Diet-Induced Vitamin D Deficiency Results in Reduced Skeletal Muscle Mitochondrial Respiration in C57BL/6J Mice

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Abstract

Vitamin D deficiency is known to be associated with symptoms of skeletal muscle myopathy including muscle weakness and fatigue. Recently, vitamin D related metabolites have been linked to the maintenance of mitochondrial function within skeletal muscle. However, current evidence is limited to *in vitro* models and the effects of diet-induced vitamin D deficiency upon skeletal muscle mitochondrial function *in vivo* have received little attention. In order to examine the role of vitamin D in the maintenance of mitochondrial function *in vivo*, we utilised an established model of diet-induced vitamin D deficiency in C57BL/6J mice. Mice were fed either a control (2,200 IU/kg) or a vitamin D deplete (0 IU/kg) diet for periods of 1-, 2- and 3-months. Skeletal muscle mitochondrial function and ADP sensitivity were assessed via high-resolution respirometry and mitochondrial protein content via immunoblotting. As a result of 3-month of diet-induced vitamin D deficiency, respiration supported via $CI+II_P$ and ETC were 35% and 37% lower when compared to vitamin D replete mice ($P < 0.05$). Despite functional alterations, the protein expression of electron transfer chain subunits remained unchanged in response to dietary intervention ($P > 0.05$). In conclusion, we report that 3-months of diet-induced vitamin D deficiency reduced skeletal muscle mitochondrial function in C57BL/6J mice. Our data, when combined with previous *in vitro* observations, suggests that vitamin D mediated regulation of mitochondrial function may underlie the exacerbated muscle fatigue and performance deficits observed during vitamin D deficiency.

Introduction

Vitamin D deficiency, characterised by serum 25(OH)D levels of <50 nmol.L^{-1}, remains prevalent across both Europe and the USA [1 2]. Although the classical actions of vitamin D within the maintenance of bone health are well established [3-5], a number of non-classical actions have recently been identified including the maintenance of skeletal muscle function [6].

Within human populations, multiple observational studies have reported a positive association between serum 25(OH)D, skeletal muscle strength and lower extremity function in older individuals [7-9]. Furthermore, the supplementation of vitamin D has also been reported to increase muscle strength within this population [10 11]. Despite these associations, studies of this design are unable to infer causality. In addition, isolating the effects of vitamin D status within older populations is often difficult given individuals may suffer from a number of pre-existing conditions that may interfere with vitamin D status [12]. These difficulties highlight the importance of model systems that allow for the manipulation and isolation of vitamin D status in order to study the precise role of vitamin D within skeletal muscle.

In order to study the impact of vitamin D deficiency on skeletal muscle function, a number of animal models have been utilised. A dysregulation of vitamin D status can be achieved via dietary means [13-16], a reduction in sunlight exposure [13] or by the administration of ethane 1-hydroxy-1, 1-diphosphonate which blocks the production of 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) [16]. Diet-induced vitamin D deficiency has been shown to result in symptoms of skeletal muscle myopathy including impaired contraction kinetics, skeletal muscle weakness, as well as

decreases in muscle force in both chicks and rats [13 14 17]. In order to isolate the effects of vitamin D alone and offset the observed hypocalcemia and hypophosphatemia that are associated with the induction of vitamin D deficiency [17], diets with increased calcium and phosphate have been utilised [15]. However, despite the administration of this rescue diet, mice still display reduced grip strength and an increase in *Myostatin* gene expression [15], a known negative regulator of muscle mass [18]. Similarly, mice fed this diet chronically (8-12 months) show similar impairments in physical performance including; reduced grip endurance, sprint speed and stride length [19].

The observed impairments in physical performance with vitamin D deficiency may be linked to skeletal muscle mitochondrial function [20 21]. *In vitro*, vitamin D related metabolites are able to increase mitochondrial function in both immortalised and primary skeletal muscle cell lines [22-25]. Furthermore, we recently observed significant impairments in mitochondrial function in Vitamin D Receptor (VDR) loss-of-function C2C12 myoblasts [26]. In humans, the supplementation of vitamin D within a cohort of severely deficient individuals resulted in a reduced phosphocreatine (PCr) recovery time, as measured non-invasively by 31-phosphorous magnetic resonance spectroscopy (31-P MRS) [20]. Whilst skeletal muscle mitochondrial content seems to remain unchanged following diet-induced vitamin D deficiency in mice [19], the functional characteristics of the mitochondria remain largely underexplored. Therefore, we aimed to determine the effects of diet-induced vitamin D deficiency upon skeletal muscle mitochondrial function in C57BL/6J mice.

Methods

Ethical Approval

Ethical approval was granted by the Garvan Institute and St. Vincent's Hospital Animal Experimentation Ethics Committee (approval number 18/19), fulfiling the requirements of the NHMRC and the NSW State Government, Australia. All animal handling was carried out by trained personnel and all procedures were carried out according to the Australian code of practice for the care and use of animals for scientific purposes 8th edition [27]. Male C57BL/6JAusb mice were received at 10-weeks of age and housed communally in a temperature controlled environment (22 ± 0.5°C) with a 12 h light-dark cycle.

Composition of Diet

Following 1-week acclimation in which mice were fed a standard chow diet, mice were placed on either a vitamin D-control diet (SF085-034, Speciality Feeds, Glen Forest, NSW) or a vitamin D-deplete diet (SF085-003, Speciality Feeds, Glen Forest, NSW) for periods of 1- (n = 10/group), 2- (n = 6/group) or 3-months (n = 6/group). The vitamin D deplete contains no vitamin D (cholecalciferol 0 IU/kg) but increased calcium (2%) and phosphorous (1.2%) in order to maintain normal mineral homeostasis. Previously, this dietary intervention has been shown to successfully induce vitamin D deficiency following 1-month of dietary intervention [15]. The vitamin D control diet contains vitamin D (cholecalciferol 2,200 IU/kg), calcium (1%) and phosphorous (0.7%).

126 **Assessment of Food Intake**

127 Food intake was assessed on a monthly basis at 1-, 2- and 3-months of dietary
128 intervention. The weight of the food within the cage was recorded and subsequently
129 re-weighed following a period of 24 h. The amount of food consumed was then
130 divided by the number of mice within the cage and reported as food intake in grams
131 per mouse.

132

133 **Assessment of Body Composition**

134 Body weight was obtained on a weekly basis throughout the dietary intervention
135 periods. In addition, prior to each measurement of body composition mice were
136 briefly weighed. Body composition was assessed upon arrival (10-weeks of age) and
137 then following 1-, 2- and 3-months of dietary intervention using the EchoMRI
138 (EchoMRI LLC, Houston, USA).

139

140 **Tissue Collection**

141 Tissue collections were completed following 1-, 2- and 3-months of dietary
142 intervention. All samples were excised from fasted (2 h) mice following isoflurane
143 (5%) anesthetization. Following collection, a blood sample was taken via cardiac
144 puncture and animal terminated via cervical dislocation. All tissues were rinsed in
145 sterile saline, blotted dry, weighed, and frozen in liquid nitrogen. A small portion (~20
146 mg) of the red gastrocnemius was removed before freezing and used for high-
147 resolution respirometry. All further tissue samples were stored at -80°C for
148 subsequent analysis.

149

150 **Tissue Processing**

Small portions of red gastrocnemius muscle (~20 mg) were removed and placed in ice-cold BIOPS buffer (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$-$6H_2O$, 20 mM Taurine, 15 mM Na_2Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, 50 mM MES Hydrate, pH 7.1, 290 mOsm). Blood samples were allowed to coagulate at room temperature for 10 minutes before being placed on ice. Blood samples were then centrifuged at 14,000 *g* for 10 minutes and the resulting supernatant was removed and stored at -80°C for further analysis.

Analysis of Serum Calcium

Serum calcium was measured using a Calcium Detection Assay kit (Abcam, Cambridge, UK). Serum samples were diluted 1:10 and manufacturers instructions were followed. The assay plate was read at 575 nm using a CLARIOstar microplate reader (BMG Labtech, Victoria, Australia). Serum calcium concentrations are reported in mM.

High-Resolution Respirometry

High-resolution respirometry was conducted in MiR05 (2 ml) with the addition of blebbistatin (25 μM) using the OROBOROS Oxygraph-2K (Oroboros Instruments, Corp., Innsbruck, AT) with stirring at 750 rpm at 37°C. Oxygen within the chamber was maintained between 150-220 μM for each experiment. Prior to the addition of the fibre bundles to the chamber, bundles were blotted dry and weighed. Bundles totalling 2.5-5.0 mg were added to each chamber. Firstly, pyruvate (10 mM) and malate (2 mM) were added in assessment of complex I related leak (CI_L). ADP was then titrated in step-wise increments (100-6000 μM) followed by the addition of glutamate (10 mM) to assess phosphorylating respiration (CI_P). The addition of

176 succinate (10 mM) followed to assess respiration support via complex II (CI+II_P).
177 Cytochrome c (cyt c) (10 μM) was added in order to check outer mitochondrial
178 membrane integrity. The partial loss of cyt c during fibre preparation may limit
179 respiration however, no fibre preparation exhibited an increase of >10%. Carbonyl
180 cyanide 3-chlorophenylhydrazone (CCCP) was titrated in a step-wise manner (0.5 to
181 2.5 μM) until the maximal capacity of the electron transport chain (ETC) was
182 reached. Finally, antimycin A (2.5 μM) was injected in order to determine non-
183 mitochondrial oxygen consumption.
184
185 The apparent K_m for ADP was determined through the Michaelis-Menten enzyme
186 kinetics – fitting model ($Y = Vmax*X/(K_m + X)$), where X = (free ADP; ADP_f), using
187 Prism (GraphPad Software, Inc., La Jolla, CA) as previously described [28]. Flux
188 control ratios (FCR) was calculated by setting CCCP stimulated respiration as 1 and
189 antimycin A respiration as 0.
190
191 **Immunoblotting**
192 Gastrocnemius samples were powdered on dry ice using a Cellcrusher™ tissue
193 pulverizer (Cellcrusher Ltd, Cork, Ireland) and homogenized via shaking in a
194 FastPrep 24 5G (MP Biochemicals, Santa Ana, California, USA) at 6.0 $m \cdot s^{-1}$ for 80 s
195 in a 10-fold mass of ice-cold sucrose lysis buffer (50 mM Tris pH 7.5; 270 mM
196 sucrose; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 50 mM sodium fluoride; 5 mM
197 sodium pyrophosphate decahydrate; 25 mM beta-glytcerolphosphate). Inhibitors
198 were added fresh on the day of use and included 1 cOmplete™ protease inhibitor
199 cocktail EDTA free tablet (Roche, Basel, Switzerland) and Phosphatase Inhibitor
200 Cocktail 3 both purchased from Sigma-Aldrich (Sigma-Aldrich, NSW, Australia).

Samples were then centrifuged for 10 min at 8,000 *g* at 4°C to remove any insoluble material. Protein concentrations were determined using the DC protein assay as per manufacturer's instructions (Bio-Rad, NSW, Australia). An equal volume of protein (30 μg) was separated by SDS-PAGE on 12.5% gels at a constant current of 23 mA per gel for ~60 minutes. Proteins were then transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) using a wet transfer system at 100 V for 1 h. Membranes were then stained in Ponceau S (Sigma-Aldrich, NSW, Australia) and imaged to check for even loading and transfer. Membranes were then blocked for 1 h in 3% dry-milk in tris-buffered saline with tween (TBS-T). Membranes were incubated overnight in primary antibodies at 4°C. Following primary antibody incubation, membranes were washed three times in TBS-T and subsequently incubated in the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Membranes were again washed three times in TBS-T prior to imaging. Images were captured using the ChemiDoc (Bio-Rad, NSW, Australia) and quantified using ImageJ.

Antibodies

MitoProfile OXPHOS antibody cocktail (110413) was from Abcam and used at a concentration of 1:1,000. Anti-mouse (7076) secondary antibody was used at a concentration of 1:10,000 in TBS-T and was from Cell Signaling Technology.

Statistical Analysis

Statistical analysis was performed using Prism version 7 (GraphPad Software Incorporated, La Jolla, CA, USA). Differences between 1-, 2- and 3-month vitamin D replete and deplete mice were determined by two-way ANOVA with Bonferroni

correction for multiple comparisons. Differences between vitamin D deplete and replete mice in mitochondrial respiration in response to ADP titration were determined by multiple t-test. All values are presented as mean ± SD. Statistical significance was set at $P < 0.05$.

Results

Body Composition Following Diet-Induced Vitamin D Deficiency

Body weight increased across time when mice were compared at the 1-, 2- and 3-month time-points ($P < 0.001$) (Fig. 1A). However, no differences in body weight were observed when comparing vitamin D replete and deplete mice ($P > 0.05$) (Fig. 1A). Further assessment of body composition revealed no differences in absolute lean mass ($P > 0.05$) (Fig. 1C) however, when expressed as a percentage of body weight, lean mass was lower when compared across dietary intervention time-points ($P < 0.001$) (Fig. 1D). Further analysis revealed that lean mass as a percentage of body weight was 16% lower at 3-months when vitamin D deplete mice were compared with the 1-month vitamin D deplete ($P < 0.001$) (Fig. 1D). Although this is indicative of a loss of lean mass with vitamin D deficiency, this effect was potentially driven by a higher lean mass as a percentage of body weight at the 1-month time-point in vitamin D deplete mice ($P = 0.039$) (Fig. 1D). Both absolute and percentage of body weight fat mass increased across time in both dietary groups ($P < 0.001$) (Fig. 1E-F). Furthermore, fat mass as a percentage of body weight was 60% higher in the vitamin D replete mice when compared with vitamin D deplete at the 1-month time point ($P = 0.044$) (Fig. 1F). Despite differences in body composition, no

difference in food intake was observed between time points ($P > 0.05$) and groups ($P > 0.05$) (Fig. 1B).

Skeletal Muscle Mass

Given the alterations in total lean mass, we determined whether individual skeletal muscle mass was effected by diet-induced Vitamin D deficiency. Crude analysis of skeletal muscle wet weight revealed no differences in the mass of the gastrocnemius in response to either dietary intervention ($P = 0.408$) or time point ($P = 0.103$) (Table 1). Overall, the mass of the quadriceps increased over time ($P = 0.004$) however, this was not changed by dietary intervention ($P = 0.951$) (Table 1). Collectively, triceps mass was higher when vitamin D replete mice were compared with deplete ($P = 0.041$) although, post-hoc analysis revealed no difference between groups at individual time points ($P > 0.05$) (Table 1).

Serum Calcium

Similar to previous reports [15], we observed no change in serum calcium irrespective of dietary group or time point ($P > 0.05$) (Fig. 2A).

Mitochondrial Function

In response to both pyruvate and malate alone, we observed no change in CI_L ($P > 0.05$) (Fig. 3A). However, following the addition of ADP, CI_P respiration increased across the 1, 2 and 3-month time points ($P = 0.048$) (Fig. 3B). Furthermore, respiration was 85% and 96% higher at the 2- ($P = 0.015$) and 3-month ($P = 0.006$) time-points respectively when compared with 1-month in vitamin D replete mice (Fig. 3B). Similarly, higher respiratory capacities during $CI+II_P$ respiration and the maximal

capacity of the ETC were observed in the 2- and 3-month vitamin D replete mice when compared with the 1-month ($P < 0.05$) (Fig. 3C-D). In addition, 3-months of diet-induced vitamin D deficiency resulted in 35% and 37% lower respiratory rates during $CI+II_P$ ($P = 0.035$) and maximal ETC capacity ($P = 0.015$) when compared to vitamin D deplete mice at the same time-point (Fig. 3C-D). We also analysed to above data as a flux control ratio which provides a method for internal normalisation [31 32]. Despite the observed changes reported above, flux control ratios revealed no differences in mitochondrial respiration supported via complex I alone ($P > 0.05$) or complex I and II in combination ($P > 0.05$) (Fig. 3E-F).

ADP Sensitivity

Commonly, the assessment of mitochondrial function is performed under saturating concentrations of ADP [31 32] which may not be biologically relevant. Therefore, we assessed mitochondrial function in response to a titration of ADP from biologically relevant to saturating concentrations [28 33]. We observed no differences in mitochondrial respiration throughout the titration of ADP between vitamin D replete and deplete mice ($P > 0.05$) (Fig. 4A-C). Furthermore, no differences were observed in the apparent K_m for ADP in response to either dietary intervention ($P > 0.05$) or time point ($P > 0.05$) (Fig. 4D).

Mitochondrial Protein Content

Finally, given the observed decrements in mitochondrial function associated with vitamin D deficiency, we sort to assess mitochondrial protein content following diet-induced vitamin D deficiency. We observed no changes in complex I (NDUFB8), complex II (SDHB) and complex IV (UQCRC2) protein content when compared

irrespective of time point or vitamin D diet ($P > 0.05$) (Fig. 5A-B, D). Interestingly, both complex III (MTCO1) and complex V (ATP5A) were decreased across time ($P < 0.05$) however, there were no differences between dietary groups ($P > 0.05$) (Fig. 5C & E).

Discussion

Vitamin D deficiency has been linked to reductions in muscle function, however the specific role of Vitamin D on mitochondrial function in less established. Therefore, the aim of the present study was to directly examine the effects of diet-induced vitamin D deficiency upon skeletal muscle mitochondrial function in C57BL/6J mice. Utilising the current gold standard method to assess mitochondrial function in permeabilised skeletal muscle fibres [29 30], we report that 3-months of diet-induced vitamin D deficiency reduces mitochondrial respiration supported via $CI+II_P$ and the maximal capacity of the ETC (Fig. 3C-D). Interestingly, despite the functional changes, we observed no differences in mitochondrial protein content following the induction of diet-induced vitamin D deficiency (Fig. 5A-F). In addition, 1-month of diet-induced vitamin D deficiency resulted in an increase in lean mass (Fig. 1D) and a decrease in fat mass (Fig. 1F) as a percentage of body weight, although these effects were transient as they did not manifest over 2- and 3-months of dietary intervention. Furthermore, diet-induced vitamin D deficiency resulted in a decrease in lean mass as a percentage of body weight across the 3-month time period (Fig. 1D). Despite this, no changes in body weight, lean mass, fat mass or food intake were apparent when comparing vitamin D replete to the deplete group following 3-months of dietary intervention (Fig. 1A-F).

The ability of vitamin D and related metabolites to increase skeletal muscle mitochondrial function in immortalised and primary cell lines has been well established [22-25]. Despite this, there is little evidence for the effects of vitamin D status upon skeletal muscle mitochondrial function *in vivo*. To date, just one study has examined skeletal muscle mitochondrial function, as measured non-invasively via 31-P MRS, in a cohort of severely deficient patients [20]. The authors reported a decrease in PCr recovery time following supplementation with vitamin D, indicative of increased oxidative phosphorylation [20]. However the study followed an open label design, making the extrapolation of this data unclear. In order to directly assess the effects of vitamin D status upon skeletal muscle mitochondrial function, we utilised a mouse model of diet-induced vitamin D deficiency. This model allows for the manipulation of vitamin D status without altering mineral homeostasis [15]. Following 3-months of diet-induced vitamin D deficiency, we report that respiration supported via $CI+II_P$ and the maximal capacity of the ETC is lower when compared to vitamin D replete mice at the same time-point. Reduced rates of respiration following 3-months of diet-induced vitamin D deficiency were mediated by increased respiration in the vitamin D replete cohort as opposed to a reduction in vitamin D deplete mice. Following internal normalisation, the above alterations in mitochondrial function were no longer apparent, suggesting the changes observed were due to alterations in mitochondrial quantity as opposed to quality. Therefore, in order to ascertain whether these increases were mediated by an increase in mitochondrial protein abundance, we assessed the protein content of ETC subunits I-V. Despite functional impairments, we observed no differences in markers of mitochondrial protein content within skeletal muscle following the induction of vitamin D deficiency. Therefore, this suggests that vitamin D deficiency alters mitochondrial function independently of

mitochondrial content. Similar observations have been made *in vitro* following either the treatment of skeletal muscle cell lines with vitamin D metabolites or the knock-down of the VDR [22 26]. In addition, within the skeletal muscle of the recently developed skeletal muscle muscle-specific VDR-KD mouse, mitochondrial protein content remained unchanged [34]. The further examination of mitochondrial function in this mouse model would help to determine whether the effects of vitamin D deficiency upon skeletal muscle mitochondrial are a direct result of vitamin D related signalling.

In addition, we also sought to determine the effects of diet-induced vitamin D deficiency upon body composition within C57BL/6J mice. Previously, no differences in body weight and lean mass were observed following 12-months of diet-induced deficiency in male C57BL/6J mice [19]. In female mice however, 12-months of diet-induced vitamin D deficiency resulted in reductions in body weight, lean mass and fat mass [35]. Similar to previous reports in male mice, we observed no differences between the vitamin D replete and deplete groups in body weight, lean mass, fat mass or food intake at the 3-month time point. We did however observe a reduction in lean mass as a percentage of body weight from 1- to 3-months in vitamin D deplete mice whereas replete mice remained stable over the same time period. This may in part be driven by the fact we also observed an increase in lean mass as a percentage of body weight following 1-month of diet-induced deficiency. Despite minimal differences in body composition, previous data suggests that vitamin D deficiency impairs physical function in mice [15 19]. Therefore, functional measures of muscle performance may be more relevant to assess the effects of vitamin D deficiency within skeletal muscle. However, it should also be noted that those with

serum concentrations of 25(OH)D <25 nmol.L^{-1} are at a greater risk of developing sarcopenia [36]. Given the effects of vitamin D deficiency and supplementation seem to be most potent in older individuals, the induction of vitamin D deficiency in aging mouse models may reveal more potent effects on skeletal muscle mass.

In conclusion, we report that mitochondrial function (CI+II_P and ETC) is reduced in C57BL/6J mice following 3-months of diet-induced vitamin D deficiency. These effects are not mediated by alterations in mitochondrial protein content suggesting vitamin D deficiency directly effects the mitochondrial respiratory machinery. Similar to others, we observed minimal differences in body composition following 3-months of diet-induced vitamin D deficiency in male C57BL/6J mice [19 37]. Finally our data provides evidence that vitamin D status is an important determinant of skeletal muscle mitochondrial function *in vivo* thereby supporting previous *in vitro* observations.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

S.P.A and A.P conceived and designed research. S.P.A, G.F, A.M.P performed experiments and S.P.A analysed data, interpreted results and prepared figures. S.P.A, P.J.A and A.P drafted the manuscript. All authors approved the final version of the manuscript.

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Figure Legends

Figure 1. Assessment of body composition and food intake in vitamin D replete and deplete C57BL/6J mice. A, increase in body weight across dietary period with no differences between differing vitamin D diets (n = 6-10/group). B, no difference in food intake was observed following dietary intervention (n = 5-6 cages/group). C-D, absolute lean mass remained unchanged whilst lean mass as a percentage of body weight was significantly lower in the 3-month vitamin D deplete when compared to the 1-month (n = 6-10/group). E-F, Absolute and percentage of body weight fat mass increased across the dietary period irrespective of dietary intervention. Data mean ± SD. [b] Main effect for time ($P < 0.05$), [c] Main effect group x time interaction ($P < 0.05$), * ($P < 0.05$).

Table 1. Assessment of skeletal muscle wet weight following the induction of diet-induced vitamin D deficiency (n = 6-10/group). Data mean ± SD.

Figure 2. Serum calcium in response to the induction of diet-induced vitamin D deficiency (n = 5-10/group). Data mean ± SD.

Figure 3. Assessment of skeletal muscle mitochondrial respiration in response to diet-induced vitamin D deficiency. A, CI_L remains unchanged irrespective of dietary intervention (n = 4-8/group). B, Respiration supported via CI_P is higher in the 2- and 3-month vitamin D replete mice when compared with the 1-month (n = 5-8/group). C-D, Respiration supported via $CI+II_P$ and the maximal capacity of the ETC is increased in the vitamin D replete mice and higher at the 3-month time point when compared to vitamin D deplete (n = 5-8/group). E-F, alteration in absolute rates of respiration are diminished when internally normalised (n = 5-8/group). Data mean ± SD. [b] Main effect for time ($P < 0.05$), [c] Main effect group x time interaction ($P < 0.05$), * ($P < 0.05$).

Figure 4. Assessment of skeletal muscle mitochondrial ADP sensitivity in response to diet-induced vitamin D deficiency. A-C, no change in respiratory capacity in response to the titration of ADP following 1-, 2- and 3-month of vitamin D deficiency (n = 5-8/group). D, no change in the apparent K_m for ADP in response to 1-, 2- and 3-month of vitamin D deficiency (n = 5-8/group). Data mean ± SD.

Figure 5. Assessment of skeletal muscle mitochondrial protein content following diet-induced vitamin D deficiency. A-B and D, no changes in expression levels of mitochondrial complex I (NDUFB8), complex II (SDHB) and complex IV (UQCRC2) following diet-induced vitamin D deficiency (n=6/group). C and E, lower expression

583 levels of mitochondrial complex III (MTCO1) and complex V (ATP5A) across the
584 dietary time period with no differences between vitamin D replete and deplete mice
585 (n = 6/group). Data mean ± SD. [b] Main effect for time ($P < 0.05$), [c] Main effect group
586 x time interaction ($P < 0.05$).
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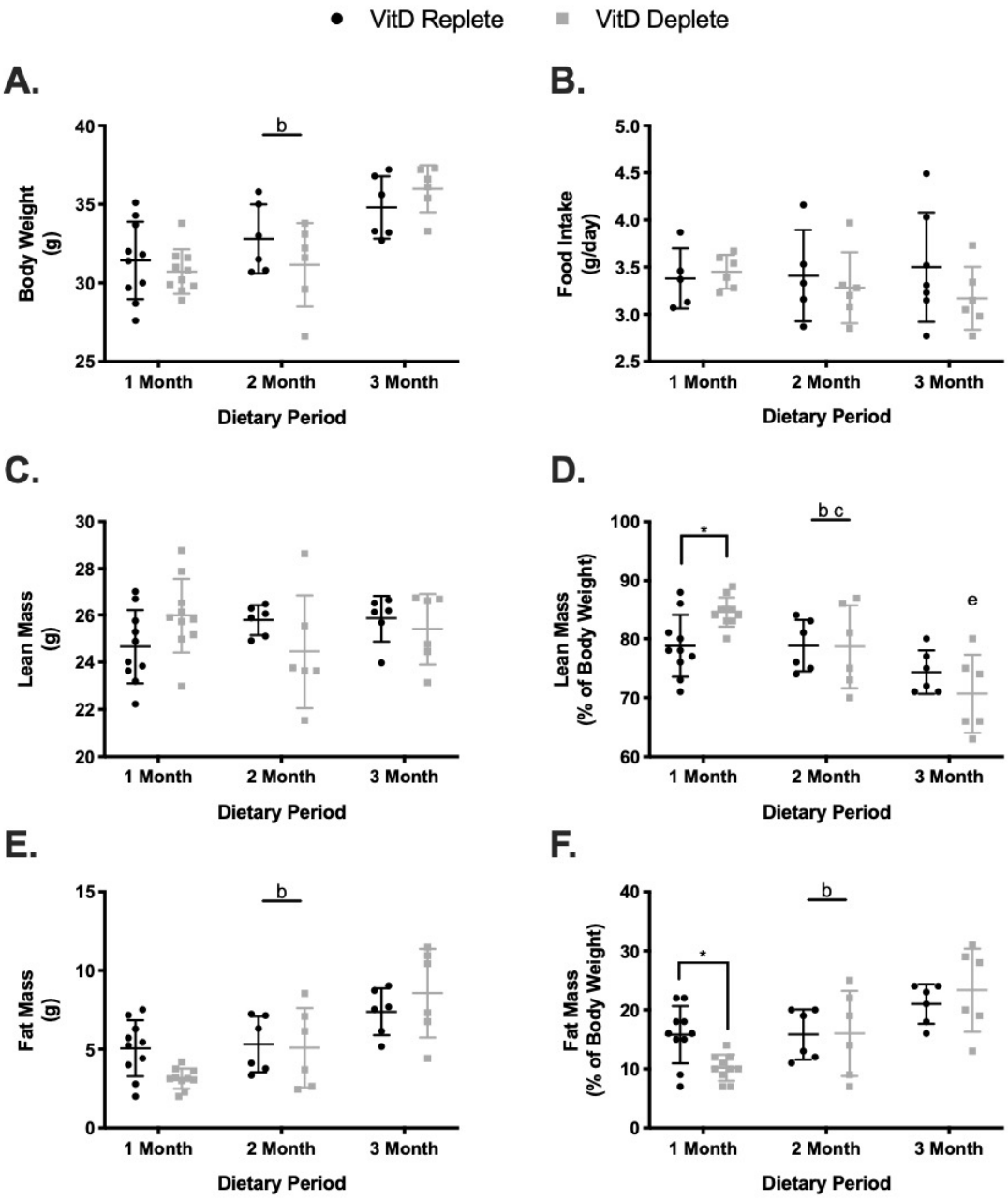
Figure 1.



600 Table 1.

	Dietary Period (Months)				
	1	2	3	*P*	
Gastrocnemius (mg)				*VitD*	*Time*
Replete	143 ± 14	166 ± 25	161 ± 14		
Deplete	152 ± 16	149 ± 12	156 ± 15	*0.408*	*0.103*
Quadriceps (mg)					
Replete	160 ± 14	189 ± 12	182 ± 20		
Deplete	169 ± 18	178 ± 22	184 ± 19	*0.951*	*0.004*
Tricep (mg)					
Replete	111 ± 20	113 ± 14	109 ± 10		
Deplete	106 ± 9	96 ± 19	101 ± 13	*0.041*	*0.695*

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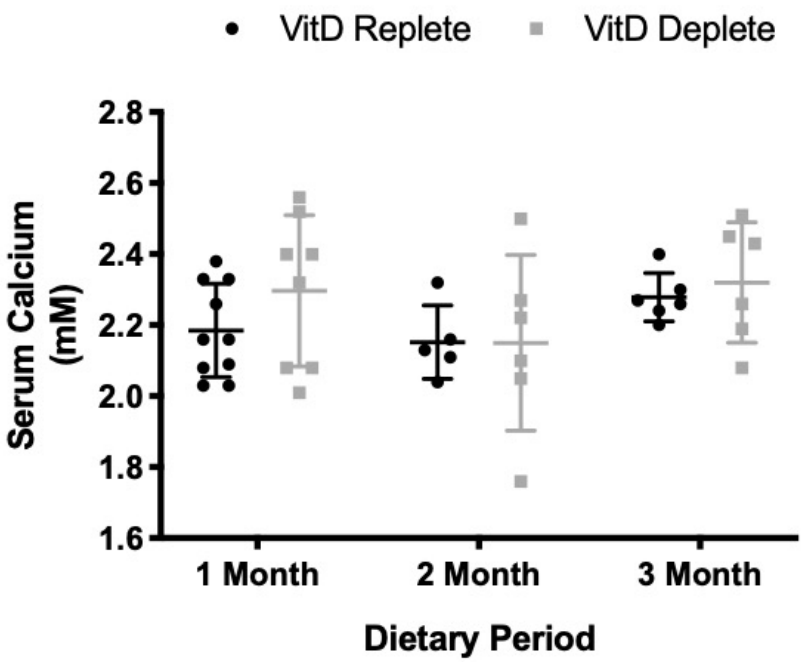
Figure 2.



642 Figure 3.

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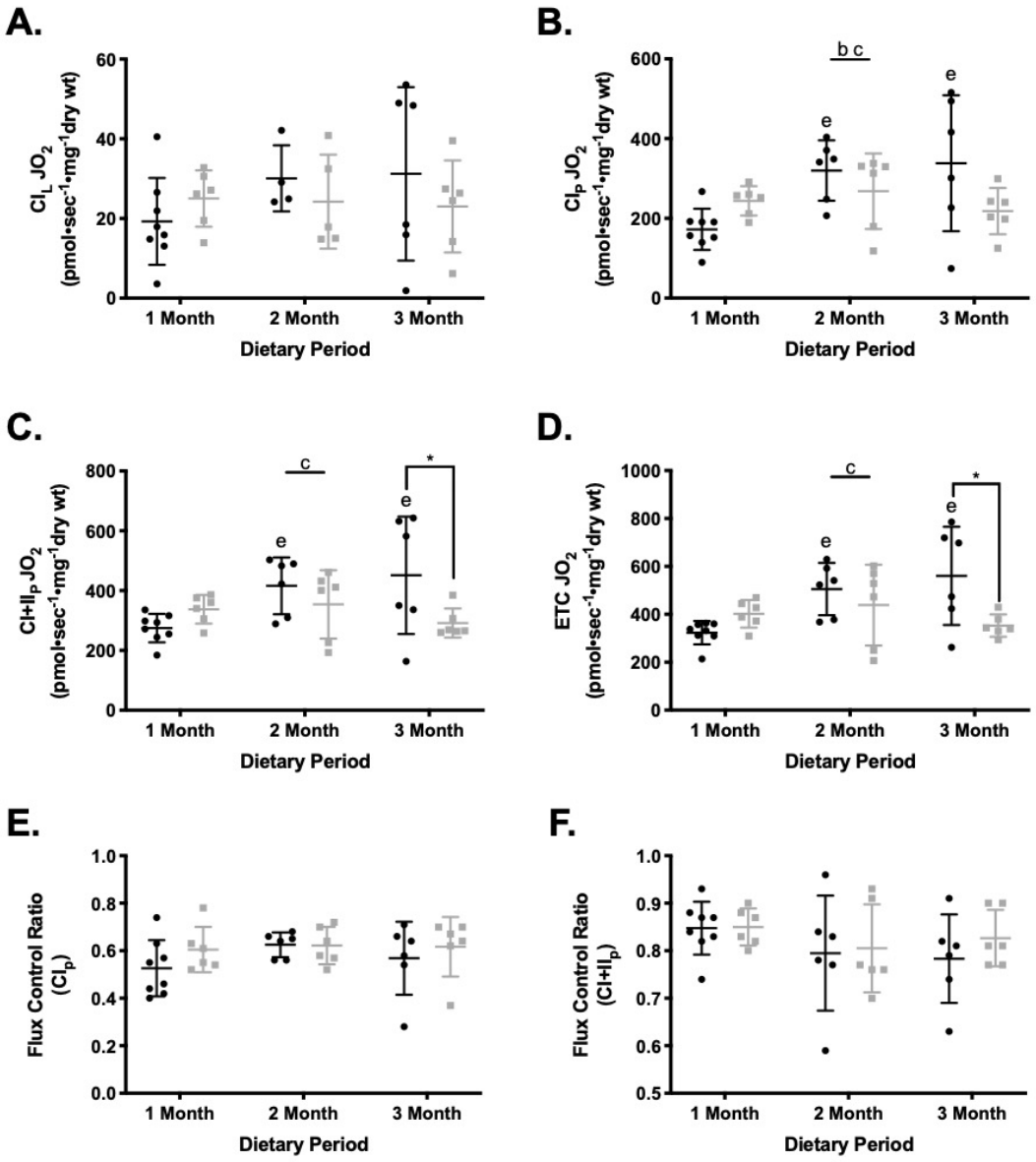

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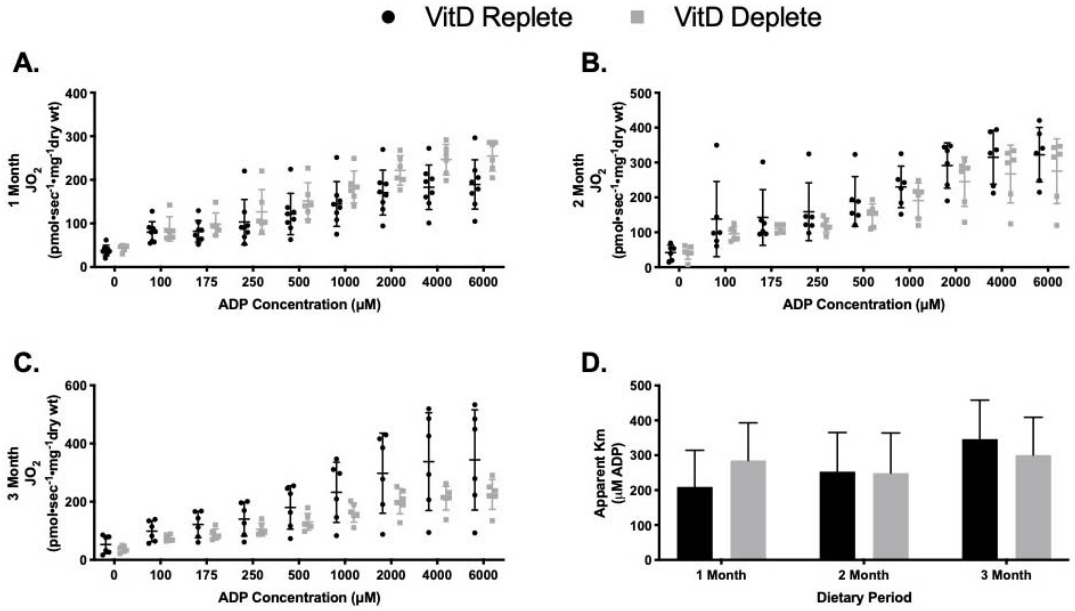
Figure 4.



664 Figure 5.