Prolonged oral coenzyme Q_{10}-β-cyclodextrin supplementation increases plasma CoQ_{10} concentration and skeletal muscle complex I+III activity in young, untrained healthy Thoroughbreds.

Short title: Coenzyme Q_{10}-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
EWH 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.

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Abstract

Coenzyme Q_{10} (CoQ_{10}) is an essential component of the mitochondrial electron transport chain (ETC). Decreased skeletal muscle CoQ_{10} content may result in decreased ETC activity and energy production. This study aimed to test the hypothesis that prolonged supplementation with oral CoQ_{10} will increase plasma CoQ_{10} concentrations and skeletal muscle CoQ_{10} content in young, healthy untrained Thoroughbreds. Nineteen Thoroughbreds (27.5±9.7 months old; 11 males, 8 females) from one farm and maintained on a grass pasture with one grain meal per day were supplemented orally once per day for 9 weeks with 1.5 mg/kg body weight of a CoQ_{10}-β-cyclodextrin inclusion complex. Whole-blood and skeletal muscle biopsies were collected before (T_0) and after (T_1) 9 weeks of supplementation. Plasma CoQ_{10} concentrations were determined via high-performance liquid chromatography. Skeletal muscle mitochondrial ETC combined complex I+III enzyme activity (an indirect measurement of CoQ_{10} content) was assessed spectrophotometrically and normalised to mitochondrial abundance. Results were analysed using a paired two-tailed Students *t*-test with $P \leq 0.05$ significant. Horses accepted supplementation with no adverse effects. The mean change in plasma CoQ_{10} concentration from T_0 to T_1 was significantly greater than zero (0.13±0.02 *vs*. 0.25±0.03 µg/ml, mean difference 0.12±0.03; $P=0.004$), although variability in absorbance resulted in only a 58% response rate. The mean change in skeletal muscle complex I+III activity from T_0 to T_1 was significantly greater than zero (0.36±0.04 *vs*. 0.59±0.05 pmol/min/mg of muscle, mean difference 0.23±0.05; $P=0.0004$), although T_1 values for 3/19 horses decreased on average by 23% below T_0 values. In conclusion, prolonged oral supplementation of the diet of young, healthy untrained Thoroughbreds with CoQ_{10} increased mean plasma CoQ_{10} concentration by 99% and mean skeletal muscle complex I+III activity by 65% with variability in absorbance among horses. Additional research is warranted investigating training and exercise effects on skeletal muscle CoQ_{10} content in CoQ_{10} supplemented and un-supplemented Thoroughbreds.

Keywords: bioavailability, CoQ_{10}-β-cyclodextrin inclusion complex, equine, skeletal muscle

Introduction

Coenzyme Q_{10} (CoQ_{10}, ubiquinone) is a small lipophilic molecule endogenously synthesised (Olson & Rudney, 1983; Tran & Clarke, 2007) in eukaryotic cells with a principal role in aerobic respiration. CoQ_{10} is a mobile component of the electron transport chain (ETC) within the mitochondrial inner membrane where it transfers electrons from NADH:ubiquinone oxidoreductase (complex I) and succinate dehydrogenase (complex II) to ubiquinol-cytochrome *c* oxidoreductase (complex III) (Mas & Mori, 2010).

In humans, chronic diseases such as chronic heart failure, hypertension and Parkinson's disease are characterised by low plasma concentration and tissue CoQ_{10} content, with CoQ_{10} supplementation shown to improve clinical responses to treatment (Hofman-Bang, Rehnqvist, Swedberg, Wiklund, & Åström, 1995; Jankowski, Korzeniowska, Cieślewicz, & Jabłecka, 2016; Mortensen et al., 2014; Yang et al., 2015). Healthy human athletes have also been found to develop CoQ_{10} deficiencies, believed to be due to increased metabolic demand (Cooke et al., 2008; M. Kon et al., 2007; Orlando et al., 2018; Zhou, Zhang, Davie, & Marshall-Gradisnik, 2005). Deficiencies in skeletal muscle CoQ_{10} are thought to result in less efficient energy transduction due to decreased ETC activity and suboptimal ATP production (Lenaz et al., 1999), resulting in reduced effective skeletal muscle contractile function and earlier onset of fatigue (Cooke et al., 2008; M. Kon et al., 2007; Michihiro Kon et al., 2008; Kwong et al., 2002; Mizuno et al., 2008). Numerous studies support CoQ_{10} supplementation in human athletes to improve exercise capacity, aerobic power and recovery after exercise (Alf, Schmidt, & Siebrecht, 2013; Bonetti, Solito, Carmosino, Bargossi, & Fiorella, 2000; Cooke et al., 2008; Leelarungrayub, Sawattikanon, Klaphajone, Pothongsunan, & Bloomer, 2010; Mizuno et al., 2008).

Approximately 50% of the total body CoQ_{10} in humans is found in the mitochondrial inner membrane (Greenberg & Frishman, 1990; Kumar, Kaur, Devi, & Mohan, 2009), with organs containing large numbers of mitochondria such as skeletal muscle having the largest amount of CoQ_{10}. In healthy people, plasma CoQ_{10} concentrations are always higher than skeletal muscle with any movement of CoQ_{10} from plasma into skeletal muscle due to simple diffusion. The rate of CoQ_{10} movement from the plasma into the mitochondrial inner membrane is limited by the large size and lipophilic nature of this molecule (Kaikkonen, Tuomainen, Nyyssönen, & Salonen, 2002; Turunen, Swiezewska, Chojnacki, Sindelar, & Dallner, 2002), with movement of exogenous CoQ_{10} into most tissues other than plasma previously believed to be low-to-absent (Svensson et al., 1999; Zhou et al., 2005). However, oral CoQ_{10} supplementation has been shown to elevate CoQ_{10} skeletal muscle mitochondrial content in rodents and humans (Cooke et al., 2008; Kamzalov, Sumien, Forster, & Sohal, 2003; M. Kon et al., 2007; Linnane et al., 2002). Although plasma CoQ_{10} concentrations can easily be measured, this only reflects the bioavailability of CoQ_{10} after oral supplementation and not the amount of uptake into skeletal muscle (Duncan et al., 2005; Zhang, Aberg, & Appelkvist, 1995). CoQ_{10} has not been extensively researched in horses, with a few studies demonstrating oral CoQ_{10} supplementation to increase plasma concentrations (Sinatra, Chopra, Jankowitz, Horohov, & Bhagavan, 2013; Sinatra, Jankowitz, Chopra, & Bhagavan, 2013).

The aim of this study was therefore to test the hypothesis that prolonged oral supplementation of CoQ_{10} to the established diet of a group of young, healthy untrained Thoroughbreds would increase plasma CoQ_{10} concentrations and skeletal muscle CoQ_{10} content.

Materials and Methods

Animals and experimental design

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

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

Nineteen clinically healthy and privately-owned Thoroughbreds from one farm (11 intact males [mean age 27.8±9.0 months], 8 females [mean age 27.1±11.1 months]) that were not currently and had never been in an exercise training programme were included into the study. Prior to and during the entire study, all horses had been/were maintained full-time in small groups of 5─6 horses on 5-acre grass pastures located next to each other. The diet of the horses at the time of entering into the study and during the study consisted of free-choice pasture grazing and one grain meal (one standard scoop of mixed oats) given in the morning. All horses had physical examinations, haematology, biochemistry and faecal evaluations performed prior to inclusion into the study. Body weight (BW) was estimated for each horse at the beginning of the study using a weight tape and formula (Carroll & Huntington, 1988): BW (kg)=[Girth^2(cm) × Length (cm)]/11,877.

Each horse acted as its own control with jugular whole-blood and skeletal muscle biopsy samples taken before (T_0) and after 9 weeks (T_1) of oral CoQ_{10} supplementation to their established diet. All horses were supplemented and sampled during the same 9-week period. A dose of approximately 1.5 mg/kg BW of a CoQ_{10}-β-cyclodextrin inclusion complex in powder form (MicroActive® CoQ_{10}, Maypro Industries, New York, USA) containing 26% CoQ_{10} w/w was used. For a 500 kg BW horse, this equated to a daily amount of approximately 200 mg of CoQ_{10}. The supplement was dissolved in water and administered via syringe immediately after the morning grain between 7–8 am. All blood and skeletal muscle biopsy samples were taken between 11 am–1 pm, 4–6 hrs after the morning grain. T_0 samples were taken the day before oral CoQ_{10} supplementation had begun with T_1 samples taken on the last day of oral CoQ_{10} supplementation.

Sample collection

Jugular venous whole-blood samples were collected from each horse into a lithium heparin vacutainer for measurement of plasma CoQ_{10} concentrations. Plasma was separated from whole-blood within 3 hrs of collection via centrifugation (1,500 g for 5 mins) and stored at -20°C until batch analysis.

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

Quantification of plasma CoQ_{10} concentrations

Plasma CoQ_{10} concentrations were measured using a validated reverse-phase high-performance liquid chromatography (HPLC) assay by CAL Ltd (Dublin, Ireland) for a randomly chosen sub-set (n=12) of the study horses (www.randomizer.org). Plasma samples were extracted by liquid:liquid extraction (ethanol:methanol; 45:55, v/v) on a Synergi C_{18} column with detection carried out at 275 nm with a UV detector. Each plasma sample was assayed in triplicate under oxidized conditions for total CoQ_{10} (ubiquinone+ubiquinol) content. Plasma CoQ_{10} concentrations were calculated from a standard curve produced by standard CoQ_{10} (Sigma-Aldrich, Co. Wicklow, Ireland) in the concentration range 0.156–2.50 µg/ml.

Quantification of skeletal muscle CoQ_{10} content

Skeletal muscle CoQ_{10} content was measured spectrophotometrically by combined complex I+III assay (indirect measure of CoQ_{10}). All reagents were purchased from Sigma-Aldrich (Co. Wicklow, Ireland) unless stated otherwise. Enzyme activity assays were performed at 30°C on a Libra S12 spectrophotometer (Biochrom Ltd., Cambridge, UK) with absorbance changes

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

Preparation of skeletal muscle homogenates

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

Citrate synthase activity assay

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

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

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

Statistical analysis

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

months (the minimum age of horses in the dataset). Horse ages were subsequently not adjusted between measurement time-points as age was not identified as a significant factor in T_0 plasma values. Where age and sex effects were deemed non-significant and excluded from the model, mean values were compared using a paired two-tailed Students *t*-test with 95% confidence intervals. Spearman's rank correlation was performed to assess correlation between plasma CoQ_{10} concentrations and skeletal muscle CoQ_{10} content. A $P \leq 0.05$ indicated significance, with all results expressed as mean ± SEM unless otherwise indicated.

Results

All horses readily accepted CoQ_{10} supplementation with no adverse effects observed. Descriptive statistics are summarised in Table 1 and 2.

Multivariable linear models were used to evaluate for interactions between age, sex and plasma CoQ_{10} values. Males had higher T_0 plasma CoQ_{10} values than females (P=0.009), with no differences in T_1 values. For females, age was only significantly associated with T_1 plasma CoQ_{10} values, with increasing age associated with increasing values (P=0.02). For males, increasing age was significantly associated with reductions in T_0 (P=0.03) and T_1 plasma CoQ_{10} values (P=0.02). These results are all tenuous, however, since a single elevated T_0 plasma CoQ_{10} value for a 13-month-old male horse skewed all statistical outcomes. For the paired differences in plasma CoQ_{10} values between T_0 and T_1, a multivariable model including age and sex as factors identified increasing age to be linked to increasing plasma CoQ_{10} values (P=0.02). However, the paired differences for plasma CoQ_{10} values between time-points were not significantly associated with sex (males P=0.07, females P=0.45). When sex was subsequently excluded from the model, the significant association between age and plasma CoQ_{10} values was lost (P=0.06). It appears that inadequate power (n=12) did not allow completely accurate statistical evaluation of sex and age effects on plasma CoQ_{10} values.

The T_0 and T_1 intra-assay coefficient of variations were 13.3% and 5.7%, respectively. The average T_1 plasma CoQ_{10} concentrations significantly increased by 99% above the average T_0 measurements (0.13±0.02 µg/ml *vs.* 0.25±0.03 µg/ml, mean difference 0.12±0.03; P=0.004; Table 1, Figure 1). Although the T_1 plasma CoQ_{10} concentrations were higher than the T_0 measurement for all horses with an average mean of the ratios (i.e., the average of each individual horse's difference between T_0 and T_1 values) showing a 162% of an increase of T_1 values above T_0 values, there was a large amount of individual variation ranging from a 0.6–617.4% of an increase above T_0 values. Using a measure of uniform bioavailability defined as at least a doubling of T_1 plasma CoQ_{10} concentrations above T_0 values, there was a 58% response rate with 7/12 horses meeting this threshold.

Multivariable linear models were used to evaluate for interactions between age, sex and skeletal muscle complex I+III activity. Age (P=0.84) and sex (P=0.06) were not significantly associated with mean T_0 skeletal muscle complex I+III activity. Age (P=0.75) and sex (P=0.30) were also not significantly associated with T_1 skeletal muscle complex I+III activity. For the paired differences in skeletal muscle complex I+III activity between T_0 and T_1, neither age (P=0.98) nor sex (P=0.81) were significantly associated with skeletal muscle complex I+III activity. These results support that any change in mean skeletal muscle complex I+III activity between time-points is independent of both age and sex.

No differences in citrate synthase activity were observed between T_0 and T_1 time-points. The average T_1 skeletal muscle CoQ_{10} content significantly increased above T_0 values by 65.1% (0.36±0.04 *vs* 0.59±0.05 pmol/min/mg of muscle protein, activity normalised to mitochondrial abundance/g muscle, mean difference 0.23±0.05; P=0.0004; Table 2, Figure 2). For 16/19 horses, T_1 skeletal CoQ_{10} content had increased on average 85% above T_0 values with a degree of variation ranging from a 13.3–420.9% of an increase above T_0 values. However, for 3/19 horses, T_1 skeletal CoQ_{10} content decreased by an average of 22.7% (range 11.4–32.4% of a

decrease) below T_0 values. There were no correlations between T_0 plasma and skeletal muscle CoQ_{10} measurements nor between T_1 plasma and skeletal muscle CoQ_{10} measurements.

Discussion

This study demonstrated that plasma CoQ_{10} concentrations and skeletal muscle CoQ_{10} content increased in young, healthy untrained Thoroughbreds after prolonged daily oral CoQ_{10} supplementation of an established diet. In the present study, T_0 plasma CoQ_{10} concentrations (0.13 µg/ml) were similar to a previous report evaluating 2 year-old Thoroughbreds in training (0.11 µg/ml) (Horohov et al., 2012; Sinatra, Chopra, et al., 2013; Sinatra, Jankowitz, et al., 2013), although other publications reported slightly higher basal plasma concentrations for Thoroughbreds of varying ages and fitness levels (0.19–2.1 µg/ml) (Sinatra, Chopra, et al., 2013; Topolovec et al., 2013). Following prolonged oral CoQ_{10} supplementation the mean plasma CoQ_{10} concentrations significantly increased as previously reported in studies using a similar oral cyclodextrin-CoQ_{10}-based delivery system (Horohov et al., 2012; Sinatra, Chopra, et al., 2013; Sinatra, Jankowitz, et al., 2013). Intestinal absorption of CoQ_{10} has been found to be faster if CoQ_{10} is given with food (Ochiai et al., 2007) which is why we chose to supplement the horses in the morning in conjunction with their grain meals. The dose of CoQ_{10} supplementation in the present study was well tolerated by the horses with no adverse effects noted, and was chosen based on a previous study using a cyclodextrin-CoQ_{10}-based delivery system (HydroQSorb, a γ-cyclodextrin [~20%] CoQ_{10} complex) (Sinatra, Chopra, et al., 2013). In humans and dogs, plasma CoQ_{10} concentrations have been found to gradually increase as the oral dosage increases (Bhagavan & Chopra, 2007), so a higher dose may have resulted in greater increases in plasma CoQ_{10} concentrations as observed in previous equine reports (Sinatra, Jankowitz, et al., 2013). However, the economic feasibility for owners and trainers were considered, as well as the fact that in humans the efficiency of oral CoQ_{10} absorption significantly decreases at extremely high doses (>300 mg) (Bhagavan & Chopra, 2007). Most researchers now believe that the formulation of oral CoQ_{10} (e.g., delivery system) is of equal if not more importance to the dosage, since this highly lipophilic molecule is typically poorly absorbed resulting in a low bioavailability despite the oral dose used as observed in humans, rats and dogs (Bank, Kagan, & Madhavi, 2011; Zhang et al., 1995; Zhang, Turunen, & Appelkvist, 1996).

CoQ_{10} is widely distributed in the body in either a reduced (i.e., ubiquinol) or oxidised (i.e., ubiquinone) form (Desbats, Lunardi, Doimo, Trevisson, & Salviati, 2015). Regardless of its form, oral CoQ_{10} is converted to ubiquinol by the enterocytes before being absorbed through the intestinal membrane, entering the systemic circulation via the lymphatic system (Bank et al., 2011) with nearly 95% of plasma CoQ_{10} present as ubiquinol (Bhagavan & Chopra, 2007). Oral CoQ_{10} bioavailability can be enhanced by altering pharmaceutical forms, with hydrophobicity of CoQ_{10} decreased by using cyclodextrin-based delivery methods (Bank et al., 2011; Jankowski et al., 2016). This delivery method significantly enhances water solubility and bioavailability (Žmitek et al., 2008) by complexing each CoQ_{10} molecule with 2 β-cyclodextrin molecules to form a water-soluble powder (Madhavi & Kagan, 2010). The oral CoQ_{10}-β-cyclodextrin inclusion complex used in this study has been previously shown to be highly bioavailable with a 100% response rate in humans (e.g., all subjects had at least a doubling of plasma concentrations) and a reduced inter-subject variance (Madhavi & Kagan, 2010). High inter-subject variance is a common problem for lipophilic compounds such as CoQ_{10} because of the poor absorption, meaning that not all subjects will have the same amount of absorbance of the product (Bank et al., 2011). In the present study, there was a degree of variability between horses as has been reported for the other equine studies (Sinatra, Chopra, et al., 2013; Sinatra, Jankowitz, et al., 2013). Although budgetary restrictions meant only 12/19 horses had plasma CoQ_{10} concentrations measured, there was a 58% response rate identified when using a uniform

bioavailability measurement defined as a minimum doubling of T_1 plasma CoQ_{10} concentrations above T_0 values.

This was a field-based study using privately-owned horses with samples not obtainable prior to the morning meal, so only the accumulation and not acute phase (0–24 hrs) of oral CoQ_{10} supplementation could be evaluated. The sample timing in the present study was based on prior studies in human subjects that demonstrated plasma concentrations to peak within 4–5 hrs of oral administration of a CoQ_{10}-β-cyclodextrin inclusion complex ((Cuomo & Rabovsky, 2000; Terao et al., 2006). The CoQ_{10}-β-cyclodextrin inclusion complex used in the present study has also been demonstrated in human subjects to have a sustained release resulting in the maintenance of plasma CoQ_{10} concentrations approximately 6 times higher than baseline for 24 hrs following oral administration (Madhavi & Kagan, 2010). This effect has been reported for other oral cyclodextrin complex CoQ_{10}-based delivery systems, although lower sustained plasma CoQ_{10} concentrations were achieved (Terao et al., 2006).

An increase in mean skeletal muscle CoQ_{10} content was observed in the current study following prolonged oral CoQ_{10} supplementation as reflected by significant increases in CoQ_{10}-dependent skeletal muscle mitochondrial function above basal activity. It has been theorised that improved CoQ_{10} absorption into the systemic circulation, elevating CoQ_{10} plasma concentrations, helps improve delivery rate into skeletal muscle (Cooke et al., 2008). The concurrent increase in plasma CoQ_{10} concentrations following supplementation found in the present study thus supports the increased skeletal muscle complex I+III activity to be a result of supplementation.

It is interesting to note that the T_1 skeletal muscle CoQ_{10} content of three horses had fallen marginally below baseline values, potentially indicating a requirement for some horses to have higher plasma concentrations to facilitate movement of CoQ_{10} into the skeletal muscle mitochondria. There were no correlations with the degree of plasma CoQ_{10} concentrations and skeletal muscle CoQ_{10} content supporting the inability to use plasma CoQ_{10} to assess skeletal muscle CoQ_{10} content. The duration of oral CoQ_{10} supplementation has been hypothesised to contribute to the limitation of how much CoQ_{10} enters skeletal muscle mitochondria (Cooke et al., 2008). One group of researchers reported skeletal muscle CoQ_{10} content in humans to increase within 2 hours of oral supplementation, but then decrease to just above baseline values after 2 weeks of oral supplementation (Cooke et al., 2008). These researchers hypothesised that CoQ_{10} uptake into skeletal muscle may be similar to creatine monohydrate, in which there appears to be a maximal limit and/or down-regulation of transporters reached after chronic supplementation leading to a plateau and/or decrease in intramuscular content over time (Guerrero-Ontiveros & Wallimann, 1998). This warrants further investigation in the horse.

A limitation of this study was an inadequate number of horses available for a control group based on power calculations for statistical validity. A control group would have verified whether changes in dietary intake of CoQ_{10} other than supplementation contributed to the observed increases in skeletal muscle CoQ_{10} content. All study horses were housed on the same farm in adjacent pastures, with changes in grass CoQ_{10} content over the 9-week study period unlikely to have occurred since plants contain an extremely small amount of CoQ_{10}, with any dietary intake for humans primarily coming from meat-based products (Kumar et al., 2009; Parmar, Jaiwal, Dhankher, & Jaiwal, 2015; Pravst, Žmitek, & Žmitek, 2010). Furthermore, even including meat-based products the typical human diet does not contain enough CoQ_{10} to significantly raise plasma CoQ_{10} concentrations above basal levels, with the daily CoQ_{10} intake from food ranging between 3–5 mg/day which is too low to significantly raise blood and tissue concentrations above basal levels (Wajda, Zirkel, & Schaffer, 2007). Since the majority of tissue CoQ_{10} content in mammals is endogenously synthesised by the mitochondria, a significantly large increase in plasma CoQ_{10} concentration is required to incite movement into tissue with human and other animal studies reporting that plasma CoQ_{10} concentrations and

skeletal muscle CoQ_{10} content will not significantly increase without exogenous influences (Bhagavan & Chopra, 2007). Although plasma CoQ_{10} concentrations in humans is typically not affected by diet alone, CoQ_{10} supplementation has been shown to increase plasma CoQ_{10} concentrations, the extent of which depends upon the dosage, duration and type of formulation (Pravst et al., 2010).

It has been hypothesised that increased skeletal muscle CoQ_{10} should result in more efficient skeletal muscle energy transduction (Lenaz et al., 1999). For horses in active exercise training this may lead to improvements in responses to exercise training, delay in the onset of fatigue and enhanced recovery following intense exercise (Cooke et al., 2008; M. Kon et al., 2007; Michihiro Kon et al., 2008; Kwong et al., 2002; Mizuno et al., 2008). During exercise, movement of plasma CoQ_{10} into skeletal muscle may increase due to increased metabolic demand (M. Kon et al., 2007; Orlando et al., 2018). This theory is supported by results from a study identifying increased post-exercise intramuscular CoQ_{10} content in human athletes orally supplemented with CoQ_{10} (Cooke et al., 2008). It has recently been shown that resting skeletal muscle CoQ_{10} content is associated with *myostatin* (*MSTN*) genotype (SNP g.66493737C>T) in untrained Thoroughbred horses (Rooney, Porter, Katz, & Hill, 2017). ETC combined complex I+III and II+III activities (indirect measures of CoQ_{10} content) were significantly lower in resting skeletal muscle from TT *MSTN* genotype horses as compared to CT and CC horses. In this same study, restoration of complex I+III and II+III activity was achieved following *in vitro* supplementation with exogenous coenzyme Q_1. Based on the observed differences in basal concentrations of skeletal muscle CoQ_{10} between *MSTN* genotypes in Thoroughbreds, oral supplementation with CoQ_{10} may have a greater efficacy in skeletal muscle of horses with the TT *MSTN* genotype, especially for TT horses training and competing in endurance-related competitions. In the present study, the number of horses with different *MSTN* genotypes was too small to assess for genotype-specific variation in plasma and skeletal muscle CoQ_{10} concentrations after supplementation, but this certainly warrants further investigation.

Conclusion

In summary, this study demonstrates that prolonged daily oral supplementation of a grass and oat diet of young, healthy untrained Thoroughbreds with a CoQ_{10}-β-cyclodextrin inclusion complex significantly increases mean plasma concentration and skeletal muscle CoQ_{10} content, although a degree of variability was identified for some horses. Additional research is warranted to investigate the effects of *MSTN* genotype, training and exercise on skeletal muscle CoQ_{10} content in CoQ_{10}-β-cyclodextrin inclusion complex supplemented and un-supplemented Thoroughbreds.

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532

533 **Table 1.** Summary statistics for plasma CoQ_{10} concentrations measured in triplicate from *n*=12
534 young, healthy untrained Thoroughbred horses before (T_0) and after (T_1) 9 weeks of daily oral
535 supplementation of the established diet with CoQ_{10} (CoQ_{10}-β-cyclodextrin complex with 26%
536 CoQ_{10}, w/w).
537

n=12	T_0 (µg/ml)	T_1 (µg/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 T_0 values (paired two-tailed Student's *t*-test, $P \leq 0.01$).
539

Table 2. Middle gluteal skeletal muscle CoQ_{10} content for 19 young, healthy untrained Thoroughbred horses before (T_0) and after (T_1) 9 weeks of daily oral supplementation of the established diet with CoQ_{10} (CoQ_{10}-β-cyclodextrin complex with 26% CoQ_{10}, w/w). CoQ_{10} content was assessed by spectrophotometrically measuring skeletal muscle mitochondrial complex I+III activity. Complex I+III activity data (pmol/min/mg of muscle protein) was normalised to mitochondrial abundance (citrate synthase activity)/g of skeletal muscle.

n=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

†denotes significant difference from T_0 values (paired two-tailed Student's *t*-test, $P \leq 0.01$).

549 **Figure Legends**
550
551 **Figure 1.** Plasma CoQ_{10} concentrations for 12 young, healthy untrained Thoroughbred horses
552 before (T_0) and after (T_1) 9 weeks of daily oral supplementation of the established diet with
553 CoQ_{10} (CoQ_{10}-β-cyclodextrin complex with 26% CoQ_{10}, w/w). *Significantly different from
554 T_0 values (paired two-tailed Student's *t*-test, $P \leq 0.01$).
555
556 **Figure 2.** Middle gluteal skeletal muscle CoQ_{10} content for 19 young, healthy untrained
557 Thoroughbred horses before (T_0) and after (T_1) 9 weeks of daily oral supplementation of the
558 established diet with CoQ_{10} (CoQ_{10}-β-cyclodextrin complex with 26% CoQ_{10}, 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 T_0 values (paired two-tailed Student's *t*-test, $P \leq 0.01$).
563

Figure 1

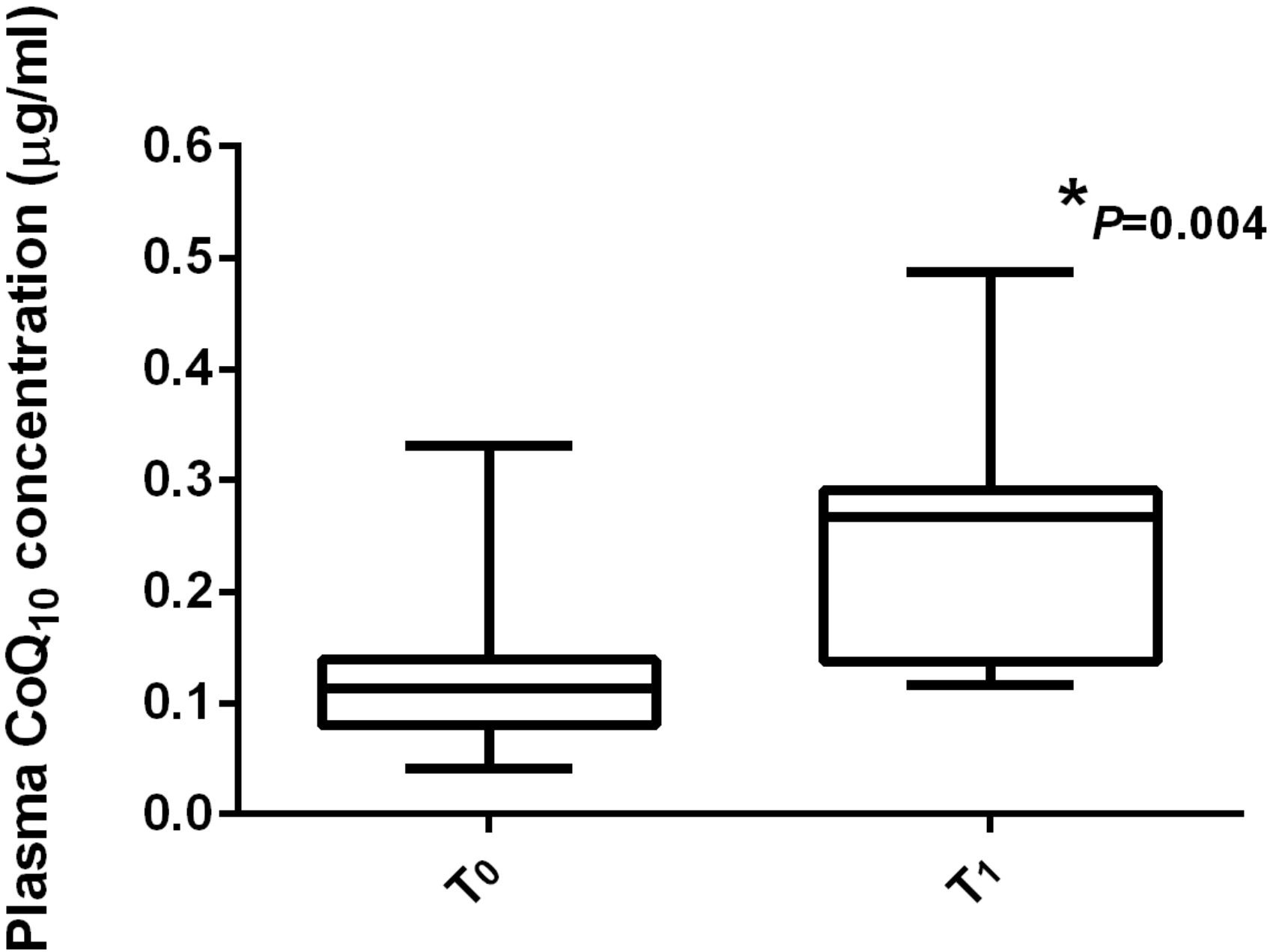
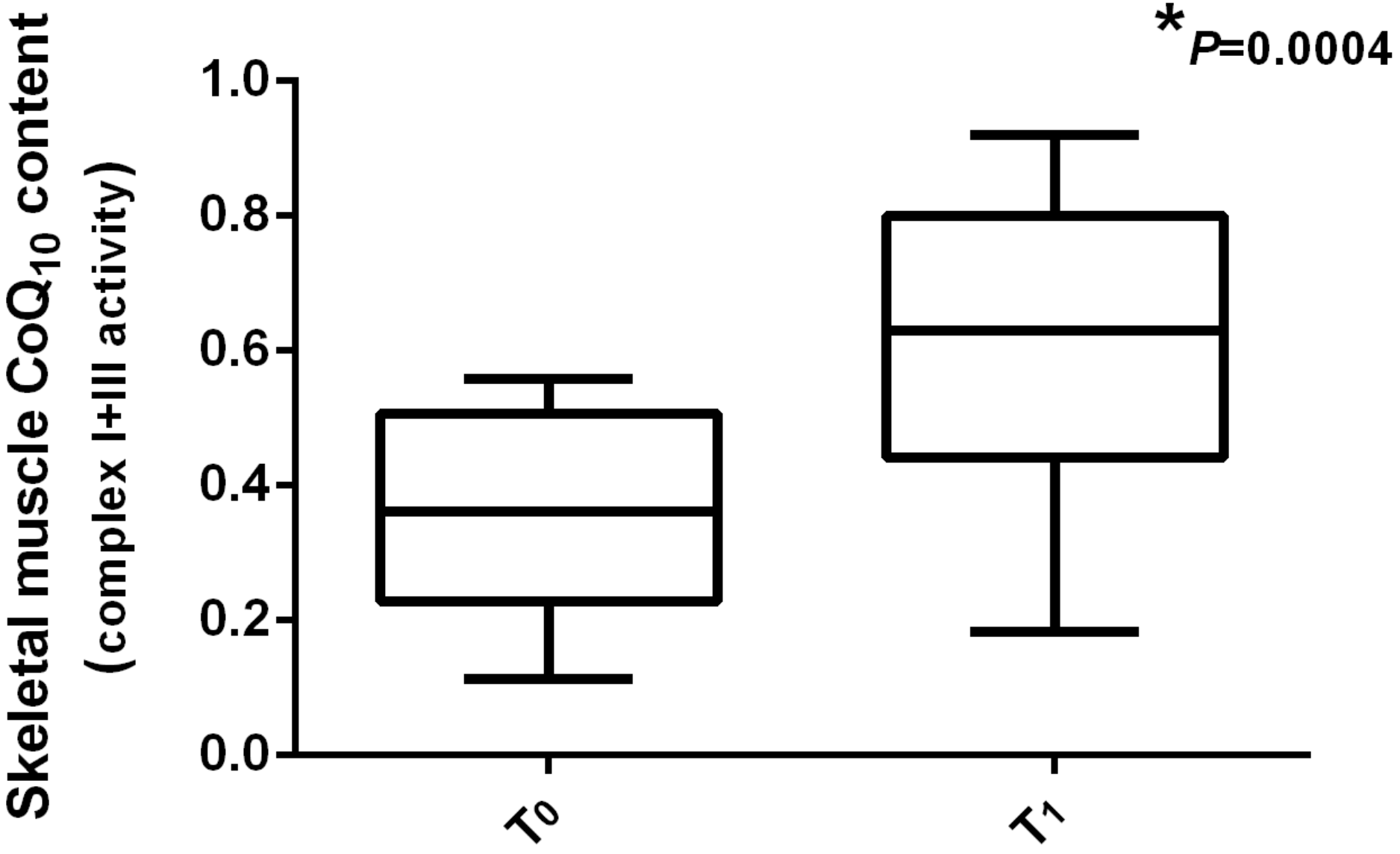

Plasma CoQ10 concentration (µg/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*P=0.004
T0
T1

Figure 2


*P=0.0004
Skeletal muscle CoQ10 content
(complex I+III activity)
1.0
0.8
0.6
0.4
0.2
0.0
T0
T1