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9 **Evidence for marsh mallow (*Malva parviflora*) toxicosis causing myocardial disease and**
10 **myopathy in four horses**

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25 **Keywords:** horse; *Malva parviflora*; mallow; myopathy; cardiomyopathy; acyl carnitines;
26 fatty acid oxidation

27

28 **Summary**

29 **Reason for performing the study**: Investigation of toxicosis caused by *Malva parviflora*
30 was required after four horses from the same farm developed severe muscle fasciculations,
31 tachycardia, sweating and periods of recumbency leading to death or euthanasia after
32 ingesting the plant.

33 **Objectives**: Describe historical, clinical, clinicopathological and pathological findings of four
34 horses with suspected *Malva parviflora* toxicosis. The role of cyclopropene fatty acids (found
35 in *Malva parviflora*) and mechanism for toxicosis are proposed.

36 **Study design**: Case series.

37 **Methods**: Historical, physical examination, clinicopathological and pathological findings are
38 reported. Due to similarities with atypical myopathy (AM) or seasonal pasture myopathy
39 (SPM) acyl carnitine profiles were performed on sera from two cases and equine controls.
40 Presence of cyclopropene fatty acids was also examined in sera of two cases.

41 **Results**: *Malva parviflora* had been heavily grazed by the horses with little other feed
42 available. Horse 1 deteriorated rapidly and was subjected to euthanasia. Horse 2 was referred
43 to hospital where severe myocardial disease and generalised myopathy was determined; this
44 horse was subjected to euthanasia 36 hours after admission. Horse 3 died rapidly, and horse 4
45 was subjected to euthanasia at onset of clinical signs. Post mortem examinations performed
46 on three horses revealed acute, multifocal cardiac and skeletal myonecrosis. Myocyte
47 glycogen accumulation was absent when examined in horse 2. Acyl carnitine profiles
48 revealed increased C14-C18 acyl carnitine concentrations in cases relative to controls.
49 Cyclopropene fatty acids were detected in sera of cases but not controls.

50 **Conclusion**: These findings suggest aetiology different to that of AM/SPM. We hypothesise
51 that cyclopropene fatty acids in *Malva parviflora* interfere with fatty acid beta-oxidation in
52 horses in negative energy balance, causing the clinical signs and abnormal acyl carnitine

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53 profiles. These equine cases suggest a pathophysiological course that closely mimics the
54 human genetic condition Very Long Chain Acyl CoA Dehydrogenase Deficiency.

55

56

57 **Introduction**

58 Marsh mallow (*Malva parviflora*), also known as small-flowered mallow, cheeseweed
59 mallow and little mallow is a common weed found in pastures of grazing animals with
60 extensive distribution including regions of Europe, Asia, North and South America, and
61 Australia and New Zealand [1] (Fig 1). It has been linked to a staggers-like syndrome in
62 horses [2], and sheep [3; 4], however reports in the literature are rare. *Malva parviflora* is a
63 member of the order Malvales, comprising several families and over 2,000 species, including
64 cotton (*Gossypium* spp.), hibiscus (*Hibiscus* spp.) and baobab (*Adansonia* spp.) [5]. Distinct
65 cyclopropene fatty acids, (long chain fatty acids containing a three carbon, double-bonded
66 (cyclopropene) ring within their structure) are found in many species within the order
67 Malvales. The two cyclopropene fatty acids; malvalic ($C_{18}H_{32}O_2$) and sterculic ($C_{19}H_{34}O_2$)
68 acids [5-9] (Fig 2), are thought to be the main toxic components of plants from this order [2].
69 Cyclopropene fatty acids are found in all parts of the *Malva parviflora* plant, but are
70 particularly concentrated in seed oils [7].

71

72 Myopathy has been described in sheep fed *Malva parviflora* [4]. In horses, ingestion of seeds
73 from some trees of the *Acer* genus containing hypoglycin A results in atypical myopathy
74 (AM) or seasonal pasture myopathy (SPM) [10; 11]. It is therefore possible that other plant
75 toxins with similar structures, such as cyclopropene fatty acids, might also cause myopathy
76 through a similar mechanism.

77

78 The purpose of this case series is to describe the historical, clinical, clinicopathologic and
79 post mortem details of four horses from the same farm that developed clinical signs of
80 myopathy and cardiomyopathy in association with grazing large quantities of marsh mallow
81 weed. A mechanism for the toxicosis is also proposed.

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83 Clinical case details

84 All four cases originated from one farm, located in the Western District of Victoria in south-
85 eastern Australia. They shared no known genetic relation. Other than one hand-reared calf,
86 these horses were the only livestock kept on the farm. The horses were kept in paddocks that
87 lacked pasture but contained extensive growth of *Malva parviflora*, which had obviously
88 been grazed by the horses. Approximately one week before the onset of clinical signs, hay
89 supplementation was suddenly reduced to approximately 0.5% of bodyweight/day. No
90 cardiac glycoside-containing plants were present in the pasture and no trees of the *Acer* genus
91 were present on the farm. The horses did not have access to grain or any ionophore-
92 containing feeds. All horses were in moderate body condition (BCS 4/9). The calf remained
93 clinically normal, but was too young to graze extensively.

94

95 *Case 1*

96 A 4-year-old Thoroughbred mare was evaluated on farm. Clinical signs began with patchy
97 sweating and progressed to include muscle fasciculations in the hindquarters. Frequent
98 vocalisation was noted by the owner. The mare was initially assessed for colic; however, no
99 gastrointestinal abnormalities were found; fluxinin meglumine (Flunixon[a] 1.1 mg/kg i.v.) was
100 administered. Haematology and biochemistry were not performed, and rectal temperature was
101 not recorded. Over approximately the next five hours, clinical signs progressed to diffuse
102 sweating and diffuse muscle fasciculations. Moderate tachycardia (64 beats/minute) with
103 regular rhythm was present at this stage. Flunixin meglumine (Flunixon[a] 1.1 mg/kg i.v.), was
104 administered again, and butorphanol (Torbugesic[b] 0.02 mg/kg i.v.) and detomidine
105 (Dormosedan[b] 0.01 mg/kg i.v.) were administered to allow abdominal palpation per rectum,
106 which was normal. Following this, severe tachycardia (120 beats/minute) with regular rhythm
107 developed. The mare developed clinical findings consistent with hypovolaemia (cool
108 extremities and prolonged jugular refill) possibly due to fluid loss through sweat with no
109 water intake. Intravenous fluid therapy was instigated; however, the mare progressed to
110 become recumbent, and was subjected to euthanasia approximately 6 hours after initial
111 presentation due to further worsening of her condition.

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113 ***Case 2***

114 A 19-year-old Quarter Horse gelding was examined on farm for acute onset of muscle
115 fasciculations and diffuse sweating the following day. This horse was referred for hospital
116 care. On presentation at the referral hospital, the horse had diffuse muscle fasciculations, was
117 sweating and quickly became recumbent. He was initially tachycardic (80 beats/minute);
118 however, this decreased to 48-52 beats/minute following a 20 ml/kg intravenous fluid bolus
119 (Hartmann's solution[c]). Rectal temperature was not obtained on presentation but was normal
120 approximately three hours after presentation. Heart rhythm was occasionally irregular on
121 auscultation, and ECG examination showed supraventricular premature depolarisations.
122 Other physical examination findings included normal mucous membrane colour, good pulse
123 quality and reduced borborygmi. Haematology and pertinent biochemistry results obtained on
124 presentation are summarised in Table 1. The increase in peripheral blood lactate
125 concentration was attributed mainly to anaerobic metabolism in skeletal myocytes.
126 Neurological examination revealed no cranial nerve deficits, and despite distress and
127 recumbency mentation was deemed normal. Gait could not be assessed. Due to the uncertain
128 cause of the clinical presentation and consistency of some signs with severe colic, work up
129 included abdominal ultrasound, passage of a nasogastric tube, abdominocentesis and
130 abdominal palpation per rectum; no abnormalities were detected. A snake venom detection
131 test was negative; however, this test has low sensitivity.

132

133 Supportive care comprised intravenous fluid therapy (Hartmann's solution[c]; 60 ml/kg/day
134 following the initial bolus), flunixin meglumine (Flunixon[a] 1.1 mg/kg i.v. q 12 hours) and a
135 lidocaine constant rate intravenous infusion (Lignomav[d] 0.05 mg/kg/min following a 1.3
136 mg/kg bolus). The horse remained recumbent other than occasional short (2-5 minute)
137 periods of standing, during which generalised muscle fasciculations would reappear and
138 resolve again once recumbent.

139

140 Approximately 24 hours after admission, the lidocaine infusion was discontinued due to
141 questionable benefit and possible contribution to prolonged recumbency. Approximately 15
142 minutes later the horse developed severe tachycardia (100 beats/minute). Electrocardiography
143 revealed multiform ventricular tachycardia that persisted despite re-instatement of lidocaine

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144 treatment. Magnesium sulphate (50 mg/kg; 25 g total dose, diluted in 1 L 0.9% sodium
145 chloride and administered i.v.) was then given, resulting in conversion to sinus rhythm.
146 Cardiac troponin I concentration was measured at this time (Advia Centaur Immunoassay[e],
147 validated for equine serum) and was markedly increased (Table 1). Unfortunately,
148 echocardiography could not be performed at any point due to the horse being able to stand for
149 only a few minutes. It was also noted that the horse had also developed pigmenturia, and CK
150 activity at this time was markedly increased (Table 1). Due to reported cases of severe
151 rhabdomyolysis caused by *Streptococcus equi* subsp. equi infection [12], a Streptococcal M
152 titre was performed. This returned a moderate positive result at 1:1600, considered
153 inconclusive for *S. equi* as a cause of myopathy. Triglyceride concentrations were measured
154 retrospectively in frozen serum and were increased (Table 1).

155

156 The horse remained recumbent for most of the time but was able to eat and drink. His clinical
157 condition did not change for the next 12 hours but he then developed a second episode of
158 marked tachycardia (100 beats/minute, no ECG performed), as well as an oesophageal feed
159 impaction. The owner elected euthanasia.

160

161 ***Case 3***

162 The day following onset of clinical signs in case 2, an 11-year-old Thoroughbred gelding was
163 presented on farm with acute onset of muscle fasciculations and sweating. This horse died
164 shortly after being loaded for transportation for hospital care.

165

166 ***Case 4***

167 Another 11-year-old Thoroughbred gelding was transported to the primary care veterinarian's
168 clinic for observation on the same day as case 3. This horse was initially asymptomatic but
169 developed muscle fasciculations and patchy sweating within 12 hours of hospitalisation.
170 Haematology revealed mild neutrophilia and lymphopenia, attributed to stress. Serum
171 biochemistry from a sample taken approximately 8 hours prior to the onset of clinical signs
172 revealed mildly increased muscle enzyme activities and hypocalcaemia (Table 1). Peripheral
173 blood lactate and glucose concentrations were not measured. Cardiac troponin I and

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174 triglyceride concentrations were normal when measured retrospectively in frozen serum
175 (Table 1). The owner elected euthanasia at the onset of clinical signs due to financial
176 constraints and expected progression of disease.

177

178 **Post mortem findings**

179 Post mortem examinations were performed on cases 2, 3 and 4. Gross examination was
180 unremarkable except for an extensive oesophageal feed impaction in case 2, occupying the
181 entire oesophageal lumen. All cases had histopathological evidence of myocardial necrosis
182 with myocardial fibre degeneration, cytoplasmic fragmentation, patchy interstitial oedema,
183 macrophage infiltration and multifocal acute interstitial haemorrhage (Fig 3a). Lesions were
184 present in both atrial and ventricular wall sections of horses 2 and 3, but were only present in
185 the atrial wall of case 4. Frozen sections from the left ventricle of case 2 were examined with
186 periodic acid-Schiff (PAS) and oil red O stains but did not reveal lipid accumulation within
187 cardiac myocytes.

188

189 Examination of skeletal muscle sections in cases 2 and 4 revealed similar but generally less
190 severe changes to those seen in the myocardial sections. No skeletal muscle abnormalities
191 were detected in case 3. Cytoplasmic fragmentation, hypereosinophilia, pallor, shrinking or
192 swelling, and interstitial oedema were seen in the diaphragm and skeletal muscle portion of
193 the oesophagus of cases 2 and 4, and in the longissimus and vastus lateralis muscles of case
194 2. The diaphragm was worst affected of all skeletal muscle sections examined of case 2 (Fig
195 3b). Frozen sections with PAS and oil red O stains showed no skeletal muscle lipid
196 accumulation. Immunohistochemistry for myosin heavy chain (Fast Type II) was performed
197 on 5 mm paraffin sections from case 2. Nonspecific antibody binding was blocked with 10%
198 (v/v) horse serum in wash buffer (PBS:0.5% Bovine Serum Albumin; 0.1% glycine). The
199 primary antibody (Clone MY32[f]) was diluted in wash buffer, added to the sections, and
200 incubated at 4°C overnight. Excess antibody was washed off in three changes of wash buffer
201 before being incubated with HRP conjugated anti-mouse IgG. Sections were washed three
202 times in wash buffer. Colour development was achieved through incubation of the sections
203 with the peroxidase substrate DAB[f], washed in distilled water and mounted using

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204 Aquamount[g]. All affected fibres were non-staining, Type I (slow twitch) fibres
205 (Supplementary Item 1). No lesions were seen in any smooth muscle sections.

206

207 Histopathological evidence of splenic congestion was also seen in case 4, while case 2 had
208 haemosiderin-laden macrophages present in the splenic red pulp and kidneys. Haemosiderin
209 was also present within the Kuppfer cells of the liver in case 2. In case 2, hepatocytes
210 displayed moderate lipid vacuolation, predominantly affecting the periacinar region and the
211 hepatic sinusoids were mildly congested. There was occasional renal tubular distension with
212 proteinaceous fluid in case 2, and rare aggregates of plasma cells and lymphocytes within the
213 renal interstitium.

214

215 Case 3 had an extensive accumulation of small brown seeds within the ventral colon, which
216 were consistent with seeds from *Malva parviflora*. An attempt was made to germinate these
217 for full identification, but was unsuccessful.

218

219 **Acyl carnitine and fatty acid analysis methods and results**

220 Acyl carnitine profiles using tandem mass spectrometry were performed on serum samples
221 from cases 2 and 4, and serum from 10 healthy control horses (8 mares and 2 geldings; 6
222 Standardbreds, one Quarter Horse, one Quarter Horse cross, one Thoroughbred and one
223 Thoroughbred cross; median age 11 years (range 3-25 years)) as previously described [13].
224 There were large increases (20 to 70 times the median of control samples) in C14-C18 acyl
225 carnitine concentrations in case samples and smaller increases (up to 8 times) in other acyl
226 carnitines such as C2-C5 (Table 2; Supplementary Item 2). The presence of malvalic,
227 sterculic, and dihydrosterculic acids in serum was analysed using gas chromatography-mass
228 spectrometry. Serum (100 μL) was hydrolysed with 500 μL of ethanol:10 mol/L sodium
229 hydroxide (8:2 v:v) in a glass tube and heated at 80°C for 1 hour. 200 μL of 6 mol/L
230 hydrochloric acid was added and fatty acids extracted into 1 mL of n-hexane which was then
231 dried under an air stream at 80°C. *Tert*-butyldimethylsilyl derivatives were formed by adding
232 50 μL of pyridine and 50 μL of N-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide
233 (MTBSTFA) and heating at 80°C for 30 minutes. 150 μL of iso-octane was added and the

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234 fatty acid derivatives were separated on a 30m HP-5 column interfaced with an Agilent 5973
235 gas chromatograph-mass spectrometer[h]. Some re-arrangement of cyclopropene fatty acids
236 occurred during the analytical process, resulting in two chromatographic peaks
237 (Supplementary Item 3). Due to lack of readily available pure standards, raw cold-pressed
238 Baobab seed oil (containing 6.2% malvalic acid, 6.5% sterculic acid and 1.3%
239 dihydrosterculic acid) [14] was used as a source of these fatty acids. Malvalic and sterculic
240 acids were present in serum from cases 2 and 4 but were undetectable in controls
241 (Supplementary Item 3).

242

243 **Discussion**

244 This case series reports suspected *Malva parviflora* toxicosis causing acute myopathy and
245 cardiomyopathy in horses associated with abnormal fatty acid oxidation, and proposes a
246 mechanism for this toxicosis. Previous reports of suspected *Malva parviflora* toxicosis in
247 horses are rare and mostly anecdotal. Previously described clinical signs associated with
248 ingestion are sweating, tachycardia, tachypnoea, muscle tremors and a stiff gait [2]. Signs of
249 toxicosis are better described in sheep, in which forced exercise results in a staggers-like
250 syndrome including collapse, tachycardia, tachypnoea, muscle fasciculations and sometimes
251 death [3; 4]. In one of these studies, similar clinical signs were also reported in one of the
252 horses used to drive the sheep [3]. Post mortem lesions described in sheep include cardiac
253 and skeletal muscle necrosis and liver lipid accumulation [3; 4], consistent with the equine
254 cases reported here. Toxicosis in the cases described here occurred when mature fruit
255 containing seeds were present on the *Malva parviflora* plant. Immature seeds of *Malva*
256 *parviflora* contain the highest concentrations of cyclopropene fatty acids (especially malvalic
257 acid), with other parts of the plant containing lower concentrations [7].

258

259 While all horses reported here died or were subjected to euthanasia, there are anecdotal
260 reports of horses with suspected clinical toxicosis surviving following ingestion of *Malva*
261 *parviflora*, and survival of some affected sheep has also been reported [2; 3]. As the owner
262 elected euthanasia of case 4 at the onset of clinical signs, it is unknown whether this horse
263 might have survived had treatment been attempted. Based on the cases described here,
264 prognosis appears guarded or poor; however, treatment might be warranted in milder cases.

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266 The marked increases in serum concentrations of cyclopropene fatty acids suggest that these
267 compounds could be responsible for toxicosis in these cases. Malvalic acid has been
268 suggested as the active toxin of *Malva parviflora* [2], but a mechanism of action has not
269 previously been elucidated. We propose that cyclopropene fatty acids found in *Malva*
270 *parviflora* impair beta-oxidation by disrupting function of the enzyme Very Long Chain Acyl
271 CoA Dehydrogenase (VLCAD, EC 1.3.8.9). VLCAD is an early enzyme in the beta-
272 oxidation spiral that cleaves C12-C18 fatty acids although affinity is highest for C14-C18
273 fatty acids [15]. Inhibition of this enzyme is expected to cause accumulation of long chain
274 (predominantly C14-C18) acyl carnitines within cells that then enter the circulation. This is
275 consistent with the results from cases 2 and 4, in which increases in serum concentrations of
276 C14-C18 acyl carnitines predominated. There were smaller increases in C2-C5 acyl carnitines
277 that may be due to minor inhibition of other acyl-CoA dehydrogenase enzymes by malvalic
278 or sterculic acids or their metabolites, as these enzymes are known to have relatively broad
279 substrate specificity.

280

281 The cyclopropene ring, especially the double bond, is thought to impart toxicity, as the
282 cyclopropane analogues dihydromalvalic and dihydrosterculic acids, that lack the double
283 bond in the three carbon ring are not toxic [6; 7]. The cyclopropene ring is destroyed during
284 hydrogenation in the refining process of seed oils from plants of the order Malvales [16]. This
285 change is thought to negate the toxicity caused by unrefined oils such as cottonseed oil [14;
286 16-22]. Further, cyclopropane fatty acids do not appear to cause any of the metabolic changes
287 reported for cyclopropene fatty acids in rats [23]. Hypoglycin A contains a cyclopropane ring
288 with an adjacent double bond and inhibits several enzymes involved in fatty acid beta-
289 oxidation. Because of the presence of the structurally similar cyclopropene ring, malvalic and
290 sterculic acids might also interfere with fatty acid beta-oxidation during times of negative
291 energy balance. This might also ultimately lead to abnormal myocyte energy handling.
292 Further work is required to confirm one or both cyclopropene fatty acids as the toxins
293 responsible for clinical signs in horses and other animals, and if so, their exact mechanism of
294 action.

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296 Our equine cases share many similarities with the human genetic condition Very Long Chain
297 Acyl CoA Dehydrogenase Deficiency (VLCADD). This is an inherited disorder of fatty acid
298 metabolism where mutations in *ACADVL*, the gene encoding VLCAD, result in abnormal
299 increases in C14-C18 acyl carnitines, with clinical signs and post mortem findings similar to
300 those seen in the equine cases reported here [24-29]. Therefore it is suspected that *Malva*
301 *parviflora* might cause an acquired form of VLCADD in horses and other animals. In
302 affected humans there are several different genetic mutations in the *ACADVL* gene, resulting
303 in different ages of onset and severity of clinical signs [30]. VLCADD can present as a severe
304 life-threatening disease in newborns that includes cardiomyopathy, hypoglycaemia, acidosis,
305 and hepatic dysfunction [26; 30]. Childhood and adult-onset forms of the disease also exist in
306 which rhabdomyolysis and myoglobinuria are more prominent features [31]. Often fatal
307 cardiac arrhythmias can occur in human disorders of fatty acid metabolism [32]. Ventricular
308 tachycardia is the most commonly reported arrhythmia in VLCADD [32], although the exact
309 pathophysiology of the cardiac manifestations is incompletely understood [32; 33].

310

311 Atypical myopathy is a disorder of fatty acid oxidation and also associated with cardiac
312 disease [33]. Premature ventricular depolarisations and paroxysmal ventricular tachycardia
313 are reported in AM [33]. Post mortem evidence of cardiomyonecrosis has also been observed
314 in some horses with SPM, although dysrhythmias were not reported [34]. Dysrhythmias were
315 documented in case 2, and are suspected to have caused the marked tachycardia in case 1 and
316 sudden death in case 3. These are presumed to be due to the myocardial lesions seen
317 histopathologically in the three horses examined. It is interesting to note that case 4 had a
318 normal CTnI in serum taken just prior to the onset of clinical signs. It is possible that the
319 cardiac lesions progress rapidly, leading to cardiomyonecrosis being evident in this horse
320 post-mortem after clinical signs developed, but had not progressed sufficiently to cause an
321 increase in CTnI in the hours prior to this.

322

323 Regardless of the mutation causing VLCADD in people and VLCAD knock-out mice,
324 episodes of clinical disease are usually precipitated by factors that result in negative energy
325 balance such as illness, fasting or physical exertion [24; 26; 31; 32; 35] [29]. Negative energy
326 balance is also important in the pathophysiology of SPM and AM in horses [10; 35]. The
327 presence of negative energy balance in *Malva parviflora* toxicosis seems key to the

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328 development of clinical toxicosis, as fat is mobilised for energy and metabolised through
329 beta-oxidation. In sheep fed *Malva parviflora*, those that showed signs of toxicosis were in
330 negative energy balance [3], while well-fed sheep were unaffected [36]. Similarly,
331 administration of cyclopropene fatty acids to rats, chickens and dairy cows in a neutral or
332 positive energy balance did not induce toxicosis, but rather caused changes in composition of
333 body, egg and milk fats [14; 17-21]. During negative energy balance, flux through beta-
334 oxidation pathways is greatly increased. Impairment of the enzymes of those pathways is
335 expected to cause the accumulation of fatty acid intermediates, including acyl carnitines, and
336 may induce acute clinical signs. Although negative energy balance appears important, it
337 should be noted that the cases described here had body condition scores of approximately 4/9.
338 Horses affected by AM or SPM are also typically in reasonable body condition [10; 37],
339 suggesting that in disorders of fatty acid oxidation, acute negative energy balance can
340 precipitate clinical disease (toxicosis?) does not need to be present long enough to generate
341 very low body condition scores. In these cases, presence of a negative energy balance in all
342 horses is assumed due to the history of sudden withdrawal of supplementary feeding
343 approximately one week prior to the onset of clinical signs. Serum triglyceride concentrations
344 reported here were measured in samples taken some time after presumed toxin ingestion, and
345 therefore do not reflect energy balance at the time of toxin ingestion.

346

347 The major differences between the cases reported here and cases of SPM or AM are the
348 different acyl carnitine profiles and more pronounced cardiac lesions present in the current
349 cases [33]. Both SPM and AM are caused by acquired Multiple Acyl CoA Dehydrogenase
350 Deficiency [38; 39], and hypoglycin A has been identified as the causative toxin [10]. Despite
351 differing acyl carnitine profiles, the clinical presentation and clinicopathological findings
352 reflecting generalised myopathy in the cases reported here were similar to those described for
353 SPM or AM cases. Necrosis of only Type 1 myofibres as demonstrated in case 2 is also a
354 feature of AM/SPM and is consistent with the clinical signs of constant movement and
355 muscle fasciculations that resolve with recumbency. The exclusive involvement of Type 1
356 myofibres is consistent with disorders of fatty acid oxidation [39] due to the higher number of
357 mitochondria and greater use of oxidative metabolism of these fibre types. The extensive
358 oesophageal feed impaction in case 2 was thought to be caused by myopathy of the skeletal
359 muscle portion of the oesophagus as has been reported in SPM [38].

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360

361 In contrast to SPM and AM, where lipid accumulation in myocytes is common [10; 40], there
362 was no evidence of lipid accumulation in any muscle sections in the one case that had these
363 sections examined with appropriate stains. In humans with VLCADD, myocyte lipid
364 accumulation occurs in approximately one third of cases and, when present, is only moderate
365 [35; 41]. The reason that lipid accumulation does not occur in all human patients is unclear;
366 however, based on our findings and the human literature, myocyte lipid accumulation should
367 not necessarily be expected to occur in animals *Malva parviflora* toxicosis.

368

369 Hepatocyte lipid vacuolation was found in case 2, and is a consistent feature of VLCADD in
370 humans [42; 43]. Hepatic lipid accumulation and increased alanine transaminase activity is
371 also described in sheep that have ingested *Malva parviflora* [3; 4]. This might reflect lipid
372 mobilisation due to negative energy balance, rather than a specific feature of toxicosis.
373 Hypoglycaemia is a feature of VLCADD, but case 2 of this series was normoglycaemic on
374 presentation. This difference might be explained by the substantial endogenous
375 catecholamine and glucocorticoid production by prey species such as horses when severely
376 distressed that might obscure blood glucose measurements. Alternatively hypoglycaemia
377 might be due to other species differences in glycogen reserves and glucose metabolism and
378 further investigation is required to determine whether it is a feature of *Malva parviflora*
379 toxicosis.

380

381 Specific treatment recommendations for AM/SPM do not exist and there is insufficient
382 information to make recommendations for horses with suspected *Malva parviflora* toxicosis.
383 Replacement of dietary long chain fatty acids with medium chain fatty acids is the mainstay
384 for management of VLCADD in people but this recommendation is not unanimously
385 accepted [44-47]. Dantrolene sodium has been reported to be beneficial in a single case of
386 VLCADD [48]. Removing animals from the source of toxin and eliminating negative energy
387 balance would appear important. However, the best source of energy to achieve this is purely
388 speculative.

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390 This case series has limitations, most importantly the small number of affected horses and
391 incomplete data for all cases. Due to the nature of clinical practice, including owner financial
392 constraints, decisions to elect euthanasia and only one case being evaluated at a referral
393 hospital, these were unavoidable. Further studies are needed to better characterise this
394 toxicosis should larger outbreaks occur in the future.

395

396 In conclusion, ingestion of *Malva parviflora* is likely related to development of acute
397 myopathy and cardiomyopathy in horses in a negative energy balance, due to effects on fatty
398 acid oxidation. This plant should be controlled in areas where horses and other species are
399 grazing, especially if adequate supplementary feed is unavailable. Acyl carnitine profiling
400 could be an effective technique to delineate *Malva parviflora* from hypoglycin A toxicosis.
401 Follow-up fatty acid analysis, using the technique described above, can be used to confirm
402 the presence of cyclopropene fatty acids. Further work involving investigation of
403 cyclopropene fatty acids in a rat model is planned to definitively determine the toxin within
404 the plant that causes clinical signs.

405

406 **Authors' declaration of interests**

407 No competing interests have been declared.

408

409 **Ethical animal research**

410 Research ethics committee oversight not required by this journal: descriptive clinical report.
411 Explicit owner informed consent for inclusion of animals in this study was not stated.

412

413 **Authorship**

414 J. Bauquier was responsible for the initiation of the study, case contribution and investigation,
415 and preparation of the manuscript. A. Stent was responsible for post mortem examination,
416 and preparation of the manuscript. I. Jerrett was responsible for case contribution, and post
417 mortem examinations. J. Gibney was responsible for case contribution, and farm
418 investigation. J. White was responsible for muscle fibre typing, and writing of the

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419 manuscript. B. Tennent-Brown contributed to the preparation of the manuscript. A. Pearce
420 was responsible for case contribution, and preparation of the manuscript. J. Pitt was
421 responsible for acyl carnitine and fatty acid analyses, and preparation of the manuscript. All
422 authors gave their final approval of the manuscript.

423

424

425 **Manufacturers' addresses**

426 [a]Norbrook, Australia.

427 [b]Zoetis, Australia.

428 [c]Baxter, New South Wales, Australia.

429 [d]Mavlab, Australia.

430 [e]Advia Centaur Immunoassay, Siemens?

431 [f]Sigma

432 [g]Thermo Scientific

433 [h]Agilent 5973, LabX?

434

435 **Table legends**

436 **Table 1:** Haematological and pertinent plasma (case 2) or serum (case 4) biochemical results
437 from the two cases for which they were measured. All values reported are from hospital
438 admission (case 2) or just prior to the onset of clinical signs (case 4) except where indicated
439 otherwise. Triglyceride values are from samples taken after the time of toxin ingestion and
440 therefore do not reflect energy balance at that time. Values outside the reference range are in
441 bold.

442 * Values from samples taken approximately 24 hours after admission

443 † Value from sample taken approximately 24 hours after admission, test performed
444 retrospectively on frozen serum.

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445 ‡ Values from tests performed retrospectively on frozen serum.

446 **Table 2:** Acyl carnitine profiles from cases 2 and 4, control horses, and horses with
447 AM/SPM. *The equine range is derived from previously published values [18; 35]. **Median
448 acyl carnitines were calculated from the combined values previously reported for individual
449 horses with AM or SPM [18; 36]. Values above the control range are in bold. Increases in
450 shorter (C2-C5) chain acyl carnitines may be due to minor inhibition of other acyl-CoA
451 dehydrogenase enzymes by malvalic or sterculic acids or their metabolites, due to relatively
452 broad substrate specificity.

453

454

455 **Figure legends**

456 **Fig 1:** *Malva parviflora*, showing mature plants and characteristic "cheesewheel" fruit. The
457 plant develops white-purple flowers prior to fruit developing.

458

459 **Fig 2:** Chemical structures of malvalic [8] (A) and sterculic [9] (B) acids.

460

461 **Fig 3:** A: Haematoxylin and eosin stained section of myocardium from case 2. Interstitial
462 oedema and infiltration of macrophages (black arrow), and fragmentation of muscle fibres
463 (white arrow) are seen. B: Haematoxylin and eosin stained section of diaphragm from case 2.
464 This section is representative of the changes in all skeletal muscle sections examined but was
465 the most severe. Swelling of muscle fibres (thick arrows) and as muscle fibre fragmentation
466 (thin arrows) are seen.

467

468 **Supplementary Information**

469 **Supplementary Item 1:** Immunohistochemistry for myosin heavy chain (Fast Type II) from
470 case 2.

471

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472 **Supplementary Item 2:** Acyl carnitine profiles of cases 2 and 4, and one control horse.

473

474 **Supplementary Item 3:** A: Extracted ion chromatograms (337 *m/z*) showing detection of
475 malvalic acid in serum from case 4 (top) compared to a control horse (bottom). Malvalic acid
476 is shown as two peaks due to separation into two compounds caused by the analysis. Absence
477 of peaks related to malvalic acid in the control horse are indicated by arrows. B: Extracted
478 ion chromatograms (351 *m/z*) showing the detection of sterculic acid in serum from case 4
479 (top) compared to a control horse (bottom). Again, sterculic acid is shown as two peaks due
480 to separation into two compounds during analysis. Absence of peaks related to sterculic acid
481 in the control horse are indicated by arrows.

482

483

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Table 1

	Value (reference range)	Value (reference range)
	Case 2	**Case 4**
Packed cell volume (%)	**51** (30-47)	37 (32-52)
Leukocytes ($x10^{-9}$)	**12.89** (4.9-11.1)	9.9 (5.5-12.5)
Neutrophils ($x10^{-9}$)	**9.82** (2.5-6.9)	**8.6** (2.7-6.7)
Monocytes ($x10^{-9}$)	0.27 (0.2-0.6)	0.3 (0.0-0.6)
Eosinophils ($x10^{-9}$)	0.17 (0.0-0.8)	0.0 (0.0-0.9)
Basophils ($x10^{-9}$)	0.03 (0.0-0.1)	0.0 (0.0-0.3)
Lymphocytes ($x10^{-9}$)	2.6 (1.5-5.1)	**1.0** (1.5-5.5)
Total protein (g/L)	71 (56-79)	74 (53-80)
CK (U/L)	**3000** (10-350) **135477** (50-400)*	**4893** (<150)
AST (U/L)	>1086 (0-600)	**790** (<300)
Calcium (mmol/L)	**2.31** (2.60-3.23)	**2.4** (2.8-3.4)
Lactate (mmol/L)	**9.2** (0.3-1.5)	Not measured
Glucose (mmol/L)	7.1 (3.56-8.34)	Not measured
Triglycerides (mmol/L)	**3.1** (0.1-0.9)†	0.2 (0.1-0.9)‡
CTnI (µg/L)	**167.5*** (<0.15)	0.09 (<0.15)‡

Haematological and pertinent plasma (case 2) or serum (case 4) biochemical results from the two cases for which they were measured. All values reported are from hospital admission (case 2) or just prior to the onset of clinical signs (case 4) except where indicated otherwise. Triglyceride values are from samples taken after the time of toxin ingestion and therefore do not reflect energy balance at that time. Values outside the reference range are in bold.

* Values from samples taken approximately 24 hours after admission

† Value from sample taken approximately 24 hours after admission, test performed retrospectively on frozen serum.

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‡ Values from tests performed retrospectively on frozen serum.

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Table 2

	Case 2	**Case 4**	**Controls (n=10); median (range)**	**Equine range***	**AM/SPM (mean)****
carnitine	**70.3**	**62.2**	20.3 (13.1-37.9)	4.3-31.3	**54.50**
C2 carnitine	**61.8**	**41.8**	7.8 (4.8-10.9)	≤18.96	**21.3**
C3 carnitine	**19.0**	**11.3**	3.1 (1.7-4.6)	≤1.41	1.94
C4 carnitine	**2.0**	**1.9**	0.6 (0.5-0.7)	≤1.06	**31.05**
C5:1 carnitine	**0.15**	0.12	0.04 (0.0-0.2)	≤0.03	0.06
C5 carnitine	**1.06**	**1.06**	0.28 (0.2-0.6)	≤0.46	**34.57**
C6 carnitine	0.3	0.17	0.05 (0.03-0.3)	≤0.12	**5.72**
C8 carnitine	0.09	0.06	0.03 (0.02-0.2)	≤0.02	**1.67**
C10 carnitine	**0.13**	**0.15**	0.03 (0.01-0.1)	≤0.03	**0.71**
C5DC carnitine	0.07	0.06	0.03 (0.02-0.1)	≤0.05	**0.71**
C12 carnitine	**0.26**	**0.28**	0.03 (0.00-0.1)	≤0.02	**0.12**
C14:2 carnitine	**0.38**	**0.43**	0.02 (0.00-0.1)	≤0.02	**0.16**
C14:1 carnitine	**1.36**	**1.24**	0.02 (0.00-0.1)	≤0.02	0.06
C14 carnitine	**0.95**	**1.05**	0.06 (0.01-0.2)	≤0.02	**0.15**
C16 carnitine	**3.32**	**2.87**	0.09 (0.03-0.2)	≤0.02	**0.38**
C18:1 carnitine	**1.84**	**1.88**	0.04 (0.02-0.1)	≤0.02	0.07
C18 carnitine	**1.69**	**1.81**	0.04 (0.02-0.1)	≤0.02	**0.38**

Acyl carnitine profiles from cases 2 and 4, control horses, and horses with AM/SPM. *The equine range is derived from previously published values [18; 35]. **Median acyl carnitines were calculated from the combined values previously reported for individual horses with AM or

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SPM [18; 36]. Values above the control range are in bold. Increases in shorter (C2-C5) chain acyl carnitines may be due to minor inhibition of other acyl-CoA dehydrogenase enzymes by malvalic or sterculic acids or their metabolites, due to relatively broad substrate specificity.

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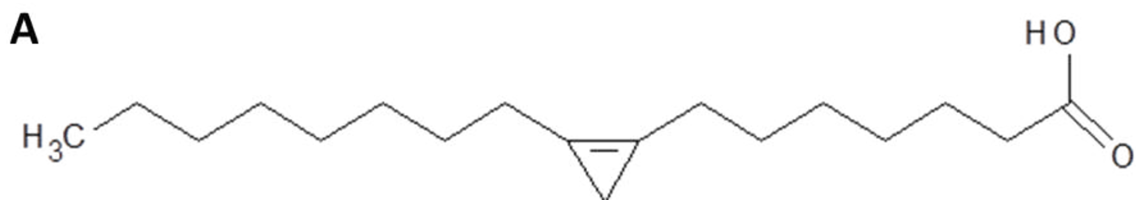

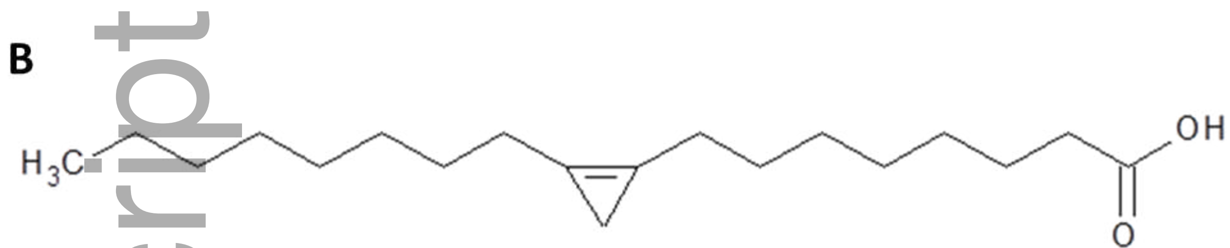

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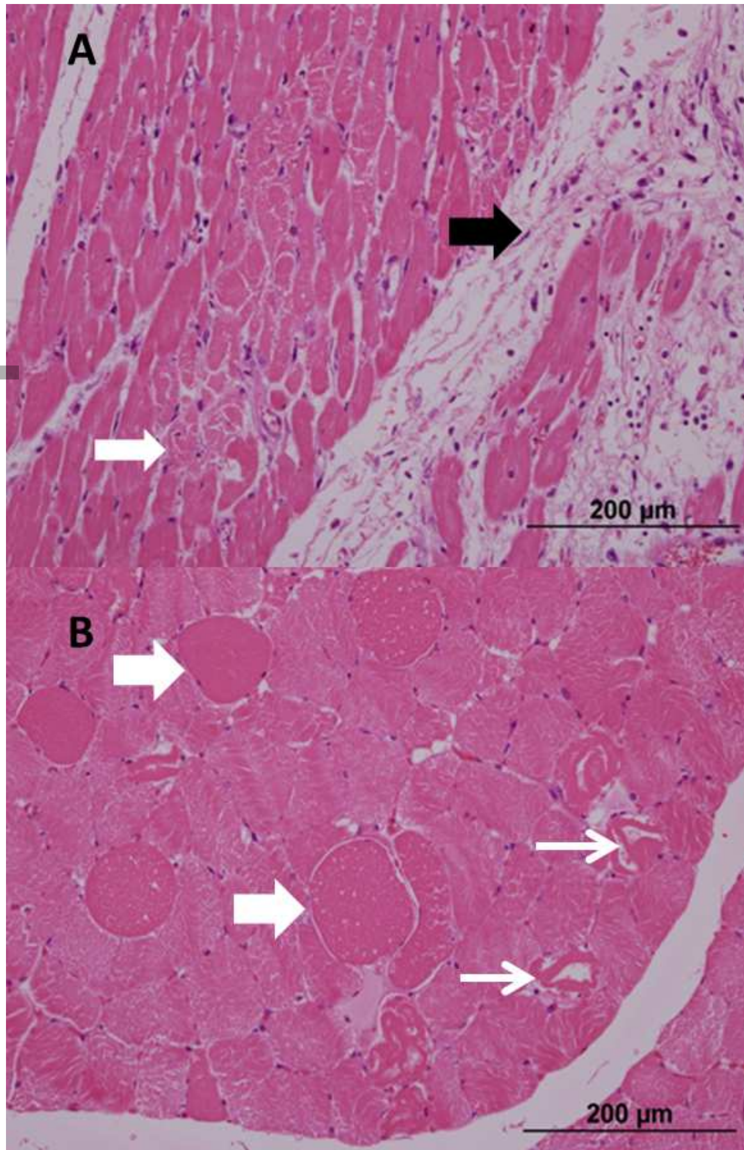

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