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Author: C.E. Medina-Torres, C.C. Pollitt, C. Underwood, E.M. Castro-Olivera, S.N. Collins, R.E. Allavena, D.W. Richardson, A.W. Eps

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1 **Equine lamellar energy metabolism studied using tissue microdialysis**
2

3 C.E. Medina-Torres [a], C.C. Pollitt [a], C. Underwood [a], E.M. Castro-Olivera [a], S.N. Collins [a],
4 R.E. Allavena [a], D.W. Richardson [b], A.W. van Eps [a,*]
5
6 [a] *Australian Equine Laminitis Research Unit, School of Veterinary Science, Faculty of*
7 *Science, University of Queensland, Gatton Campus, Gatton, QLD, 4343, Australia*
8 [b] *New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, 382 West*
9 *Street Road, Kennett Square, PA 19348-1692, USA*
10
11
12
13
14 * Corresponding author. Tel.: +61 7 5460 1799
15 *E-mail address:* a.vaneps@uq.edu.au (A.W. van Eps).
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19 **Abstract**

20 Failure of lamellar energy metabolism may contribute to the pathophysiology of equine
21 laminitis. Tissue microdialysis has the potential to dynamically monitor lamellar energy
22 balance over time. The objectives of this study were to develop a minimally invasive lamellar
23 microdialysis technique and use it to measure normal lamellar energy metabolite
24 concentrations over 24 h. Microdialysis probes were placed (through the white line) into
25 either the lamellar dermis (LAM) (n = 6) or the sublamellar dermis (SUBLAM) (n = 6) and
26 perfused continuously over a 24 h study period. Probes were placed in the skin dermis
27 (SKIN) for simultaneous comparison to LAM (n = 6). Samples were collected every 2 h and
28 analysed for glucose, lactate, pyruvate, urea and glycerol concentrations. LAM was further
29 compared with SUBLAM by simultaneous placement and sampling in four feet from two
30 horses over 4 h. Horses were monitored for lameness, and either clinically evaluated for 1
31 month after probe removal (n = 4) or subjected to histological evaluation of the probe site (n
32 = 10).

33

34 There were no deleterious clinical effects of probe placement and the histological
35 response was mild. Sample fluid recovery and metabolite concentrations were stable for 24 h.
36 Glucose was lower (and lactate:glucose ratio higher) in LAM compared with SUBLAM and
37 SKIN (P < 0.05). Pyruvate was lower in SUBLAM than SKIN and urea was lower in LAM
38 than SKIN (P < 0.05). These differences suggest lower perfusion and increased glucose
39 consumption in LAM compared with SUBLAM and SKIN. In conclusion, lamellar tissue
40 microdialysis was well tolerated and may be useful for determining the contribution of
41 energy failure in laminitis pathogenesis.

42

43 *Keywords:* Horse; Laminitis; Lamellae; Bioenergetics; Urea clearance.

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45 **Introduction**

46 Failure of lamellar energy metabolism may be an important contributor to laminitis
47 development (Belknap et al., 2009; French and Pollitt, 2004; Pass et al., 1998; van Eps et al.,
48 2010). In vitro lamellar glucose starvation causes hemidesmosome loss with resultant
49 weakening of the dermo-epidermal connection and lamellar separation (French and Pollitt,
50 2004; Pass et al., 1998). Despite the potential importance of bioenergetic disturbances in the
51 pathogenesis of laminitis, very little is known about normal lamellar energy metabolism.
52
53 Tissue microdialysis is a minimally invasive technique used to assess local energy
54 metabolism through measurement of energy metabolites such as glucose, pyruvate and
55 lactate. A probe with a semi-permeable membrane, introduced into the tissue of interest, is
56 slowly and continuously perfused allowing molecules to diffuse across the membrane down
57 their concentration gradient (de Lange, 2013). Therefore, metabolite concentrations in the
58 microdialysis sample (dialysate) are reflective of the metabolite concentration in the
59 interstitial fluid of the studied tissue (Klaus et al., 2004). Microdialysis is used in clinical and
60 research settings to study tissue bioenergetic disturbances (ischaemic and non-ischaemic)
61 particularly in the brain (Benveniste et al., 1989; Hillered et al., 2006; Klaus et al., 2004;
62 Oddo et al., 2012a,b; Schulz et al., 2000). The addition of a known (high) concentration of
63 urea to the perfusate allows for determination of urea clearance, an estimate of local
64 perfusion (Farnebo et al., 2010).
65
66 In horses, microdialysis has been used in pharmacokinetic studies and for assessment of
67 muscle energy metabolism (Chou et al., 2001; Edner et al., 2005, 2009; Ingvastlarsson et al.,
68 1992; Murchie et al., 2006; Vickroy et al., 2008). Lamellar microdialysis has been described
69 for pharmacokinetic studies (Nourian et al., 2010a, b). However, the previously reported

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70 technique necessitated removal of a 2 cm diameter portion of hoof wall over the insertion
71 site, potentially introducing heat artefact and interfering with normal mechanical forces
72 acting on the sampled area.
73
74 The primary objectives of the current study were: (1) to develop and evaluate a lamellar
75 microdialysis technique without hoof wall disruption at the probe membrane site, and (2) to
76 measure and compare energy metabolites in microdialysate from the lamellar, sublamellar
77 and skin dermis of normal horses over 24 h.
78
79 **Materials and methods**

80 *Animals and experimental design*

81 Fourteen clinically normal Standardbred horses (12 geldings and 2 mares; mean age, 6.5
82 ± 3.03 years; mean bodyweight, 461.2 ± 49.3 kg) with radiographically normal forefeet were
83 used. The project was approved by the University of Queensland Animal Ethics Committee
84 (approval number: SVS/257/11/GJRF) that monitors compliance with the Animal Welfare
85 Act (2001) and The Code of Practice for the care and use of animals for scientific purposes
86 (current edition).
87
88 Three experiments were performed. In experiment 1, linear microdialysis probes were
89 inserted in the lamellar dermis (LAM) of one forelimb (Figs. 1A and B) and the skin dermis
90 (SKIN) over the tail base in six horses. Simultaneous samples were obtained every 2 h for
91 comparison of metabolites over a 24 h period with horses confined to stocks. Four of these
92 horses then had the probes removed and were monitored for 1 month afterwards to assess
93 recovery. In experiment 2, dialysate metabolite concentrations in the sublamellar dermis and
94 lamellar dermis were compared by inserting two coaxial microdialysis probes into each

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95 forelimb of two horses: one directed into the sublamellar dermis (SUBLAM) and the other
96 into the LAM (Figs. 1C and D). Simultaneous hourly sampling was performed for 4h in
97 stocks. Experiment 3 was performed to further assess dialysate metabolite concentrations in
98 the SUBLAM: coaxial microdialysis probes were placed in the SUBLAM of one foot (Fig.
99 1C) in six horses and sampling was performed every 2 h for 24 h with horses confined to
100 stocks. In two of these horses, sampling was continued every 6 h for an additional 96 h in a
101 stall.
102
103 *Microdialysis probe placement and perfusion*
104 For lamellar (LAM and SUBLAM) placement, keratinized tissue was resected from the
105 white line region (on midline, dorsal to the point of the frog) with an electric rotating burr
106 (Die-Grinder, Makita) to create an ovoid 1.5 × 1.0 cm hole, leaving the majority of the
107 stratum medium of the distal hoof wall intact. The depth was slowly increased until the white
108 line was easily depressed with forceps. Bilateral abaxial sesamoid perineural anaesthesia was
109 performed with 2% lignocaine (Ilium Lignocaine 20, Troy Laboratories).
110
111 After aseptic preparation of the hoof and pastern, the hoof was covered with a sterile
112 adhesive drape (Ioban, 3M). Spinal needles (15 cm) were used as introducers for lamellar
113 probe placement. A 20 G needle (Becton-Dickinson) was used for linear probes (CMA66,
114 CMA-Microdialysis) and an 18 G needle (Lochimed) for coaxial probes (CMA20, CMA-
115 Microdialysis). The needles were inserted at the white line and advanced proximally (parallel
116 with the dorsal hoof wall for LAM placement and at a ~5º angle toward the third phalanx for
117 SUBLAM placement) until the tip appeared through the skin above the coronet. The stylet
118 was removed and a microdialysis probe with a 10 × 0.5 mm, 100 kDa cut-off membrane was

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119 inserted to position the probe membrane in the mid lamellar region. The needle was removed
120 leaving the probe in place (Figs. 1A and C).
121
122 The inlet/outlet tubing was passed through a 2 mm hole drilled in the distal hoof wall
123 defect to emerge on the dorsal surface of the hoof. The outlet tubing was connected to a
124 custom made microdialysis vial holder (Fig. 1B) secured to the dorsal hoof wall, and the inlet
125 tubing was connected to a precision pump (CMA107, CMA-Microdialysis) housed in a
126 custom limb boot over the metacarpus. The defect at the toe was filled with silicone putty
127 (Hoof-Life).
128
129 For SKIN probe placement, a linear microdialysis probe with a 10 × 0.26 mm, 55 kDa
130 cut-off membrane (CMA31, CMA-Microdialysis) was inserted into the dermis over the tail
131 base using a 21 G, 38 mm needle (Becton-Dickinson), after aseptic preparation and
132 subcutaneous infiltration with local anaesthetic (2% lignocaine). The inlet tubing was
133 connected to a precision pump (CMA107) and the outlet tubing to a microdialysis vial
134 (Microvial, CMA-Microdialysis), both secured to the tail.
135
136 All probes were perfused with an isotonic, polyionic sterile perfusion fluid (T1, CMA-
137 Microdialysis) containing 40 g/L dextran-70 (Sigma-Aldrich) to prevent perfusate loss
138 (Rosdahl et al., 1997). The perfusate in experiments 2 and 3 contained urea (20 mmol/L) for
139 determination of urea clearance (Farnebo et al., 2010). All probes were perfused for 30 min
140 prior to implantation, then continuously at a rate of 1 μL/min throughout the experiments.
141 The perfusion rate was decreased to 0.3 μL/min during the additional 96 h sampling period in
142 stalls for two horses in experiment 3.
143

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144 *Sample collection and analysis*

145 After probe insertion, a 2 h stabilization period was allowed before commencement of
146 sample collection. Dialysate samples were simultaneously collected every 2 h from the LAM
147 and SKIN probes (experiment 1), hourly from SUBLAM and LAM probes (experiment 2)
148 and every 2 h from SUBLAM probes in experiment 3. The collected dialysate samples were
149 weighed and fluid recovery calculated as a percentage of the perfused volume during the
150 sampling period (120 µL). Glucose, lactate, pyruvate, urea and glycerol concentrations were
151 determined immediately (analysed within 3 min of sample collection) using a commercially
152 available, dedicated microdialysis analyser (IscusFlex, CMA-Microdialysis). Standard
153 indices of energy metabolism (lactate:glucose [L:G] and lactate:pyruvate [L:P]) were
154 calculated.

155

156 *Lameness evaluation*

157 Digital pedometers (Digiwalker sw700, Yamax) were taped to the distal antebrachium
158 of both forelimbs in all horses to record weight shifting frequency across the entire sampling
159 period (counts recorded and reset every 2 h). Lameness evaluations were performed
160 immediately prior to and at the conclusion of the entire sampling period in each experiment.
161 Each horse was walked and trotted towards and away from the observer and circled to the
162 right and left. Lameness was graded on a 0 – 5 scale following the AAEP guidelines[1]. In the
163 four horses from experiment 1 where the probes were removed, lameness evaluations and
164 visual inspection of the hooves were performed weekly for 4 weeks.

165

166 *Histology*

[1] American Association of Equine Practitioners; see: www.aaep.org

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167 Two horses from experiment 1, and all horses in experiments 2 (*n* = 2) and 3 (*n* = 6)
168 were euthanased with pentobarbital sodium (20 mg/kg IV) (Lethabarb, Virbac) and lamellar
169 tissue harvested and prepared for light microscopy as previously described (Pollitt, 1996).
170 The area of skin containing the SKIN probe was also harvested. The tissue sections were
171 blindly assessed by a specialist veterinary pathologist (REA) and scored using a semi-
172 quantitative method (Table 1).

173

174 *Statistical analysis*

175 The data failed normality testing therefore non-parametric tests were used. The
176 concentrations of dialysate analytes, ratios (L:G and L:P), fluid recovery, and pedometer
177 counts were compared over time using Friedman analysis with Dunn's post-tests, and also
178 between LAM and SKIN at each time point in experiment 1 using Wilcoxon signed-rank
179 tests. The median concentrations of analytes and calculated ratios were compared between
180 LAM and SKIN (experiment 1), and SUBLAM (experiment 3) using Mann-Whitney tests.
181 Wilcoxon signed-rank tests were also used to compare pedometer count frequencies between
182 limbs (experiments 1 and 3). Data from experiment 2 were analysed descriptively. Statistics
183 were performed using GraphPad Prism v6.00 for Windows (GraphPad Software) with
184 significance set at $P < 0.05$. Data are expressed as median with interquartile range (IQR).

185

186 **Results**

187 *Probe performance and clinical effects*

188 In each horse the microdialysis probes remained in position and were functional
189 throughout the 24-h study periods whilst confined to stocks. Pedometer count frequency did
190 not differ significantly between limbs and was not significantly different over time in any
191 experiment (data not shown). The lameness score was 0 at each observation interval for all

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192 horses, including over the 1-month follow-up period (n = 4; experiment 1) and there were no
193 visible changes in external hoof wall and sole morphology during this period.
194
195 *Dialysate analysis*
196 Fluid recovery did not vary significantly over time (Fig. 2), nor was there a difference
197 between LAM (100.1%, [97.0-102.4%]), SKIN (95.7%, [90.7-99.3%]) and SUBLAM
198 (101.7%, [99.6-103.9%]) recovery. When considering the median dialysate analyte values
199 from all time-points during experiments 1 and 3 (Table 2), LAM glucose concentrations were
200 lower and LAM L:G higher than both SKIN and SUBLAM, LAM endogenous urea
201 concentrations were lower than SKIN, and SUBLAM pyruvate concentrations were higher
202 than SKIN ($P < 0.05$). SUBLAM and LAM urea concentrations were not compared due to
203 the addition of urea to the dialysate for urea clearance studies in experiment 3. In all three
204 experiments, median concentrations of glucose, lactate, pyruvate and urea, as well as L:G and
205 L:P did not differ significantly over time in LAM, SKIN and SUBLAM dialysate, whereas
206 glycerol concentration decreased significantly ($P < 0.05$) in each (Fig. 2).
207
208 In experiment 2, glucose and pyruvate concentrations appeared to be lower, and L:G
209 higher in LAM vs. SUBLAM dialysate; however the data were not statistically analysed (Fig.
210 3). Urea (mmol/L) also appeared to be higher in LAM (13.53, [13.2-13.6]) vs. SUBLAM
211 (11.18, [10.11–12.03]). The perfusate urea concentration was not measured in experiment 2.
212 The urea concentration (mmol/L) in SUBLAM dialysate in experiment 3 was 14.83 [13.95-
213 14.92] with a measured perfusate concentration of 17.92 [17.54-18.36].
214
215 Extended sampling (96 h in stalls) in two horses (H5 and H6) from experiment 3 yielded
216 an overall fluid recovery of 98.84% [91.32-100.8]; however breakage of the vial holders (H5:

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217 18 h and H6: 60 h) and disruption of tubing connections (H5: 36 h and H6: 12 h; 24 h)
218 occurred. Metabolite concentrations were similar to the initial 24-h period in stocks and
219 appeared stable until time point 48 h (H5) and 66 h (H6), after which a rapid decrease in
220 glucose and increase in lactate concentrations was observed (data not shown). Glucose
221 concentrations (mmol/L) were 0.06 (H5) and 0.0 (H6) at the last sampling time point. Other
222 metabolite values remained stable.
223
224 *Probe location and host reaction*
225 Histological examination confirmed that probe membrane placement was in the desired
226 LAM, SKIN and SUBLAM position in all tissue sections (Fig. 4). With LAM placement,
227 damage to adjacent primary epidermal lamellae (PEL) was observed (Fig. 4A), whereas no
228 evidence of damage to PEL was apparent with SUBLAM probes (Fig.4B). There was
229 evidence of mild to moderate inflammation and cellular debris, mild endothelial reactivity,
230 oedema and haemorrhage around each probe. Neutrophils were the predominant cell type
231 with rare macrophages and lymphocytes. The histological scores for each location are
232 presented in Table 3. Overall, the host response to microdialysis probe implantation was
233 mild, regardless of probe location, and consistent with tissue damage due to probe placement
234 and wound healing responses relative to the time the probe was in situ (24 h vs. 120 h).
235
236 **Discussion**
237 Lamellar microdialysis allowed for consistent recovery of dialysate samples and was well
238 tolerated. Fluid recovery from LAM, SUBLAM and SKIN probes was similar and consistent
239 throughout the study. The minor disruption of the distal hoof wall and sole that was required
240 for probe insertion was remote from the sampling site (microdialysis membrane).
241 Maintaining tissue integrity around the membrane is likely to be important when studying the

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242 effects of weight bearing and limb load cycling on lamellar perfusion and bioenergetics, as
243 mechanical forces operating on the soft tissue between hoof wall and distal phalanx may
244 affect perfusion (Redden, 2003; van Eps et al., 2010).
245
246 Metabolite concentrations were stable for 24 h and there was minimal histological
247 response to the probes, suggesting that analyte recovery was unlikely to be affected by
248 fibrous capsule formation or host response to the probe. These findings suggest that
249 microdialysis is suitable for evaluating disturbances in lamellar energy metabolism over time,
250 particularly during laminitis development; however, it should be noted that sampling in the
251 current study was confined to a single region (dorsal lamellae), which may not be
252 representative of lamellar tissue in other regions of the foot. Sampling in two horses moving
253 freely in a stall for 96 h was achieved, but a decrease in glucose followed breakage of the
254 microdialysis system in both horses. The decrease was likely due to bacterial contamination
255 of the microdialysis system itself (a recognised cause of low glucose concentration artefact)
256 (Heller and Feldman, 2013), since there was minimal histological evidence of a foreign body
257 reaction (the other major cause of low glucose artefact in microdialysis) (Nichols et al.,
258 2011). A more robust collection system is required for ambulatory studies and studies of
259 longer duration.
260
261 The lower glucose and endogenous urea concentrations in LAM compared with SKIN
262 may be due to the presence of lower blood flow in lamellar tissue, increased glucose uptake
263 by surrounding cells, or a combination of both. Interstitial glucose concentrations may also be
264 influenced by blood glucose concentration, but this would be expected to equally affect
265 simultaneous LAM and SKIN samples. Both glucose and urea must be delivered to the
266 lamellae and skin haematogenously (Hall, 2011; Sorg et al., 2005), but only glucose is

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267 consumed. Therefore, the lower LAM endogenous urea concentration compared with SKIN
268 supports the existence of comparatively lower perfusion in the lamellar dermis. The lower
269 LAM glucose concentration compared to SUBLAM and SKIN supports the existence of
270 higher glucose consumption at the site of LAM probe placement (between avascular, but
271 glucose-consuming epidermal lamellae). A high rate of glucose consumption has been
272 demonstrated in the equine digit and this probably reflects the rate of glycolysis, since there is
273 no local means of glycogen storage in lamellar tissue (Wattle and Pollitt, 2004).
274
275 Lamellar perfusion was assessed further in experiments 2 and 3 using urea clearance
276 (Farnebo et al., 2010). In experiment 3 (SUBLAM), urea concentrations remained stable over
277 time, indicating the method may be useful for documenting changes in perfusion over time.
278 In experiment 2 there appeared to be lower urea concentrations (higher urea clearance) in
279 SUBLAM compared to simultaneous LAM samples, which may suggest lower local
280 perfusion in the LAM region. Urea clearance was not used in experiment 1 because an
281 objective was to determine the endogenous dialysate concentrations of all analytes (including
282 urea). Further evaluation of the urea clearance technique is required to determine whether
283 changes in lamellar perfusion can be detected.
284
285 Increased dialysate L:G has been observed with hypoxia and ischaemia in brain tissue,
286 where it is the consequence of a combination of increased extracellular lactate and reduced
287 glucose (Goodman et al., 1999; Meierhans et al., 2010). The high LAM L:G in this study was
288 due to the significantly lower LAM dialysate glucose concentrations (rather than higher
289 lactate concentrations), since LAM dialysate lactate concentrations were similar to those of
290 the SKIN, SUBLAM and previously reported tissues such as brain (Langemann et al., 2001)
291 and muscle (Edner et al., 2005). Therefore, the high L:G in normal LAM (more than twice

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292 that of SKIN, SUBLAM and other previously studied tissues) suggests the presence of a high
293 glycolytic rate in the lamellae (glucose consumption with production of lactate), which is
294 typical of epidermal tissues (Ronquist et al., 2003; Wattle and Pollitt, 2004). The lack of
295 lactate accumulation suggests that lamellar tissue is adapted to the high rate of glycolysis,
296 with the lactate produced being either utilised as an energy source via lactate dehydrogenase
297 (LDH; richly present in lamellar epidermis) (Wattle and Pollitt, 2004) or removed efficiently
298 via a lactate shuttle (Gladden, 2004).
299
300 Other metabolite values (lactate, pyruvate, glycerol) were similar in all sampled tissues
301 and also similar to values reported for the healthy human brain under similar microdialysis
302 conditions (Reinstrup et al., 2000). In the current study, the perfusion rate of 1 μL/min was
303 chosen as it allows for collection of adequate volumes of dialysate over short sampling
304 intervals (≤15 min), a requirement for ongoing studies in the authors' laboratory. This
305 relatively high flow rate reduces metabolite recovery (Benveniste and Huttemeier, 1990);
306 therefore measured dialysate metabolite concentrations were assumed to be much lower than
307 the absolute values in the interstitial fluid. The pattern of initially high glycerol
308 concentrations, decreasing over 24 h in the current study was also observed in equine muscle
309 (Edner et al., 2005), and is likely the result of cell membrane damage caused by probe
310 insertion. Measurement of other markers of cell membrane damage such as LDH (Glick,
311 1969) is also possible in microdialysis fluid and should be considered in future studies.
312
313 The L:P measured at all sites in the current study resembled values previously reported
314 for brain (Zhang and Natowicz, 2013) and muscle (Edner et al., 2005). The L:P is indicative
315 of tissue redox status (which is dependent on mitochondrial function and tissue oxygenation),
316 and an increase in L:P is the most reliable indicator of energy metabolism dysfunction

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317 (ischaemic and non-ischaemic) in well studied tissues (Hillered et al., 2006; Setala et al.,
318 2004; Waelgaard et al., 2012). In fact L:P is considered a sensitive marker of cellular
319 metabolic dysfunction (Vespa et al., 2005) and is used in the diagnostic evaluation of
320 disorders of energy metabolism in a range of tissues (Marcoux et al., 2008; Zhang and
321 Natowicz, 2013). Therefore, lamellar dialysate L:P may be useful for detecting the presence
322 of bioenergetic failure during the development of different forms of equine laminitis. The L:P
323 in lamellar dialysate in the present study was similar to that of SKIN and also brain and
324 muscle in previous studies, suggesting a similar resting redox state. This appears to be at odds
325 with the findings of Pawlak et al. (2014), who concluded that the equine epidermal lamellar
326 tissue is comparatively hypoxic.
327
328 In the current study, the contribution of epidermal cell metabolism (basal cells and deeper
329 epidermal cell layers) to the dialysate composition is not clear since the probes were placed in
330 the dermis (either between PEL (LAM) or adjacent to the PEL tips (SUBLAM)). The
331 sampling area of a microdialysis probe may depend on the tissue and the analyte. However, it
332 has been estimated to be 50 times the membrane thickness (Benveniste et al., 1989) or
333 calculated by mathematical models to be 0.85 ± 0.25 mm for neuroactive substances in the
334 brain (Diczfalusy et al., 2011), suggesting that in the present study, both epidermal and
335 dermal lamellar tissues should have influenced the dialysate regardless of probe position.
336 However, immediately adjacent tissues are likely to have more influence on dialysate
337 composition, which may account for the differences observed between LAM and SUBLAM.
338
339 The limitations of the present study include the use of different microdialysis probes and
340 the lack of ambulation (having the horses restrained in stocks). Membrane characteristics
341 were however similar for all probes, with CMA20 and CMA66 probes having identical

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342 membrane characteristics, and the CMA31 differing only in pore size (55 kDa). Net transport
343 of metabolites across the microdialysis membrane (recovery) depends mainly on perfusate
344 flow rate, membrane size (area) and how diffusible each metabolite is in the sampled tissue
345 (Hamrin et al., 2002; Rosdahl et al., 1998). Therefore, the difference in membrane pore sizes
346 is likely negligible, particularly considering the molecular weight of the largest metabolite
347 measured in the present study (glucose) was 0.18 kDa. Although it is possible that the
348 lamellar bioenergetic profile in stationary horses differs from that of ambulating horses,
349 restraining the study subjects in stocks was necessary for frequent sampling and protection of
350 the equipment. Sampling was only performed in two freely moving horses to assess if long-
351 term lamellar microdialysis was achievable, and a different perfusion rate was used for
352 convenience (6-h sampling interval), preventing comparisons between ambulating and
353 standing static horses from being performed.
354
355 This study is the first to evaluate with histology the tissue response to microdialysis probe
356 placement in horses. The scoring system was designed to represent the range of pathology
357 present in the samples scored, rather than to act as a comparison with other foreign body
358 responses. Overall the histological changes were benign and not suggestive of a foreign body
359 response driven by the microdialysis membrane.
360

361 **Conclusions**

362 Tissue microdialysis is a suitable method for energy metabolite sampling in the equine
363 lamellar and skin dermis. Recovery of dialysate was consistent and the concentrations of
364 measured metabolites were stable for 24 h. There were no adverse clinical effects of probe
365 implantation and histological reactions were mild. In the standing, static horse, the lamellar
366 dialysate had lower glucose and urea concentrations and a higher L:G compared to skin

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367 (consistent with lower perfusion and/or higher glucose consumption), but lamellar L:P, the
368 most useful marker of energy homeostasis, was similar to skin and previously studied tissues.
369 Probe position (lamellar vs. sublamellar dermis) affected microdialysate metabolite
370 concentrations, which is likely to be a factor of proximity to the avascular, but metabolically
371 active epidermis.

372

373 **Conflict of interest statement**

374 None of the authors of this paper has a financial or personal relationship with other
375 people or organizations that could inappropriately influence or bias the content of the paper.

376

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381

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554 **Table 1:** Semi-quantitative histological scoring system applied to the lamellar and skin
555 dermis tissue slides. The scoring system was designed to represent the range of
556 pathology present in the samples scored and is not a comparison to other foreign body
557 responses. HPF, high power field.
558

Score	**0**	**1**	**2**	**3**
Epidermal basal cell and parabasal cell hyperplasia (fold increase in thickness of epidermal cell layer)	Absent	2 fold	3 fold	≥ 3 fold
Flattening of secondary epidermal lamellae (SEL): expressed as % of length of unaffected SEL remaining	100%	66-99%	50-66%	<50%
Mitotic figures: per HPF (200X)	0	1	2	≥3
Inflammatory cell count around the probe per HPF at 200X	0	1-50	50-100	>100
Oedema	Absent	Mild	Moderate	Marked
Haemorrhage	Absent	Mild	Moderate	Marked
Fibroplasia: thickness of the fibrous tissue around the probe	Absent	Mild	Moderate	Marked
Collagen bundle formation	Absent	Mild	Moderate	Marked
Cellular debris	Absent	Mild	Moderate	Marked
Endothelial reactivity: number of vessels with reactive endothelium per HPF (200X)	0	1-20	21-50	>50
Tissue necrosis	Absent	Mild	Moderate	Marked

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561 **Table 2:** Median and interquartile range (IQR) concentrations of energy metabolites, urea,
562 glycerol and standard calculated ratios in lamellar dermis (LAM) and skin dermis (SKIN)
563 dialysate in experiment 1, and sublamellar dermis (SUBLAM) dialysate in experiment 3 for
564 the entire 24 h sampling period.

	LAM	**SKIN**	**SUBLAM**
Glucose (mmol/L)	0.45 [1,2] (0.38-0.50)	1.23 [1] (1.08-1.39)	0.83 [2] (0.73-1.13)
Lactate (mmol/L)	1.32 (1.09-1.52)	1.45 (1.34-1.50)	1.22 (1.11-1.31)
Pyruvate (μmol/L)	65.45 (50.90-76.64)	72.10 [3] (69.35-74.88)	54.04 [3] (43.51-59.65)
Glycerol (μmol/L)	5.04 (3.79-8.05)	3.10 (2.37-4.38)	7.38 (4.61-12.06)
Urea (mmol/L)	2.63 [1] (2.30-2.81)	3.72 [1] (3.55-4.06)	14.83 [4] (13.95-14.92)
Lactate:Glucose	2.84 [1,2] (2.64-3.63)	1.21 [1] (1.04-1.34)	1.44 [2] (1.12-1.77)
Lactate:Pyruvate	20.33 (18.07-22.82)	21.34 (19.36-23.15)	25.57 (23.41-28.59)

565

566 [1] Statistically significant ($P < 0.05$) difference between LAM and SKIN. [2] Statistically
567 significant ($P < 0.05$) difference between LAM and SUBLAM. [3] Statistically significant ($P <$
568 0.05) difference between SKIN and SUBLAM. [4] Perfusate contained 20 mmol/L of urea.

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570 **Table 3:** Median and interquartile range (IQR) histological score in the tissue surrounding
571 the probe for LAM, SKIN, SUBLAM, and for different implantation duration (24 h vs. 120
572 h). Medians are ≤ 1 for all subcolumns except after 120 h in situ, indicative of a mild host
573 response to microdialysis probe implantation. The scoring system represents the range of
574 pathology present in the samples scored. The histological changes were not suggestive of a
575 foreign body response driven by the microdialysis membrane. EBC, epidermal cell
576 hyperplasia; SEL, secondary epidermal lamellae; PMN, polymorphonuclear neutrophils.
577

Parameter	**Experiment 1**		**Experiment 2**		**Experiment 3**	
	LAM	**SKIN**	**LAM**	**SUBLAM**	**24 h**	**120 h**
EBC hyperplasia	**0** (0-0)	N/A	**0** (0-0)	**0** (0-0)	**0** (0-0)	**1.5** (0-3)
Flattening of SEL	**0** (0-0)	N/A	**0** (0-0)	**0** (0-0)	**0** (0-0)	**1.5** (0-3)
Mitotic figures	**0.5** (0-1)	**0** (0-0)	**0** (0-0)	**0** (0-0)	**0** (0-0)	**0** (0-0)
Inflammatory cell count	**1** (1-1)	**1.5** (1-2)	**3** (1.5-3)	**2** (2-2.75)	**1.5** (1-2.75)	**3** (3-3)
- *cell type*	*PMN*	*PMN*	*PMN*	*PMN*	*PMN*	*PMN*
Oedema	**2** (2-2)	**1** (1-1)	**1** (1-2.5)	**1** (1-2.5)	**1.5** (0.25-2)	**0.5** (0-1)
Haemorrhage	**1** (1-1)	**1** (1-1)	**1** (1-1)	**1.5** (1.25-2)	**1.5** (0.25-2.75)	**0.5** (0-1)
Fibroplasia	**0** (0-0)	**0** (0-0)	**0** (0-0)	**0** (0-0)	**0** (0-0)	2 (2-2)
Collagen bundles	**0** (0-0)	**0** (0-0)	**0** (0-0)	**0** (0-0)	**0** (0-0)	**1** (1-1)
Cell debris	**2** (2-2)	**1.5** (1-2)	**2.5** (1.25-3)	**1.5** (1-2.75)	**2** (0.5-2.75)	**3** (3-3)
Endothelial reactivity	**1.5** (1-2)	**1** (1-1)	**1** (0.25-2.5)	**2** (1-3)	**0.5** (0-2.5)	**2** (1-3)
Tissue necrosis	**1.25** (0.5-2)	**0** (0-0)	**0.5** (0.5-0.86)	**1.5** (0.62-2.75)	**1** (1-1.75)	**1.5** (1-2)
Total median (IQR)	**1 (0-1.5)**	**1 (0-1.25)**	**0.5 (0-1)**	**1 (0-1.5)**	**0.5 (0-1.5)**	**1.5 (0.5-2)**

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580 **Figure legends**

581

582 Fig. 1. Schematic representation of the tissue microdialysis linear probe system used in
583 experiment 1 (A): the microdialysis membrane is located within the mid-dorsal lamellar
584 tissue, the outlet tubing is connected to a collection vial within a custom-made vial holder on
585 the dorsal hoof wall (B). Schematic representation of a tissue microdialysis coaxial probe
586 system used in experiments 2 and 3 (C): the microdialysis membrane is located within the
587 mid-dorsal lamellar tissue: the inlet and outlet tubing emerge through the white line and via a
588 hole in the hoof wall are connected to a microdialysis pump and a collection vial on the
589 dorsal hoof wall, respectively. For Experiment 2, two tissue microdialysis coaxial probes
590 were placed 1 cm lateral and medial of midline for simultaneous comparison of dialysate
591 from LAM (probe introducer inserted parallel to hoof wall) and SUBLAM (probe introducer
592 inserted at a ~5º angle towards the third phalanx) (D). The dotted lines depict the internal
593 location of the probes.

594

595 Fig. 2. Median ± interquartile range (IQR) concentrations of energy metabolites, urea,
596 glycerol, L:G, L:P and dialysate recovery in LAM, SKIN (experiment 1), and SUBLAM
597 (experiment 3) vs. time. Glycerol was the only analyte that varied significantly over time,
598 decreasing compared to baseline (2 h) in the dialysate from each tissue. The concentrations of
599 glucose, and urea were significantly lower in LAM compared to SKIN ($P<0.05$). The L:G
600 was significantly higher in LAM compared SKIN ($P < 0.05$). Fluid recovery is expressed as a
601 percentage of the infused volume for each sampling interval (120 μL in 2 h). Fluid recovery
602 did not vary significantly over time.

603 [1], significant difference ($P < 0.05$) between LAM and SKIN in experiment 1 at this time
604 point; [2], significant difference ($P < 0.05$) vs. 2 h for LAM dialysate in experiment 1 at this

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605 time point; [3], significant difference ($P < 0.05$) vs. 2 h for SKIN dialysate in experiment 1 at
606 this time point; [4], significant difference ($P < 0.05$) vs. 2 h for SUBLAM dialysate in
607 experiment 3 at this time point. [5], perfusate contained 20 mmol/L of urea.
608
609 Fig. 3. Median and interquartile range (IQR) concentrations of energy metabolites, urea,
610 glycerol and standard calculated ratios in simultaneous LAM and SUBLAM dialysate
611 samples in four feet from two horses (experiment 2) over a 4-h period. A lower concentration
612 of glucose and pyruvate, lower urea clearance, and a higher lactate:glucose (L:G) was
613 apparent in LAM vs. SUBLAM dialysate (descriptive analysis only; $n = 2$).
614
615 Fig. 4: Haematoxylin and eosin-stained light microscopy at 4x magnification showing the
616 position of the microdialysis probe membranes (P) in representative sections from LAM (A);
617 SUBLAM (B); and SKIN (C). There was damage to primary epidermal lamellae (white
618 asterisks) neighbouring the probe in all of the LAM sections, but not in SUBLAM sections.
619 White arrow heads, skin hair follicles.
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