Citrulline Malate fails to improve German volume training performance in healthy young men and women

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Title: Citrulline Malate fails to improve German Volume Training performance in healthy young men and women.

Short Running Head: Citrulline fails to improve exercise performance
Abstract

Background: Citrulline malate (CM) is purported to buffer lactic acid, enhance oxygen delivery, and attenuate muscle soreness. Anaerobic exercise trials with CM have produced conflicting results. **Objective:** The aim of the current investigation was to test the efficacy of CM on resistance training (RT) with the hypothesis that CM would improve performance. **Design:** A double-blind, counter-balanced, randomised control trial was utilised to assess the effects of CM on RT. 19 subjects (8 female) (25.7 ± 7.7 years), regularly engaged in RT consumed either 8 g of CM (1.1 : 1 ratio) or a placebo (6 g citric acid). Subjects attempted to perform a German Volume Training (GVT) protocol comprising 10 sets of 10 repetitions of barbell curls at 80% of their one repetition maximum. **Results:** Repeated ANOVA suggested no effect of CM on RT performance (treatment × time × order $p = 0.217$). There was no difference ($p = 0.320$) in the total number of reps over the ten sets (CM median = 57, IQR 45 to 73; placebo median = 61, IQR 51 to 69). Blood lactate and creatine kinase did not differ between CM and placebo ($p > 0.05$). Finally, total muscle soreness was reduced significantly in CM compared to placebo (treatment × time × order $p = 0.004$). **Conclusions:** These results require corroboration; an ergogenic benefit is yet to be established and weight trainers should exercise caution when assessing the efficacy of CM. Future research should focus on the potential effects of loading doses of CM. **Keywords:** Resistance Training, Supplementation, Muscle Soreness, Arginine, Creatine Kinase, NOS
**Introduction**

Citrulline (CIT) is a non-essential amino acid, which acts as a nitrogen ion acceptor from carbamoyl-phosphate within the urea cycle. The dietary supplement citrulline malate (CM) has been purported to improve aerobic and anaerobic exercise performance via a variety of mechanisms including improved ammonia, arginine and lactic acid metabolism, alongside an increase in ATP production (da Silva et al., 2018). Increasing muscle CIT may attenuate ammonia accumulation resulting in a lactic acid buffer effect, which might enhance subsequent recovery, attenuate fatigue and reduce post exercise muscle soreness (Ochia et al., 2012; Cutufrello et al., 2015). Ammonia influences fatigue by stimulating phosphofructokinase, leading to an increased rate of glycolysis (Takeda et al., 2011). This may increase blood lactic acid during exercise inhibiting pyruvate oxidation leading to hydrogen ion accumulation, and a reduction in muscle pH, and contractile potential (Cutufrello et al., 2015).

Citrulline is also adjacent to arginine succinate in the urea cycle, and can be directly synthesised to arginine. Studies have demonstrated that supplementation with CIT increases circulating levels of arginine, which may simultaneously acting as a lactic acid buffer, and vasodilator via nitric oxide synthase (Takeda et al., 2011; Cutufrello et al., 2015; Martinez-Sanchez et al., 2017). Trials have demonstrated that blood arginine increases to a greater extent with oral CIT than arginine due to splanchnic extraction (Moinard et al., 2008; Sureda et al., 2010; Takeda et al., 2011; Wijnands et al., 2015). By way of comparison, a 0.75 g twice daily oral dose of CIT resulted in similar increase in blood arginine compared to a 1.6 g twice daily dose of arginine (Schwedhelm et al., 2007). Finally, malate acid is combined with CIT to form the salt CM. Malate is an intermediate in the TCA cycle, and increasing the pool size of
intermediates may theoretically increase oxidative ATP (Thomas et al., 2004), although to date, no trials have investigated the performance enhancing effects of malate.

Citrulline malate as an ergogenic aid and has become of interest to exercise scientists in recent years, with studies suggesting conflicting effects on exercise performance (Bendehan et al., 2002; Perez-Guisda and Jakeman 2010; Wax et al., 2015; Wax et al., 2016; Farney et al., 2017; Glenn et al., 2017; da Silva et al., 2018). However, some evidence suggests an acute dose of 6-8 g of CM improves resistance training (RT) performance (Perez-Guisda and Jakeman 2010; Wax et al., 2015; Wax et al., 2016; Glenn et al., 2017) and aerobic energy production (Bendehan et al., 2002). More recently however, studies using similar dosages and comparable RT protocols have found no effect (Farney et al., 2017; da Silva et al., 2018). The aim of the current investigation was to assess the effects of CM on German Volume Training (GVT) performance to elucidate its efficacy of this potential ergogenic aid. We hypothesised that supplementation with CM would increase the total amount of work performed during repeated bouts of exercise.

**Subjects and Methods**

**Subjects**

Twenty one subjects volunteered to participate in this study. Two females dropped out, without citing a reason for doing so, leaving 12 males and 7 females who completed the study. Table 1 displays the subject characteristics. Subjects were recruited from Sheffield Hallam University campus via posters, and word of mouth. All subjects were healthy, non-smokers, free from injury and any underlying health
conditions, and not currently using medication or taking supplements containing CM.

Subjects were advised to follow their normal diet and supplement regime for the duration of their involvement in the trial, to avoid strenuous exercise 48 h pre and post laboratory visits and to maintain their regular exercise schedule post 48 h. All subjects were at least "moderately resistance trained" defined as following a structured RT programme at least twice per week, for the last 6 months. Written consent was obtained from all subjects prior to taking part in the trial. The Sheffield Hallam University Business School Food Research Ethics Committee approved the trial.

(Table one approx. here)

Experimental Design

A randomised double-blind placebo cross-over design was implemented. Each subject reported to the laboratory on three separate occasions 7 days apart. Subjects were randomised to either CM or placebo using a random number generator and an independent researcher (JG) performed concealment. The treatments were counterbalanced and blinding was revealed on completion of the trial. On the first visit subjects had, the GVT protocol and the muscle soreness visual analogue scale (VAS) explained to them. Subjects then performed a bicep barbell curl one rep maximum (1RM) test, used to calculate 80% 1RM used for the GVT.

On the second and third visits, subjects arrived fasted to the laboratory, provided a blood sample and then consumed either 8 g of CM or a placebo which were both provided as sports drinks. Subjects then completed a 24 h dietary recall and baseline muscle soreness VAS was recorded. One hour after consuming the sports drink,
subjects performed the GVT barbell curl protocol (10 sets to failure with a maximum of 10 repetitions per set, one-minute rest between sets). The total number of repetitions was counted across each set, and failure was determined when a full range of motion could no longer be completed. Following the GVT a second blood sample was taken, subjects then completed the muscle soreness VAS and then again at 24, 48 and 72 h. The following week subjects completed the trial under the opposite treatment condition.

**Anthropometrics and Muscle Soreness Scale**

Height and weight were assessed using a stadiometer (Holtain, Crymych, United Kingdom) and column scale (Seca, Birmingham, United Kingdom), body composition via bioelectrical impedance (Bodystat 1500, Douglas, Isle of Man) and body mass index (BMI) was calculated kg/m². Muscle soreness was scored using a 100 mm VAS at four sites on the upper and lower arm (Biceps brachii: long and short head and Brachioradialis and Flexor carpi radialis). On the VAS 0 indicated no pain, and 100 indicated the worst pain imaginable. The sum of the four sites was combined and used to represent total soreness at each time point. Muscle soreness was recorded immediately pre and post exercise under supervision and self-assessed at 24, 48 and 72 h following GVT. A demonstration of how to self-palpate and score the muscle soreness was provided by the researchers along with an instruction sheet with diagrams detailing the points of palpation. The research team reminded the subjects to complete muscle soreness VAS at the same time of day using both text message and email prompts.
Supplementation Protocol

Subjects were provided with either 8 g of CM (1.1:1 ratio, 4.2 g citrulline, 3.8 g malate, Bulk Powders, United Kingdom), or a placebo 6 g of citric acid (Sigma-Aldrich, Dorset, United Kingdom). Both placebo and supplement drinks were mixed in 70 ml of fruit cordial and 150 ml of water and consumed within 5 minutes. The dosages and timing of the CM were based on research showing that peak CIT levels occur 1 h after administration (Moinard et al., 2008). The CM and placebo drinks were both well tolerated, and subjects reported no side effects.

Assessment of Supplement Quality

Determination of the supplement quality was assessed using nuclear magnetic resonance (NMR) spectroscopy. The CM utilised in the present investigation had a purported ratio of 2:1 citrulline to malate (Bulk Powders, United Kingdom). Briefly, a standard NMR tube containing 100 mg of the CM supplement powder was added to 1 mL of D$_2$O (Fisher Scientific, UK). The sample was then warmed to 40 °C and agitated to ensure complete dissolution of the solid for analysis of the total organic fraction. Once the solid was dissolved, the solution was cooled to room temperature and analysed on a Bruker Avance DPX-400 NMR spectrometer operating at resonance frequencies of 400 MHz (1H) and 100 MHz (13C). All 1H and 13C NMR chemical environments in both CIT and malic acid were unambiguously assigned using 1H, 1H–COSY, 13C, DEPT–135, HSQC and HMBC experiments. The 1H NMR signals were then integrated, with the signal resulting from H9 on malic acid (at $\delta = 4.37$ ppm) being calibrated as 1. The integration values for the triplicate runs were then calculated. All possible comparisons of integral values between chemical
environments in CIT and those in malate were then performed and the mean nuclear
ratios for each individual replicate were then compiled into overall mean and
standard deviation values, which represent the citrulline:malate molar ratio calculated
from 8 individual data comparisons and three total experimental repeats.

**German Volume Training and One Rep Max Testing**

The 1RM testing protocol was carried out in accordance with the recommendations
of the National Strength and Conditioning Association (2015). 48 hours prior to and
48 h after each visit to the laboratory subjects were asked to refrain from all
strenuous exercise. Subjects fasted and avoided caffeine intake on the morning of
testing. Both GVT sessions were performed at the same time of day (± 1 h), 7 days
apart, and under the same laboratory environmental conditions (21°C, 45 - 55%
Relative Humidity). One hour after consuming the sports drink subjects completed a
warm-up (5 min brisk walk followed by 3 sets of barbell curls with no weight on the
bar) before proceeding with the GVT consisting of 10 sets, of 10 repetitions of barbell
curls at 80 % of the subjects 1RM, with 60 s rest between sets. The numbers of
complete repetitions performed for each set were recorded with the set terminating
when the subject could no longer complete a full repetition, full repetitions were
determined by one of the investigators (TS).

**Blood Sampling, Lactate and Creatine Kinase Analysis**

Prior to the GVT exercise protocol, subjects reported to the laboratory and rested for
10 minutes in a seated position. Immediately after the GVT a second blood sample
was obtained. Blood samples were obtained via finger prick using a lancet (Accu-
chek, Safe-T-Pro). A 20 µl blood sample was taken with a capillary tube and added to
an Eppendorf containing heparin and saline lactate was then read immediately using
a Biosen C-line (EKF Diagnostics, Ebendorfer, Germany). Creatine kinase (CK) was
measured via a 30 µL capillary sample collected pre and post-exercise in Microsafe
Collection and dispensing tube (Inverness Medical, Cheshire, UK) and applied
immediately to a Reflotron Creatine kinase strip (Reflotron Plus clinical chemistry
analyser; Woodley Laboratory Diagnostics, Bolton, United Kingdom).

Dietary Intake and Analysis

Subjects were instructed to consume similar foods the day before testing and refrain
from starting/stopping any new dietary/supplement regimes for the duration of their
involvement in the trial. Prior to taking part in the GVT subjects completed a 24 h
dietary recall. Nutritics (version 2017, Dublin, Ireland) dietary analysis software was
used to analyse the data (see Table 2). Nutrition data was adjusted for bodyweight
and expressed as grams and calories per kg of bodyweight.

Statistics

All data were analyzed using SPSS (IBM, version 24). The main outcome measure,
RT performance during GVT, was analysed using a 2 condition (CM and placebo)
repeated measures analysis of variance to determine any differences in performance
over the ten sets (treatment × time). Treatment order was added to the model as a
covariant (treatment × time × order). Muscle soreness was analysed in the same way,
using the sum of the 4-site muscle soreness VAS for each time point. Mauchly’s test of sphericity was applied to determine sphericity, where this was violated; the Greenhouse-Geisser estimate was used. Data for blood lactate, CK, total reps (the sum of all repetitions across all 10 GVT sets), and dietary intake were assessed for normality using the Kolmogorov-Smirnov test. A paired sample t-test or Wilcoxon Signed Rank test was used as appropriate, when data met the requirements for parametric or non-parametric testing. Statistical significance was established at $p < 0.05$. Where data was normally distributed it is presented as means, standard deviation and 95% confidence interval (CI). For non-normally distributed data the median and the inter-quartile range were presented.

**Results**

Analysis of dietary recall indicated no difference ($p > 0.05$) in macronutrient or energy intake, between CM and placebo conditions (Table 2). Subjects confirmed that they did not stop or start any new supplements over the duration of the trial. Determination of the citrulline to malate ratio revealed the supplement contained a 1.1:1 ratio citrulline to malate rather than the purported 2:1 ratio advertised on the back of the packaging. Resistance training performance during the GVT is presented in Figure 1. There was no significant difference, between the CM and placebo condition, on GVT performance, measured by repetitions performed per set (treatment $\times$ time $p = 0.174$). Treatment order had no effect on GVT performance (treatment $\times$ time $\times$ order $p = 0.217$). There was a significant effect of time ($p < 0.001$) on GVT performance, e.g. the number of repetitions performed declined as sets progressed (mean repetitions set 1, placebo $9.8 \pm 0.7$ vs CM $9.5 \pm 1.1$; set 10, placebo $4.4 \pm 2.9$ vs CM $4.6 \pm 2.4$).
There was no statistically significant difference ($Z = 0.995, p = 0.320$) in the total number of repetitions achieved over the ten sets of GVT between the CM (median = 57, IQR 45 - 73) and placebo conditions (median = 61, IQR 51 - 69).

(Table 2, and Figure 1 approx. here)

Blood lactate data is presented in Figure 2. Lactate increased significantly ($p < 0.001$) post GVT in both the CM (mean lactate pre, 1.9 ± 0.6, post 4.7 ± 2.0 mM) and placebo conditions (mean lactate pre, 1.8 ± 0.6, post 4.9 ± 1.7 mM). There was however, no difference ($t = 0.434, p = 0.670$) in the magnitude of change in lactate post GVT between CM and placebo with a mean increase of 3.01 mM (95% CI 2.41 - 3.62). There was no difference in CK levels post GVT under the CM treatment (pre, 172 I/U IQR 48.5 - 222.9; post, 179.00 IQR 149.5 - 370.4, $Z = 1.552, p = 0.121$), however there was a difference under the placebo condition (pre, 145 I/U IQR 52.9 - 333.0; post, 219 IQR 91.1 - 423.2, $Z = 1.991, p = 0.046$). The median difference of the differences the subjects for CK pre to post exercise did not differ ($Z = 0.052, p = 0.959$) between CM (median = 66.1 I/U IQR 34.3 - 152.6) or the placebo condition (median = 60.4 I/U IQR 19.3 - 150.0).

(Figure two approx. here)

The effect of CM on 72 h muscle soreness is detailed in Figure 3. Muscle soreness was significantly higher in the placebo compared to the CM treatment over 72 h (treatment × time $p = 0.004$). The statistical difference was maintained when treatment order was included in the model (treatment × time × order $p = 0.008$).

(Figure three and table two approx. here)

Discussion
In the current investigation, we hypothesised that an acute dose of CM prior to an
exercise session would increase the total amount of work performed during GVT.
Citrulline malate had no effect on GVT performance, blood lactate or CK compared to
a placebo, although subjects did report an overall reduction in muscle soreness. The
results of the present investigation accept the null hypothesis and are in agreement
with recent findings showing no effect of CM on RT performance (Farney et al., 2017;
Chappell et al. 2018; da Silva et al., 2018). Moreover, these findings are in contrast
to others who have identified an effect of CM with RT (Wax et al., 2015; Wax et al.,
2016; Glenn et al., 2017). Specifically, Perez-Guisda and Jakeman (2010) and Wax
et al., (2016) reported a 13 and 12.5 % respectively, increase in the number of
repetitions achieved after consecutive bouts of RT following CM supplementation.
By way of comparison, a similar increase would have amounted to an 8-repetition
difference in the total repetitions between the CM and placebo condition in the
present investigation.

The reduction in soreness identified with CM was accompanied by a lack of
difference in the total amount of work performed between treatment groups. This
could lead to the conclusion that less soreness was elicited from the same workload
when the subjects consumed CM. Although we note that subjects did not report high
values for muscle soreness pre or post exercise, which may reflect the nature of the
exercise i.e only small muscle groups were involved in our protocol. da Silva et al.
2017 and Chappell et al. 2018, both found a greater degree of soreness, this seems
to be commensurate with protocols involving larger muscle groups. We also note a
lack of any immediate difference in CK or lactate levels between conditions, which
might be expected to accompany any difference in soreness. The lack of change, in
these markers or muscle damage and exercise intensity is in agreement with recent
trials utilising CM during RT protocols (Wax et al., 2015; Wax et al., 2016; da Silva et al., 2017; Farney et al., 2017). Effects of CM on muscle soreness and RT have so far produced mixed results, with both reductions in soreness (Perez-Guisado & Jakeman 2010) and no difference when compared to placebo (da Silva et al., 2017; Chappell et al., 2018). Curiously, trials of aerobic exercise have so far indicated a reduction in soreness utilising CM (Tarozona-Diaz et al., 2013, Martinez-Sanchez et al., 2017). Potential explanations for a reduction in soreness therefore may be attributed to CIT’s role in the urea cycle and the augmented clearance of ammonia (Callis et al., 1991).

Finally, we sought to corroborate the level of the active ingredient used in the present investigation with the manufacturer’s labelling. The data suggests that the amounts of active ingredient in the supplement varied, by almost half, from the manufacturers labelling. The dosage consumed by subjects was 4.2 g CIT and 3.8 g malate (1.1:1 ratio) instead of the intended 6 g CIT and 2 g malate (2:1 ratio). A previous investigation reported similar findings from five "over the counter" CM supplements (Chappell et al., 2018). The disparity between the actual and intended dosage may account for a lack of significant findings. Moinard et al., (2008) however, reported a significant increase in plasma CIT using a similar dosage to the one used in the present investigation. Moreover, the data highlights the need for researchers to conduct analyses of supplement quality prior to publishing trials. Caution therefore needs to be taken when assessing any dose-response study using commercial CM supplements. Commercial supplements, however, are the products recreationally active individuals and athletes alike use in an attempt to aid recovery and improve performance.
Strengths and Limitations

A counterbalanced design was employed to account for a potential training effect, and statistical analysis identified that subjects did not perform better on their second GVT session compared to the first. There was also no difference in dietary intake for the 24 hours preceding testing, between the CM and placebo conditions. Therefore any lack of statistical finding may not be attributed to a disparity in diet (e.g. an increased carbohydrate or energy intake between conditions).

The investigation did not include a measure of ammonia to accompany the data gathered on lactate and CK to corroborate the difference in muscle soreness. Furthermore, although we advised subjects to rest 48 h prior to commencing the trial, several subjects had elevated CK suggesting engagement in prior exercise. Increases in CK levels in response to exercise are known to be highly variable and increase significantly in days following exercise (Ehlers et al. 2002). Creatine kinase was only measured immediately post exercise and a measurement taken alongside muscle soreness over 72 h would have elucidated the recovery process more clearly. Moreover, measurements of muscle soreness were self-reported between the 24-72 h measures potentially and supervised measures would have been preferable. The subjects involved in the present investigation were not a homogenous group. Training experience varied and no controls were put in place to account for the impact of menstrual cycling. Finally, we utilised citric acid as a placebo condition. We only found a single study focused on the effect of citric acid on exercise. Sugino et al. (2007) utilised an 8 day loading protocol combined with aerobic exercise, the authors found no effect on performance, blood citric acid or lactate levels. The placebo was effective at replicate the taste of CM and was well tolerated.
Subjects completed the trial fasted to eliminate the influence of feeding on performance. We could alternatively have included a standardised breakfast in the protocol to reflect the probability that exercise is often not carried out in a fasted state. Finally, we asked the participants not to stop or start any other supplement intake for the duration of the trial and a condition of participation was that they weren’t taking any supplements containing CM. Although we acknowledge the potential effects of other supplements on performance the crossover design of this study to some extent mitigates against this. Secondly we are reminded of the real-world nature of supplement intake whereby in reality people will not postpone ongoing supplementation regimes when they begin new ones.

Conclusion

An acute dose of 8 g CM (1:1:1 ratio) did not confer any benefits to the main outcome measure of RT performance, in a moderately trained group. Total muscle soreness was reduced over the 72 h following CM treatment however; both CK and lactate were unaffected immediately following exercise. This is the first study to date to test the effectiveness of CM using a GVT free weight exercise protocol. Further trials on acute and chronic use of CM are warranted to confirm or deny its effectiveness as an ergogenic aid. Athletes and coaches should proceed with caution when deciding whether to utilise CM as part of a supplementation protocol; the present study and current literature is mixed and recommendation of CM as an anaerobic ergogenic aid is not warranted based on the present results, although we note with caution there may be a positive effect on muscle soreness. Finally, in light of our findings, future
researchers should endeavour to carry out chemical analysis to ensure the validity of treatment dose.

Acknowledgements

The study was designed and conducted by TNS and AJC: data was collected by TNS, and AJC, analysis of the data and interpretation was carried out by TNS and AJC. Dr. Daniel M Allwood carried out analysis of the dietary supplement via NMR and contributed to the method section of the manuscript. Manuscript preparation was undertaken by TNS and AJC. All authors approved the final version of the paper. The authors would like to acknowledge Dr. Jeanette Gittens for blinding and counterbalancing the supplement/placebo in this investigation. The authors would also like to acknowledge Mr. Adrien Parry for his assistance during the data collection. The authors declare no conflict of interest. This study had prior approval from the Sheffield Hallam University Food Research Ethics Committee No; SBS - 252.
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# Tables

**Table 1 Participant Characteristics (n - 19, 12 male, 7 female)**

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Height (m)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
<th>Bodyfat (%)</th>
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<tbody>
<tr>
<td>25.7 ± 7.7</td>
<td>1.7 ± 0.1</td>
<td>75.3 ± 13.7</td>
<td>24.8 ± 2.9</td>
<td>18.3 ± 5.8</td>
</tr>
</tbody>
</table>

Abbreviations: m, metres; kg, kilogram; BMI, body mass index.

All values are mean ± SD.
Table 2. 24 Hour Dietary Intakes Preceding the German Volume Training

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Citrulline Malate</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate (g / kg BW)</td>
<td>3.8 ± 1.8</td>
<td>3.2 ± 1.7</td>
<td>0.285</td>
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<tr>
<td>Protein (g / kg BW)</td>
<td>2.2 ± 1.5</td>
<td>1.8 ± 0.9</td>
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<td>Fat (g / kg BW)</td>
<td>1.0 ± 0.4</td>
<td>0.9 ± 0.4</td>
<td>0.223</td>
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<tr>
<td>Energy (Kcal / kg BW)</td>
<td>34.6 ± 10.7</td>
<td>28.8 ± 10.7</td>
<td>0.730</td>
</tr>
</tbody>
</table>

Abbreviations: g, grams; kg, kilograms; BW, bodyweight; Kcal, calories.

(Paired t-test: Fat; Wilcoxon Signed Rank Carbohydrate, Protein and Energy). All values are means ± SD,
Legends for Figures

Figure 1 - The effect of citrulline malate on resistance training performance. Exercise performance with the placebo is represented by the solid black line ($n = 19$); citrulline malate is represented by the dashed line ($n = 19$). (repeated measures ANOVA time $p < 0.05$; treatment $\times$ time $\times$ order $p > 0.05$) The data presented is the mean ± SD.
Figure 2 - The effect of citrulline malate on blood lactate following resistance training. Pre-exercise lactate values are represented by the grey bars ($n = 18$); post-exercise values are represented by the black bars ($n = 18$); the white bars represent the magnitude of change between placebo and supplement ($n = 18$). Results analysed using a paired t-test, * indicates significant difference pre to post exercise. The data presented is the mean ± SD.
Figure 3 - The effect of citrulline malate on muscle soreness following resistance training. Muscle soreness with the placebo is represented by the solid black line \((n = 19)\); citrulline malate is represented by the dashed line \((n = 19)\). Over time muscle soreness was significantly higher in the placebo compared to citrulline malate treatment (repeated measures ANOVA: treatment \(\times\) time \(\times\) order \(p = 0.008\)). The data presented is the mean \(\pm\) SD.