to chance; a significant response to A might well be lost in an overall test of significance such as F . Thus in a 2^2 factorial with 3 degrees of freedom for treatments, the sum of squares for the real A effect with its 1 degree of freedom may easily be lost when averaged with the sums of squares for the nonreal B and AB with their 2 degrees of freedom. Calculation of the sum of squares for treatments is more often used as part of a computational procedure than to supply the numerator of an F -test.

All units of the factorial experiment are involved in measuring any one main effect or interaction. This is apparent from Eqs. (15.1) to (15.3). Thus it appears that in a factorial experiment as opposed to several single-factor experiments, all units are devoted to measuring A , and in turn to B , and to AB , and so on, when there are more factors; nothing is lost either in replication or in measuring main effects; something is gained in that we do measure any one factor at various levels of other factors and, in terms of treatment comparisons, we are also able to measure interactions and test hypotheses concerning them.

The results of a factorial experiment lend themselves to a relatively

simple explanation because of the variety and nature of treatment comparisons. If the factors are largely independent, the table of treatment means and analysis of variance summarize the data well. When the factors are not independent, the data require a detailed study with the possibility of further experimentation. Here the difficulty lies in the complex nature of the situation and not in the factorial approach to it. The factorial experiment has indicated the complexity, a fact that might well have been missed had a single-factor approach been used.

15.3 The 2×2 Factorial Experiment: An Example

Wilkinson (15.17) reports the results of an experiment to study the influence of time of bleeding, factor A , and diethylstilbestrol (an estrogenic compound), factor B , on plasma phospholipid in lambs. Five lambs were assigned at random to each of four treatment groups; treatment combinations are for morning and afternoon times of bleeding with and without diethylstilbestrol treatment. The data are shown in Table 15.2.

Table 15.2 The influence of time of bleeding and diethylstilbestrol on phospholipid in lambs

	Treatment groups		Totals	
	$a_1, b_1 =$ control 1	$a_1, b_2 =$ treated 1	$a_2, b_1 =$ control 2	$a_2, b_2 =$ treated 2
8.53	17.53	39.14	32.00	
20.53	21.07	26.20	23.80	
12.53	20.80	31.33	28.87	
14.00	17.33	45.80	25.06	
10.80	20.07	40.20	29.33	
$\sum Y$	66.39	96.80	182.67	139.06
$\sum Y^2$	963.88	1,887.02	6,913.63	3,912.17
$\frac{\sum Y^2}{Y}$	13.28	19.36	36.53	27.81
	Treatment totals			484.92
				13,676.70
				24.25

Factor	$A = \text{time}$				Totals
	Level	$(a_1) = \text{A.M.}$	$(a_2) = \text{P.M.}$		
$B = \text{estrogen}$	$(b_1) = \text{control}$	66.39	182.67	249.06	
	$(b_2) = \text{treated}$	96.80	139.06	235.86	
Totals		163.19	321.73	484.92	

DESIGN AND ANALYSIS OF EXPERIMENTS

in the Animal and Medical Sciences

J O H N L . G I L L

VOLUME I

John L. Gill is professor of biometry in the Department of Dairy Science at Michigan State University. He received the M.S. and Ph.D. degrees in animal genetics and statistics at Iowa State University. Besides this book, he is author of many articles in the journals of his fields, and he has had long experience as a teacher of applied statistics and statistical consultant in the biological sciences.

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2.4.1. Advantages and Disadvantages of Factorial Experiments

If the effects of 2 or more factors on an observable trait are not independent, i.e., if the factors interact synergistically or antagonistically, the existence and nature of the interaction cannot be determined unless the factors are studied in combination in the same experiment. Ignorance of interaction often results in too broad, unwarranted generalizations or inferences. Even if factors studied simultaneously do not interact, factorial experiments save time and resources because the *main* (mean) effect of each factor is estimated with as much precision as if the entire experiment were devoted to one factor.

However, factorial experiments involving a large number of treatment combinations require extensive resources. In some cases one may be able to obtain the most important information by sacrificing information on higher order interactions (among 3 or more factors) to formulate fractionally replicated designs. Higher order interactions usually are small in effect relative to the magnitude of main effects and first order interactions (between only 2 factors at a time) and often are difficult to interpret if they are not small. An occasional 3-way interaction is clearly very strong. For example, Thatcher and Tucker (1970) found that the DNA content of mammary glands of intensely suckled rats was increased by injection of growth hormone with prolactin but without cortisol-21-acetate; but DNA content was unchanged by injection of growth hormone with cortisol-21-acetate regardless of the presence or absence of injected prolactin. The interpretability of such higher order interactions usually depends on the existence of a theoretical body of knowledge, such as the feedback mechanisms of the endocrine system. Graphical aids may be helpful in interpretation (see Sec. 2.4.5).

Large factorial experiments may lead to increased standard error per experimental unit because the large number of units required may be rather heterogeneous in ways affecting the primary trait. Of course this problem may be partially overcome by forming blocks; or if heterogeneity of units within complete blocks (as many units as treatments) still presents a problem, by sacrificing information on one or more higher order interactions as a basis for constructing incomplete blocks (fewer units than treatments).

2.4.2. Fixed, Mixed, and Random Models; Formulation of Sums and Squares and Expected Mean Squares

The Australian statistician, E. J. Williams, has written that "data collection is meaningful only in relation to some model, even if only a rudimentary one, of the characteristics of the population being studied. The mathematical model of any phenomenon makes a contribution only if it leads to conclusions that can be checked against observational or experimental evidence. What is important in a mathematical model is not its validity (which is illusory), but its capacity to generalize to different physical situations, its ability to predict observable consequences, and its aid to the imagination in the formulation of new concepts."

A factor is said to have *fixed effects* if the levels of treatment or classes of interest are selected deliberately by the experimenter and the primary objective of the analysis is estimation and comparison of means of those levels or classes. In designed experiments most factors are considered fixed.

A factor may be said to have *random effects* if the classes of interest are chosen randomly from some larger set (e.g., as in sampling only certain groups from large collections of similar classes in survey or field work) or if the classes observed may be considered representative of a larger set for

teract, and the combination means (\bar{y}_{ij}) rather than the main effect means ($\bar{y}_{i..}$ and $\bar{y}_{.j}$) are the critical quantities. For factors with more than 2 levels, more specific tests may be desired. Orthogonal contrasts for one factor, say A , may be calculated separately within each level of the other factor, B , using totals of observations for appropriate combinations. Such contrasts represent orthogonal partition of $ss_A + ss_{AB}$. Contrasts for B within each level of A , i.e., partition of $ss_B + ss_{AB}$, are *not* independent of the first group because ss_{AB} is involved in both groups. Alternatively one may devise orthogonal contrasts among the set of treatment combination means. Nonorthogonal designed contrasts among treatment combination means and contrasts contrived a posteriori are tested by Bonferroni t statistics and Scheffé's procedure, respectively. In the latter procedure, the t of (2.67) is the number of treatment combinations (i.e., $t = ab$).

For quantitative factors with 3 or more levels, one may partition the degrees of freedom for interaction by using orthogonal polynomials. For example, if factors A and B each have 3 quantitative levels so linear (L) and quadratic (Q) terms may be examined, the 4 df for a significant AB interaction may be partitioned into contrasts for components A_L^B , $A_L^B Q$, A_Q^B , and $A_Q^B Q$, each with 1 df. Coefficients for the 9 treatment combination totals for each contrast may be determined by finding the products of the arrays of coefficients (App. A.7) for the polynomial effects combined. For example, the coefficients for totals $y_{1..}$, $y_{2..}$, $y_{3..}$ for the linear effect of A are -1, 0, +1, respectively, and those for $y_{.1}$, $y_{.2}$, $y_{.3}$ for a second degree curve for B are +1, -2, +1, respectively. The 9 products of coefficients (-1, +2, -1, 0, 0, 0, +1, -2, +1) are the proper coefficients for the treatment combination totals (y_{11} , y_{12} , y_{13} , y_{21} , y_{22} , y_{23} , y_{31} , y_{32} , y_{33}) in the contrast for interaction component $A_L^B Q$. A significant $A_L^B Q$ component could occur if consecutive unit increments of A produce equal increments of response within any level of B , but the increments of response to consecutive unit increments of B diminish at differing rates for the different levels of A (Fig. 2.3). A more sophisticated approach for interacting quantitative factors involves the design and analysis of response surfaces (Chap. 9).

The use of all possible pair-wise comparisons among means of treatment combinations cannot be recommended because the only unconfounded (unambiguous) comparisons are those made among the means sharing the same level of A or the same level of B (Cicchetti 1969).

If the hypothesis $H: (\alpha\beta)_{ij} = 0$ (for all i and j) is accepted, one may assume that if interaction exists at all, it may be negligible or small relative to the main effects. Also, tests concerning general and specific differences among the means for levels of each factor may be performed independently of the other factor. Mead et al. (1975) have shown that one should not pool an "insignificant" interaction with error to test main effects unless the degrees of freedom for error are few and less than the degrees of freedom for interaction (an uncommon situation). Note from Table 2.15 that if effects of A and B both are fixed, ms_E is an appropriate denominator of f statistics to test $H: \alpha_i = 0$ (for all i) and $H: \beta_j = 0$ (for all j). However, if only one factor has fixed effects, say A , a test of $H: \alpha_i = 0$ (for all i) will involve ms_{AB} in the denominator of the test statistic. Conversely, if both A and B have random