

Prolonged oral coenzyme Q₁₀-β-cyclodextrin supplementation increases plasma CoQ₁₀ concentration and skeletal muscle complex I+III activity in young, untrained healthy Thoroughbreds.

Short title: Coenzyme Q₁₀-β-cyclodextrin supplementation in Thoroughbreds.

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Ethical Considerations

University College Dublin Animal Research Ethics Committee approval, a Health Products Regulatory Authority license and explicit owner/trainer informed consent were all obtained for the use of the horses in this study.

Conflict of interest statement/declaration

EWB is a shareholder in Plusvital Ltd, an equine nutrition and genetic testing company. Plusvital is the commercial developer of EnerGene-Q10, an equine nutritional supplement product containing MicroActive® CoQ10. Plusvital has a licence with Maypro Industries (New York, USA) for the equine use of MicroActive® CoQ10. LMK received remuneration for consulting on this project. MEG was employed by Plusvital Ltd during the project.

1 **Prolonged oral coenzyme Q₁₀- β -cyclodextrin supplementation increases plasma CoQ₁₀**
2 **concentration and skeletal muscle complex I+III activity in young, untrained healthy**
3 **Thoroughbreds.**

4

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6 **Abstract**

7 Coenzyme Q₁₀ (CoQ₁₀) is an essential component of the mitochondrial electron transport chain
8 (ETC). Decreased skeletal muscle CoQ₁₀ content may result in decreased ETC activity and
9 energy production. This study aimed to test the hypothesis that prolonged supplementation with
10 oral CoQ₁₀ will increase plasma CoQ₁₀ concentrations and skeletal muscle CoQ₁₀ content in
11 young, healthy untrained Thoroughbreds. Nineteen Thoroughbreds (27.5±9.7 months old; 11
12 males, 8 females) from one farm and maintained on a grass pasture with one grain meal per
13 day were supplemented orally once per day for 9 weeks with 1.5 mg/kg body weight of a
14 CoQ₁₀-β-cyclodextrin inclusion complex. Whole-blood and skeletal muscle biopsies were
15 collected before (T₀) and after (T₁) 9 weeks of supplementation. Plasma CoQ₁₀ concentrations
16 were determined via high-performance liquid chromatography. Skeletal muscle mitochondrial
17 ETC combined complex I+III enzyme activity (an indirect measurement of CoQ₁₀ content) was
18 assessed spectrophotometrically and normalised to mitochondrial abundance. Results were
19 analysed using a paired two-tailed Students *t*-test with $P \leq 0.05$ significant. Horses accepted
20 supplementation with no adverse effects. The mean change in plasma CoQ₁₀ concentration
21 from T₀ to T₁ was significantly greater than zero (0.13±0.02 vs. 0.25±0.03 µg/ml, mean
22 difference 0.12±0.03; $P=0.004$), although variability in absorbance resulted in only a 58%
23 response rate. The mean change in skeletal muscle complex I+III activity from T₀ to T₁ was
24 significantly greater than zero (0.36±0.04 vs. 0.59±0.05 pmol/min/mg of muscle, mean
25 difference 0.23±0.05; $P=0.0004$), although T₁ values for 3/19 horses decreased on average by
26 23% below T₀ values. In conclusion, prolonged oral supplementation of the diet of young,
27 healthy untrained Thoroughbreds with CoQ₁₀ increased mean plasma CoQ₁₀ concentration by
28 99% and mean skeletal muscle complex I+III activity by 65% with variability in absorbance
29 among horses. Additional research is warranted investigating training and exercise effects on
30 skeletal muscle CoQ₁₀ content in CoQ₁₀ supplemented and un-supplemented Thoroughbreds.

31
32 **Keywords:** bioavailability, CoQ₁₀-β-cyclodextrin inclusion complex, equine, skeletal muscle

33
34

35 **Introduction**

36 Coenzyme Q₁₀ (CoQ₁₀, ubiquinone) is a small lipophilic molecule endogenously synthesised
37 (Olson & Rudney, 1983; Tran & Clarke, 2007) in eukaryotic cells with a principal role in
38 aerobic respiration. CoQ₁₀ is a mobile component of the electron transport chain (ETC) within
39 the mitochondrial inner membrane where it transfers electrons from NADH:ubiquinone
40 oxidoreductase (complex I) and succinate dehydrogenase (complex II) to ubiquinol-
41 cytochrome *c* oxidoreductase (complex III) (Mas & Mori, 2010).

42 In humans, chronic diseases such as chronic heart failure, hypertension and Parkinson's
43 disease are characterised by low plasma concentration and tissue CoQ₁₀ content, with CoQ₁₀
44 supplementation shown to improve clinical responses to treatment (Hofman-Bang, Rehnqvist,
45 Swedberg, Wiklund, & Åström, 1995; Jankowski, Korzeniowska, Cieślewicz, & Jabłeczka,
46 2016; Mortensen et al., 2014; Yang et al., 2015). Healthy human athletes have also been found
47 to develop CoQ₁₀ deficiencies, believed to be due to increased metabolic demand (Cooke et
48 al., 2008; M. Kon et al., 2007; Orlando et al., 2018; Zhou, Zhang, Davie, & Marshall-Gradisnik,
49 2005). Deficiencies in skeletal muscle CoQ₁₀ are thought to result in less efficient energy
50 transduction due to decreased ETC activity and suboptimal ATP production (Lenaz et al.,
51 1999), resulting in reduced effective skeletal muscle contractile function and earlier onset of
52 fatigue (Cooke et al., 2008; M. Kon et al., 2007; Michihiro Kon et al., 2008; Kwong et al.,
53 2002; Mizuno et al., 2008). Numerous studies support CoQ₁₀ supplementation in human
54 athletes to improve exercise capacity, aerobic power and recovery after exercise (Alf, Schmidt,
55 & Siebrecht, 2013; Bonetti, Solito, Carosino, Bargossi, & Fiorella, 2000; Cooke et al., 2008;
56 Leelarungrayub, Sawattikanon, Klaphajone, Pothongsunan, & Bloomer, 2010; Mizuno et al.,
57 2008).

58 Approximately 50% of the total body CoQ₁₀ in humans is found in the mitochondrial inner
59 membrane (Greenberg & Frishman, 1990; Kumar, Kaur, Devi, & Mohan, 2009), with organs
60 containing large numbers of mitochondria such as skeletal muscle having the largest amount
61 of CoQ₁₀. In healthy people, plasma CoQ₁₀ concentrations are always higher than skeletal
62 muscle with any movement of CoQ₁₀ from plasma into skeletal muscle due to simple diffusion.
63 The rate of CoQ₁₀ movement from the plasma into the mitochondrial inner membrane is limited
64 by the large size and lipophilic nature of this molecule (Kaikkonen, Tuomainen, Nyysönen, &
65 Salonen, 2002; Turunen, Swiezewska, Chojnacki, Sindelar, & Dallner, 2002), with movement
66 of exogenous CoQ₁₀ into most tissues other than plasma previously believed to be low-to-
67 absent (Svensson et al., 1999; Zhou et al., 2005). However, oral CoQ₁₀ supplementation has
68 been shown to elevate CoQ₁₀ skeletal muscle mitochondrial content in rodents and humans
69 (Cooke et al., 2008; Kamzalov, Sumien, Forster, & Sohal, 2003; M. Kon et al., 2007; Linnane
70 et al., 2002). Although plasma CoQ₁₀ concentrations can easily be measured, this only reflects
71 the bioavailability of CoQ₁₀ after oral supplementation and not the amount of uptake into
72 skeletal muscle (Duncan et al., 2005; Zhang, Aberg, & Appelkvist, 1995). CoQ₁₀ has not been
73 extensively researched in horses, with a few studies demonstrating oral CoQ₁₀ supplementation
74 to increase plasma concentrations (Sinatra, Chopra, Jankowitz, Horohov, & Bhagavan, 2013;
75 Sinatra, Jankowitz, Chopra, & Bhagavan, 2013).

76 The aim of this study was therefore to test the hypothesis that prolonged oral supplementation
77 of CoQ₁₀ to the established diet of a group of young, healthy untrained Thoroughbreds would
78 increase plasma CoQ₁₀ concentrations and skeletal muscle CoQ₁₀ content.

79

80 **Materials and Methods**

81 **Animals and experimental design**

82 This study took place from the last week of May – end of July 2017 in the Republic of Ireland.
83 Approval was obtained from the University College Dublin Animal Research Ethics

84 Committee with informed owner consent. The project was licenced under the Health Products
85 Regulatory Authority (Ireland).

86 Nineteen clinically healthy and privately-owned Thoroughbreds from one farm (11 intact
87 males [mean age 27.8±9.0 months], 8 females [mean age 27.1±11.1 months]) that were not
88 currently and had never been in an exercise training programme were included into the study.
89 Prior to and during the entire study, all horses had been/were maintained full-time in small
90 groups of 5–6 horses on 5-acre grass pastures located next to each other. The diet of the horses
91 at the time of entering into the study and during the study consisted of free-choice pasture
92 grazing and one grain meal (one standard scoop of mixed oats) given in the morning. All horses
93 had physical examinations, haematology, biochemistry and faecal evaluations performed prior
94 to inclusion into the study. Body weight (BW) was estimated for each horse at the beginning
95 of the study using a weight tape and formula (Carroll & Huntington, 1988): BW
96 (kg)=[Girth²(cm) × Length (cm)]/11,877.

97 Each horse acted as its own control with jugular whole-blood and skeletal muscle biopsy
98 samples taken before (T₀) and after 9 weeks (T₁) of oral CoQ₁₀ supplementation to their
99 established diet. All horses were supplemented and sampled during the same 9-week period. A
100 dose of approximately 1.5 mg/kg BW of a CoQ₁₀-β-cyclodextrin inclusion complex in powder
101 form (MicroActive® CoQ₁₀, Maypro Industries, New York, USA) containing 26% CoQ₁₀ w/w
102 was used. For a 500 kg BW horse, this equated to a daily amount of approximately 200 mg of
103 CoQ₁₀. The supplement was dissolved in water and administered via syringe immediately after
104 the morning grain between 7–8 am. All blood and skeletal muscle biopsy samples were taken
105 between 11 am–1 pm, 4–6 hrs after the morning grain. T₀ samples were taken the day before
106 oral CoQ₁₀ supplementation had begun with T₁ samples taken on the last day of oral CoQ₁₀
107 supplementation.

108

109 **Sample collection**

110 Jugular venous whole-blood samples were collected from each horse into a lithium heparin
111 vacutainer for measurement of plasma CoQ₁₀ concentrations. Plasma was separated from
112 whole-blood within 3 hrs of collection via centrifugation (1,500 g for 5 mins) and stored at -
113 20°C until batch analysis.

114 Skeletal muscle biopsies were taken from the middle gluteal muscle from standing un-
115 sedated horses as previously described by Ledwith and McGowan (2004). Once collected, all
116 samples were immediately stored on dry ice for transport to the laboratory (within 3 hrs of
117 collection) and subsequently stored at -70°C until analysis.

118

119 **Quantification of plasma CoQ₁₀ concentrations**

120 Plasma CoQ₁₀ concentrations were measured using a validated reverse-phase high-
121 performance liquid chromatography (HPLC) assay by CAL Ltd (Dublin, Ireland) for a
122 randomly chosen sub-set (*n*=12) of the study horses (www.randomizer.org). Plasma samples
123 were extracted by liquid:liquid extraction (ethanol:methanol; 45:55, v/v) on a Synergi C₁₈
124 column with detection carried out at 275 nm with a UV detector. Each plasma sample was
125 assayed in triplicate under oxidized conditions for total CoQ₁₀ (ubiquinone+ubiquinol) content.
126 Plasma CoQ₁₀ concentrations were calculated from a standard curve produced by standard
127 CoQ₁₀ (Sigma-Aldrich, Co. Wicklow, Ireland) in the concentration range 0.156–2.50 µg/ml.

128

129 **Quantification of skeletal muscle CoQ₁₀ content**

130 Skeletal muscle CoQ₁₀ content was measured spectrophotometrically by combined complex
131 I+III assay (indirect measure of CoQ₁₀). All reagents were purchased from Sigma-Aldrich (Co.
132 Wicklow, Ireland) unless stated otherwise. Enzyme activity assays were performed at 30°C on
133 a Libra S12 spectrophotometer (Biochrom Ltd., Cambridge, UK) with absorbance changes

134 measured using an attached chart recorder. The activity of each enzyme was measured in
135 triplicate on the same homogenate for each sample.

136

137 *Preparation of skeletal muscle homogenates*

138 Skeletal muscle homogenates were prepared from tissue stored at -70°C . Any fat/connective
139 tissue was removed from the sample before it was weighed using a fine balance (ME104
140 Mettler Toledo [Mason Technology, Dublin, Ireland], 0.08 mg repeatability). The tissue was
141 then homogenised using an Ultra Turrax T25 (Janke & Kunkel IKA-Labortechnik, Staufen,
142 Germany) in sucrose muscle homogenisation buffer (20mM tris-HCl, 40mM KCl, 2 mM
143 EGTA, 250 mM sucrose, 1 mM ATP, 5 mM MgCl_2 , pH 7.4). An aliquot of the sample was
144 used to perform protein determination using the bicinchoninic acid assay as described by Smith
145 et al. (1985).

146

147 *Citrate synthase activity assay*

148 Citrate synthase enzyme activity (a measure of mitochondrial abundance) was measured
149 spectrophotometrically by a coloured coupled reaction, using a method adapted from Sreer
150 (1969). The activity of citrate synthase was determined by monitoring the rate of production of
151 thionitrobenzoic acid at a wavelength of 412 nm. Skeletal muscle homogenate (approximately
152 $5\ \mu\text{g}$) was incubated in a 1 ml cuvette with tris buffer (0.2 M, pH 8.1) with reaction components
153 5,5'-dithiobis-(2-nitrobenzoic acid) (0.1 mM), acetyl coenzyme A (0.3 mM) and Triton X
154 (0.1%) added. A blank rate was measured for 2 mins before oxaloacetate (0.5 mM) was added
155 to initiate the reaction with any increase in absorbance monitored for 3 mins. Specific enzyme
156 activity was expressed as pmol/min/mg of muscle protein using the molar extinction coefficient
157 13,600 L/mol/cm for citrate synthase at 412 nm.

158

159 *NADH cytochrome c oxidoreductase (Complex I+III) activity assay*

160 The activity of NADH cytochrome *c* oxidoreductase (Complex I+III) is an indirect measure of
161 CoQ_{10} . As part of the Q cycle in mitochondria, CoQ_{10} transfers electrons from complex I and
162 complex II to complex III. Thus, measurement of combined complex I+III activity gives an
163 indirect measure of CoQ_{10} content, as the activity of these two complexes in combination is
164 dependent on CoQ_{10} (Lerman-Sagie et al., 2001; Leshinsky-Silver et al., 2003). Complex I+III
165 activity was determined in the present study by monitoring the reduction of cytochrome *c* at
166 550 nm as per the method described by Powers et al. (2007). Homogenate samples
167 (approximately $20\ \mu\text{g}$) were incubated in distilled H_2O in a 1 ml cuvette to allow osmotic shock
168 to occur. After 2 mins incubation, the reaction components potassium phosphate pH 7.5 (50
169 mM), oxidised cytochrome *c* ($50\ \mu\text{M}$), KCN (0.3 mM), and fatty-acid free BSA (1 mg/ml) were
170 added; a blank rate was measured for 2 mins. NADH (0.2 mM) was then added to initiate the
171 reaction with any increase in absorbance monitored for 3 mins. Following this, rotenone (10
172 μM) was added and the rate monitored for a further 2 mins. Complex I+III combined specific
173 activity was taken as the rotenone-sensitive activity determined by subtracting the rotenone-
174 resistant activity from the total activity. Specific enzyme activity for complex I+III was
175 expressed as pmol/min/mg of muscle protein using the molar extinction coefficient 18,500
176 L/mol/cm for reduced cytochrome *c* at 550 nm. Complex I+III activity was subsequently
177 expressed as a ratio to citrate synthase activity to account for the mitochondrial enrichment of
178 the skeletal muscle homogenates.

179

180 **Statistical analysis**

181 Statistical analyses were performed using R 3.3.2 (R Foundation for Statistical Computing,
182 Vienna, Austria). The effects of sex and age were investigated using multivariable linear
183 regression models with interaction effects included for age and sex. Baseline age was set at 13

184 months (the minimum age of horses in the dataset). Horse ages were subsequently not adjusted
185 between measurement time-points as age was not identified as a significant factor in T₀ plasma
186 values. Where age and sex effects were deemed non-significant and excluded from the model,
187 mean values were compared using a paired two-tailed Students *t*-test with 95% confidence
188 intervals. Spearman's rank correlation was performed to assess correlation between plasma
189 CoQ₁₀ concentrations and skeletal muscle CoQ₁₀ content. A $P \leq 0.05$ indicated significance,
190 with all results expressed as mean \pm SEM unless otherwise indicated.

191

192 **Results**

193 All horses readily accepted CoQ₁₀ supplementation with no adverse effects observed.
194 Descriptive statistics are summarised in Table 1 and 2.

195 Multivariable linear models were used to evaluate for interactions between age, sex and
196 plasma CoQ₁₀ values. Males had higher T₀ plasma CoQ₁₀ values than females ($P=0.009$), with
197 no differences in T₁ values. For females, age was only significantly associated with T₁ plasma
198 CoQ₁₀ values, with increasing age associated with increasing values ($P=0.02$). For males,
199 increasing age was significantly associated with reductions in T₀ ($P=0.03$) and T₁ plasma
200 CoQ₁₀ values ($P=0.02$). These results are all tenuous, however, since a single elevated T₀
201 plasma CoQ₁₀ value for a 13-month-old male horse skewed all statistical outcomes. For the
202 paired differences in plasma CoQ₁₀ values between T₀ and T₁, a multivariable model including
203 age and sex as factors identified increasing age to be linked to increasing plasma CoQ₁₀ values
204 ($P=0.02$). However, the paired differences for plasma CoQ₁₀ values between time-points were
205 not significantly associated with sex (males $P=0.07$, females $P=0.45$). When sex was
206 subsequently excluded from the model, the significant association between age and plasma
207 CoQ₁₀ values was lost ($P=0.06$). It appears that inadequate power ($n=12$) did not allow
208 completely accurate statistical evaluation of sex and age effects on plasma CoQ₁₀ values.

209 The T₀ and T₁ intra-assay coefficient of variations were 13.3% and 5.7%, respectively. The
210 average T₁ plasma CoQ₁₀ concentrations significantly increased by 99% above the average T₀
211 measurements (0.13 ± 0.02 $\mu\text{g/ml}$ vs. 0.25 ± 0.03 $\mu\text{g/ml}$, mean difference 0.12 ± 0.03 ; $P=0.004$;
212 Table 1, Figure 1). Although the T₁ plasma CoQ₁₀ concentrations were higher than the T₀
213 measurement for all horses with an average mean of the ratios (i.e., the average of each
214 individual horse's difference between T₀ and T₁ values) showing a 162% of an increase of T₁
215 values above T₀ values, there was a large amount of individual variation ranging from a 0.6–
216 617.4% of an increase above T₀ values. Using a measure of uniform bioavailability defined as
217 at least a doubling of T₁ plasma CoQ₁₀ concentrations above T₀ values, there was a 58%
218 response rate with 7/12 horses meeting this threshold.

219 Multivariable linear models were used to evaluate for interactions between age, sex and
220 skeletal muscle complex I+III activity. Age ($P=0.84$) and sex ($P=0.06$) were not significantly
221 associated with mean T₀ skeletal muscle complex I+III activity. Age ($P=0.75$) and sex ($P=0.30$)
222 were also not significantly associated with T₁ skeletal muscle complex I+III activity. For the
223 paired differences in skeletal muscle complex I+III activity between T₀ and T₁, neither age
224 ($P=0.98$) nor sex ($P=0.81$) were significantly associated with skeletal muscle complex I+III
225 activity. These results support that any change in mean skeletal muscle complex I+III activity
226 between time-points is independent of both age and sex.

227 No differences in citrate synthase activity were observed between T₀ and T₁ time-points. The
228 average T₁ skeletal muscle CoQ₁₀ content significantly increased above T₀ values by 65.1%
229 (0.36 ± 0.04 vs 0.59 ± 0.05 pmol/min/mg of muscle protein, activity normalised to mitochondrial
230 abundance/g muscle, mean difference 0.23 ± 0.05 ; $P=0.0004$; Table 2, Figure 2). For 16/19
231 horses, T₁ skeletal CoQ₁₀ content had increased on average 85% above T₀ values with a degree
232 of variation ranging from a 13.3–420.9% of an increase above T₀ values. However, for 3/19
233 horses, T₁ skeletal CoQ₁₀ content decreased by an average of 22.7% (range 11.4–32.4% of a

234 decrease) below T_0 values. There were no correlations between T_0 plasma and skeletal muscle
235 CoQ₁₀ measurements nor between T_1 plasma and skeletal muscle CoQ₁₀ measurements.

236

237 Discussion

238 This study demonstrated that plasma CoQ₁₀ concentrations and skeletal muscle CoQ₁₀ content
239 increased in young, healthy untrained Thoroughbreds after prolonged daily oral CoQ₁₀
240 supplementation of an established diet. In the present study, T_0 plasma CoQ₁₀ concentrations
241 (0.13 µg/ml) were similar to a previous report evaluating 2 year-old Thoroughbreds in training
242 (0.11 µg/ml) (Horohov et al., 2012; Sinatra, Chopra, et al., 2013; Sinatra, Jankowitz, et al.,
243 2013), although other publications reported slightly higher basal plasma concentrations for
244 Thoroughbreds of varying ages and fitness levels (0.19–2.1 µg/ml) (Sinatra, Chopra, et al.,
245 2013; Topolovec et al., 2013). Following prolonged oral CoQ₁₀ supplementation the mean
246 plasma CoQ₁₀ concentrations significantly increased as previously reported in studies using a
247 similar oral cyclodextrin-CoQ₁₀-based delivery system (Horohov et al., 2012; Sinatra, Chopra,
248 et al., 2013; Sinatra, Jankowitz, et al., 2013). Intestinal absorption of CoQ₁₀ has been found to
249 be faster if CoQ₁₀ is given with food (Ochiai et al., 2007) which is why we chose to supplement
250 the horses in the morning in conjunction with their grain meals. The dose of CoQ₁₀
251 supplementation in the present study was well tolerated by the horses with no adverse effects
252 noted, and was chosen based on a previous study using a cyclodextrin-CoQ₁₀-based delivery
253 system (HydroQSorb, a γ -cyclodextrin [\sim 20%] CoQ₁₀ complex) (Sinatra, Chopra, et al., 2013).
254 In humans and dogs, plasma CoQ₁₀ concentrations have been found to gradually increase as
255 the oral dosage increases (Bhagavan & Chopra, 2007), so a higher dose may have resulted in
256 greater increases in plasma CoQ₁₀ concentrations as observed in previous equine reports
257 (Sinatra, Jankowitz, et al., 2013). However, the economic feasibility for owners and trainers
258 were considered, as well as the fact that in humans the efficiency of oral CoQ₁₀ absorption
259 significantly decreases at extremely high doses (>300 mg) (Bhagavan & Chopra, 2007). Most
260 researchers now believe that the formulation of oral CoQ₁₀ (e.g., delivery system) is of equal
261 if not more importance to the dosage, since this highly lipophilic molecule is typically poorly
262 absorbed resulting in a low bioavailability despite the oral dose used as observed in humans,
263 rats and dogs (Bank, Kagan, & Madhavi, 2011; Zhang et al., 1995; Zhang, Turunen, &
264 Appelkvist, 1996).

265 CoQ₁₀ is widely distributed in the body in either a reduced (i.e., ubiquinol) or oxidised (i.e.,
266 ubiquinone) form (Desbats, Lunardi, Doimo, Trevisson, & Salviati, 2015). Regardless of its
267 form, oral CoQ₁₀ is converted to ubiquinol by the enterocytes before being absorbed through
268 the intestinal membrane, entering the systemic circulation via the lymphatic system (Bank et
269 al., 2011) with nearly 95% of plasma CoQ₁₀ present as ubiquinol (Bhagavan & Chopra, 2007).
270 Oral CoQ₁₀ bioavailability can be enhanced by altering pharmaceutical forms, with
271 hydrophobicity of CoQ₁₀ decreased by using cyclodextrin-based delivery methods (Bank et al.,
272 2011; Jankowski et al., 2016). This delivery method significantly enhances water solubility and
273 bioavailability (Žmitek et al., 2008) by complexing each CoQ₁₀ molecule with 2 β -cyclodextrin
274 molecules to form a water-soluble powder (Madhavi & Kagan, 2010). The oral CoQ₁₀- β -
275 cyclodextrin inclusion complex used in this study has been previously shown to be highly
276 bioavailable with a 100% response rate in humans (e.g., all subjects had at least a doubling of
277 plasma concentrations) and a reduced inter-subject variance (Madhavi & Kagan, 2010). High
278 inter-subject variance is a common problem for lipophilic compounds such as CoQ₁₀ because
279 of the poor absorption, meaning that not all subjects will have the same amount of absorbance
280 of the product (Bank et al., 2011). In the present study, there was a degree of variability between
281 horses as has been reported for the other equine studies (Sinatra, Chopra, et al., 2013; Sinatra,
282 Jankowitz, et al., 2013). Although budgetary restrictions meant only 12/19 horses had plasma
283 CoQ₁₀ concentrations measured, there was a 58% response rate identified when using a uniform

284 bioavailability measurement defined as a minimum doubling of T_1 plasma CoQ₁₀
285 concentrations above T_0 values.

286 This was a field-based study using privately-owned horses with samples not obtainable prior
287 to the morning meal, so only the accumulation and not acute phase (0–24 hrs) of oral CoQ₁₀
288 supplementation could be evaluated. The sample timing in the present study was based on prior
289 studies in human subjects that demonstrated plasma concentrations to peak within 4–5 hrs of
290 oral administration of a CoQ₁₀- β -cyclodextrin inclusion complex ((Cuomo & Rabovsky, 2000;
291 Terao et al., 2006). The CoQ₁₀- β -cyclodextrin inclusion complex used in the present study has
292 also been demonstrated in human subjects to have a sustained release resulting in the
293 maintenance of plasma CoQ₁₀ concentrations approximately 6 times higher than baseline for
294 24 hrs following oral administration (Madhavi & Kagan, 2010). This effect has been reported
295 for other oral cyclodextrin complex CoQ₁₀-based delivery systems, although lower sustained
296 plasma CoQ₁₀ concentrations were achieved (Terao et al., 2006).

297 An increase in mean skeletal muscle CoQ₁₀ content was observed in the current study
298 following prolonged oral CoQ₁₀ supplementation as reflected by significant increases in CoQ₁₀-
299 dependent skeletal muscle mitochondrial function above basal activity. It has been theorised
300 that improved CoQ₁₀ absorption into the systemic circulation, elevating CoQ₁₀ plasma
301 concentrations, helps improve delivery rate into skeletal muscle (Cooke et al., 2008). The
302 concurrent increase in plasma CoQ₁₀ concentrations following supplementation found in the
303 present study thus supports the increased skeletal muscle complex I+III activity to be a result
304 of supplementation.

305 It is interesting to note that the T_1 skeletal muscle CoQ₁₀ content of three horses had fallen
306 marginally below baseline values, potentially indicating a requirement for some horses to have
307 higher plasma concentrations to facilitate movement of CoQ₁₀ into the skeletal muscle
308 mitochondria. There were no correlations with the degree of plasma CoQ₁₀ concentrations and
309 skeletal muscle CoQ₁₀ content supporting the inability to use plasma CoQ₁₀ to assess skeletal
310 muscle CoQ₁₀ content. The duration of oral CoQ₁₀ supplementation has been hypothesised to
311 contribute to the limitation of how much CoQ₁₀ enters skeletal muscle mitochondria (Cooke et
312 al., 2008). One group of researchers reported skeletal muscle CoQ₁₀ content in humans to
313 increase within 2 hours of oral supplementation, but then decrease to just above baseline values
314 after 2 weeks of oral supplementation (Cooke et al., 2008). These researchers hypothesised that
315 CoQ₁₀ uptake into skeletal muscle may be similar to creatine monohydrate, in which there
316 appears to be a maximal limit and/or down-regulation of transporters reached after chronic
317 supplementation leading to a plateau and/or decrease in intramuscular content over time
318 (Guerrero-Ontiveros & Wallimann, 1998). This warrants further investigation in the horse.

319 A limitation of this study was an inadequate number of horses available for a control group
320 based on power calculations for statistical validity. A control group would have verified
321 whether changes in dietary intake of CoQ₁₀ other than supplementation contributed to the
322 observed increases in skeletal muscle CoQ₁₀ content. All study horses were housed on the same
323 farm in adjacent pastures, with changes in grass CoQ₁₀ content over the 9-week study period
324 unlikely to have occurred since plants contain an extremely small amount of CoQ₁₀, with any
325 dietary intake for humans primarily coming from meat-based products (Kumar et al., 2009;
326 Parmar, Jaiwal, Dhankher, & Jaiwal, 2015; Pravst, Žmitek, & Žmitek, 2010). Furthermore,
327 even including meat-based products the typical human diet does not contain enough CoQ₁₀ to
328 significantly raise plasma CoQ₁₀ concentrations above basal levels, with the daily CoQ₁₀ intake
329 from food ranging between 3–5 mg/day which is too low to significantly raise blood and tissue
330 concentrations above basal levels (Wajda, Zirkel, & Schaffer, 2007). Since the majority of
331 tissue CoQ₁₀ content in mammals is endogenously synthesised by the mitochondria, a
332 significantly large increase in plasma CoQ₁₀ concentration is required to incite movement into
333 tissue with human and other animal studies reporting that plasma CoQ₁₀ concentrations and

334 skeletal muscle CoQ₁₀ content will not significantly increase without exogenous influences
335 (Bhagavan & Chopra, 2007). Although plasma CoQ₁₀ concentrations in humans is typically
336 not affected by diet alone, CoQ₁₀ supplementation has been shown to increase plasma CoQ₁₀
337 concentrations, the extent of which depends upon the dosage, duration and type of formulation
338 (Pravst et al., 2010).

339 It has been hypothesised that increased skeletal muscle CoQ₁₀ should result in more efficient
340 skeletal muscle energy transduction (Lenaz et al., 1999). For horses in active exercise training
341 this may lead to improvements in responses to exercise training, delay in the onset of fatigue
342 and enhanced recovery following intense exercise (Cooke et al., 2008; M. Kon et al., 2007;
343 Michihiro Kon et al., 2008; Kwong et al., 2002; Mizuno et al., 2008). During exercise,
344 movement of plasma CoQ₁₀ into skeletal muscle may increase due to increased metabolic
345 demand (M. Kon et al., 2007; Orlando et al., 2018). This theory is supported by results from a
346 study identifying increased post-exercise intramuscular CoQ₁₀ content in human athletes orally
347 supplemented with CoQ₁₀ (Cooke et al., 2008). It has recently been shown that resting skeletal
348 muscle CoQ₁₀ content is associated with *myostatin* (*MSTN*) genotype (SNP g.66493737C>T)
349 in untrained Thoroughbred horses (Rooney, Porter, Katz, & Hill, 2017). ETC combined
350 complex I+III and II+III activities (indirect measures of CoQ₁₀ content) were significantly
351 lower in resting skeletal muscle from TT *MSTN* genotype horses as compared to CT and CC
352 horses. In this same study, restoration of complex I+III and II+III activity was achieved
353 following *in vitro* supplementation with exogenous coenzyme Q₁. Based on the observed
354 differences in basal concentrations of skeletal muscle CoQ₁₀ between *MSTN* genotypes in
355 Thoroughbreds, oral supplementation with CoQ₁₀ may have a greater efficacy in skeletal
356 muscle of horses with the TT *MSTN* genotype, especially for TT horses training and competing
357 in endurance-related competitions. In the present study, the number of horses with different
358 *MSTN* genotypes was too small to assess for genotype-specific variation in plasma and skeletal
359 muscle CoQ₁₀ concentrations after supplementation, but this certainly warrants further
360 investigation.

361

362 **Conclusion**

363 In summary, this study demonstrates that prolonged daily oral supplementation of a grass and
364 oat diet of young, healthy untrained Thoroughbreds with a CoQ₁₀-β-cyclodextrin inclusion
365 complex significantly increases mean plasma concentration and skeletal muscle CoQ₁₀ content,
366 although a degree of variability was identified for some horses. Additional research is
367 warranted to investigate the effects of *MSTN* genotype, training and exercise on skeletal muscle
368 CoQ₁₀ content in CoQ₁₀-β-cyclodextrin inclusion complex supplemented and un-supplemented
369 Thoroughbreds.

370

371

372 **References**

373

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532

533 **Table 1.** Summary statistics for plasma CoQ₁₀ concentrations measured in triplicate from $n=12$
534 young, healthy untrained Thoroughbred horses before (T₀) and after (T₁) 9 weeks of daily oral
535 supplementation of the established diet with CoQ₁₀ (CoQ₁₀- β -cyclodextrin complex with 26%
536 CoQ₁₀, w/w).
537

$n=12$	T ₀ ($\mu\text{g/ml}$)	T ₁ ($\mu\text{g/ml}$)
Minimum	0.04	0.12
25% percentile	0.08	0.14
Median	0.11	0.27
75% percentile	0.14	0.29
Maximum	0.33	0.49
Mean	0.13	0.25 [†]
Standard deviation	0.08	0.11
Standard error of the mean	0.02	0.03
95% Confidence Intervals	0.08–0.17	0.18–0.32

538 [†]denotes significant difference from T₀ values (paired two-tailed Student's t -test, $P \leq 0.01$).
539

540 **Table 2.** Middle gluteal skeletal muscle CoQ₁₀ content for 19 young, healthy untrained
541 Thoroughbred horses before (T₀) and after (T₁) 9 weeks of daily oral supplementation of the
542 established diet with CoQ₁₀ (CoQ₁₀- β -cyclodextrin complex with 26% CoQ₁₀, w/w). CoQ₁₀
543 content was assessed by spectrophotometrically measuring skeletal muscle mitochondrial
544 complex I+III activity. Complex I+III activity data (pmol/min/mg of muscle protein) was
545 normalised to mitochondrial abundance (citrate synthase activity)/g of skeletal muscle.

546

<i>n</i> =19	T ₀ (pmol/min/mg muscle protein)	T ₁ (pmol/min/mg muscle protein)
Minimum	0.11	0.18
25% percentile	0.23	0.44
Median	0.36	0.63
75% percentile	0.51	0.8
Maximum	0.56	0.92
Mean	0.36	0.59 [†]
Standard deviation	0.16	0.22
Standard error of the mean	0.04	0.05
95% Confidence Intervals	0.28–0.43	0.49–0.7

547 [†]denotes significant difference from T₀ values (paired two-tailed Student's *t*-test, $P \leq 0.01$).

548

549 **Figure Legends**

550

551 **Figure 1.** Plasma CoQ₁₀ concentrations for 12 young, healthy untrained Thoroughbred horses
552 before (T₀) and after (T₁) 9 weeks of daily oral supplementation of the established diet with
553 CoQ₁₀ (CoQ₁₀-β-cyclodextrin complex with 26% CoQ₁₀, w/w). *Significantly different from
554 T₀ values (paired two-tailed Student's *t*-test, $P \leq 0.01$).

555

556 **Figure 2.** Middle gluteal skeletal muscle CoQ₁₀ content for 19 young, healthy untrained
557 Thoroughbred horses before (T₀) and after (T₁) 9 weeks of daily oral supplementation of the
558 established diet with CoQ₁₀ (CoQ₁₀-β-cyclodextrin complex with 26% CoQ₁₀, w/w). CoQ₁₀
559 content was assessed by spectrophotometrically measuring skeletal muscle mitochondrial
560 complex I+III activity. Complex I+III activity data (pmol/min/mg of muscle protein) was
561 normalised to mitochondrial abundance (citrate synthase activity)/g of skeletal muscle.
562 *Significantly different from T₀ values (paired two-tailed Student's *t*-test, $P \leq 0.01$).

563

Figure 1

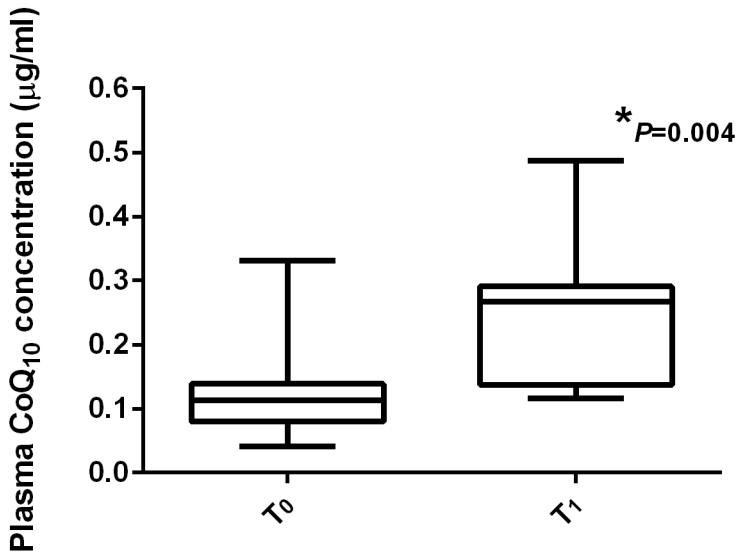


Figure 2

Skeletal muscle CoQ₁₀ content
(complex I+III activity)

