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1 **Oral contraceptive pill phase does not influence muscle protein synthesis or myofibrillar**

2 **proteolysis at rest or in response to resistance exercise**

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18 **Running title:** Oral Contraceptives and Muscle Protein Turnover

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24 **ABSTRACT**

25 There is speculation that oral contraceptive pill (OCP) use affects skeletal muscle biology and
26 protein turnover in response to resistance exercise; however, research in this area is scarce. We
27 aimed to assess, using stable isotope tracers and skeletal muscle biopsies, how second-generation
28 OCP phase affected muscle protein synthesis and whole-body proteolysis. Participants (n=12)
29 completed two 6-day study phases in a randomized order: an active pill phase (Active; week two
30 of a monthly active OCP cycle) and an inactive pill phase (Inactive; final week of a monthly
31 OCP cycle). Participants performed unilateral resistance exercise in each study phase, exercising
32 the contralateral leg in the opposite phase in a randomized, counterbalanced order. The Active
33 phase myofibrillar protein synthesis (MPS) rates were 1.44 ± 0.14 %•d^{-1} in the control leg and
34 1.64 ± 0.15 %•d^{-1} in the exercise leg ($p < 0.001$). The Inactive phase MPS rates were 1.49 ± 0.12
35 %•d-1 %/d in the control leg and 1.71 ± 0.16 %•d^{-1} in the exercise leg ($p < 0.001$), with no
36 interaction between phases ($p = 0.63$). There was no significant effect of OCP phase on whole-
37 body myofibrillar proteolytic rate (active phase $k = 0.018 \pm 0.01$; inactive phase $k = 0.018 \pm$
38 0.006; $p = 0.55$). Skeletal muscle remains equally as responsive, in terms of stimulation of MPS,
39 during Active and Inactive OCP phases; hence, our data does not support a pro-anabolic or
40 catabolic, based on myofibrillar proteolysis, effect of OCP phase on skeletal muscle in females.

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42 **NEW AND NOTEWORTHY**

43 We discovered that women taking a second-generation oral contraceptive pill (OCP) showed no
44 difference in integrated daily muscle protein synthesis or whole-body myofibrillar proteolysis in
45 the active or placebo pill phases of the pill cycle. Our data show that OCP phase does not
46 influence skeletal muscle protein turnover in females and does not support a marked pro-
47 catabolic or anabolic effect.

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48 **INTRODUCTION**

49 Premenopausal females are frequently excluded from exercise physiology research, with
50 an often-cited reason being the potential for the effects of menstrual cycle (MC) hormones or
51 oral contraceptive pills (OCP) on the outcomes (1). Although the primary purpose of ovarian
52 hormones (estradiol [E2] and progesterone [P4]) is for reproductive function, it has been
53 proposed that E2 may be pro-anabolic and involved in pathways and processes that influence
54 muscular adaptations to exercise (2). In contrast, the presence of P4 has been proposed to
55 antagonize the action of E2 (3). Despite this speculation, we recently showed that MC phase
56 does not affect rates of MPS or whole-body myofibrillar proteolysis (4). We have also pointed
57 out that P4 is more androgenic than E2, which conflicts with the notion that high P4 in the luteal
58 phase creates a catabolic environment (3, 5, 6).

59 Hansen et al. studied the effects of OCP on myofibrillar protein synthesis (MPS) (7) and
60 tendon and muscle connective tissue synthesis (8). These authors reported lower MPS and
61 tendon as well as muscle (and possibly bone based on indirect biomarkers) connective tissue
62 protein synthesis in OCP users (8). When data were split into those taking second- versus third-
63 generation OCP, Hansen et al. concluded that the lower MPS was predominantly due to third-
64 generation OCP users (n=7) who showed a marked lowering of MPS versus those taking second-
65 generation OCP (n=4). Notably, rates of myofibrillar proteolysis were also determined using
66 microdialysis and reported to be no different between OCP users and controls and were
67 unaffected by OCP generation (7). These were short-term studies lasting several hours, and the
68 exercise used was a high-intensity single-leg kicking (7, 8); thus, the effects over longer periods
69 and with a more anabolic exercise stimulus, such as resistance exercise training (RET), are less
70 well known. Given that the net balance of MPS and muscle protein breakdown (MPB), along

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with other processes (9), contribute to RET-induced hypertrophy, there is an important research gap.

It is unknown how OCP use may affect RET outcomes such as hypertrophy and strength; however, speculation is that since OCP use downregulates the production of endogenous E2 and P4, this suppression may affect molecular mechanisms that are important in hypertrophy and somehow suppress normal adaptation (2, 10). RET studies involving females taking OCP have produced mixed results (11-15); however, generally, no significant differences were observed between OCP users and non-users regarding RET-induced muscle hypertrophy. Nolan et al. (10) systematically reviewed how OCP affects RET outcomes and found no consistent effect on hypertrophy, power, or strength.

The purpose of this study was to investigate MPS and myofibrillar proteolysis in response to resistance exercise in females on OCP. Subjects were assessed during their active pill phase and the inactive phase of second-generation OCP. We aimed to test the hypothesis that muscle protein synthesis would increase in response to resistance exercise in both phases but with no differences between phases.

METHODS

The study was approved by the Hamilton Integrated Research Ethics Board (project number: 14067) and conformed to the standards for the use of human subjects in research as outlined by the Canadian Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans – TCPS 2, 2022 (https://ethics.gc.ca/eng/policy-politique_tcps2-eptc2_2022.html) and the Declaration of Helsinki (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/). Each participant was informed of

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94 the purpose of the study, experimental procedures, and potential risks before written informed
95 consent was obtained. The trial was registered with the National Institutes of Health at
96 http://www.clinicaltrials.gov repository as NCT05347667.

97 *Participants.* Healthy young females (n=12) were recruited for the study. Eligible participants
98 were between the ages of 18 and 30 and in good health (as determined by a medical screening
99 questionnaire). All participants reported taking second-generation OCP (Allese or Alysena) for at
100 least 6 mo prior to taking part in the study. Participants were excluded if they: i) suffered from an
101 orthopedic, cardiovascular, pulmonary, renal, liver, infectious disease, immune, metabolic or
102 gastrointestinal disorder likely to impact study outcomes; ii) took medications known to affect
103 protein metabolism (i.e., corticosteroids, non-steroidal anti-inflammatory drugs, or high strength
104 acne medication, testosterone replacement); iii) used tobacco or cannabis or tobacco/cannabis-
105 related products (smoking or vaping); iv) had been previously diagnosed with a menstrual cycle
106 disorder, polycystic ovarian syndrome, or endometriosis. Participants' characteristics are shown
107 in Table 1.

108 A sample size of 9 subjects, using a crossover design, was determined based on an *a*
109 *priori* power analysis calculated using G*power (Version 3.1.9.6, Franz Faul, Kiel University,
110 Germany) based on our previous trial (4) (target alpha of 0.05 and power of 0.80) with a small
111 effect size of 0.2 to be sufficient to detect a change of ~25% in muscle protein synthesis, which
112 we deemed to be physiologically relevant. To protect power and account for any dropouts, we
113 included 12 subjects.

114 *Study Overview.* Participants completed two 6-day study phases in a randomized order during
115 each phase of their OCP cycle: Active pill phase (days 9-14) and Inactive pill phase (days 23-
116 28). A schematic of the study protocol is shown in Figure 1.

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117 Participants completed a general health questionnaire to indicate their current health
118 status and medication use to ensure eligibility for the study. Height and body mass were assessed
119 using a calibrated stadiometer and scale. Participants underwent a dual X-ray absorptiometry
120 (DXA; GE-Lunar iDXA; Aymes Medical, Toronto, ON) scan to assess body composition. DXA-
121 derived lean mass was used to determine D_2O dosing. Unilateral knee extension 10 repetition
122 maximum (RM) was assessed for each leg to determine the starting load for subsequent study
123 visits.

124 Much of the details of the protocol and methods we employed, such as RET protocol and
125 the calculation of the rates of MPS and whole-body myofibrillar proteolysis, were reported in our
126 previous paper (4); thus, we provide only relevant details here. The salient difference between
127 our previous protocol (4) and this study was the timing of the study protocols, which was
128 completed within one OCP cycle and took place during the Active and Inactive pill phases.
129 Participants arrived for the first study visit after an overnight fast. Following a pregnancy test,
130 subjects provided a baseline saliva sample (to obtain baseline body water enrichment), a baseline
131 urine sample (to measure D_3-creatinine enrichment for muscle mass; see below), a blood sample
132 (to assess serum hormones), and a baseline muscle biopsy from the *vastus lateralis* of the control
133 leg. The control (non-exercised) leg was randomly determined for phase one, and the
134 contralateral leg served as the control for phase two. On the priming dose day, participants were
135 given three (1.25 ml•kg^{-1} lean mass) aliquots of 70 atom % D_2O to consume 30 min apart. An
136 oral dose of 30 mg D_3-Cr was included in the third aliquot of D_2O to assess skeletal muscle mass
137 as previously described in detail (6, 16). Participants performed three sets of 10 unilateral knee
138 extensions to volitional fatigue, defined as an inability to complete a repetition through the full

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139 range of motion. If the participant completed more than 12 or less than eight repetitions, the load
140 was adjusted, up or down, accordingly.

141 Participants returned to the laboratory 48h after Visit 1 to provide a urine sample and
142 perform three additional sets of unilateral knee extensions as outlined above. Participants
143 returned to the laboratory 72h after Visit 1 to provide a urine sample and 18h prior to the
144 scheduled Visit 5 to consume 10mg of D_3-3-methyl-histidine (3MH) dissolved in water. After an
145 overnight fast, participants reported to the laboratory for the final visit. Muscle biopsies were
146 taken from the exercise and control legs. Blood samples were collected hourly for five hours to
147 assess plasma D_3-3MH and measure whole-body myofibrillar proteolysis as described (4, 6, 16,
148 17).

149 *Blood Analysis.* Blood samples were analyzed using the Ortho Vitros MicroWell, using the
150 VITROS 5600 Integrated System that provides enhanced chemiluminescence detection for serum
151 estradiol (E2; pmol/L; by competitive immunoassay; inter-assay CV <4%), progesterone (P4;
152 nmol/L; by competitive immunoassay; inter-assay CV <6%), and luteinizing hormone (LH;
153 IU/L; by non-competitive immunometric assay; inter-assay CV <5%) by Hamilton Regional
154 Laboratory Medicine and D_3-3-methyl-histidine enrichment as described previously (4, 6, 16,
155 17).

156 *Deuterium Oxide.* The incorporation of deuterium (as D_2O) into muscle protein-bound alanine
157 was assessed to quantify MPS rates (6, 16-18). The protocol consisted of one loading day and
158 four maintenance days with the goal of enriching and maintaining the body water pool.

159 *Muscle Biopsies.* Muscle biopsy samples were obtained on 7 occasions using a 5mm Bergstrom
160 needle modified for manual suction under 1% xylocaine local anesthesia. The first biopsy site
161 was approximately 15 cm above the patella, and subsequent biopsy sites were spaced ~3-5cm

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162 apart. Biopsies were taken from the control limb pre-exercise (phase [randomized to Active or
163 Inactive] 1, visit 1) and the control and exercise limbs (phase 1, visit 5; phase 2, visit 1; and
164 phase 2, visit 5). Visible connective and adipose tissue were dissected from each specimen prior
165 to being snap-frozen in liquid nitrogen and stored at −80°C.

166 *Saliva Analysis.* Saliva samples were obtained by gently chewing on a cotton swab for 2–3 min
167 until completely saturated with saliva. Salivettes were centrifuged at 1500g for 10 min and
168 diluted in doubly distilled water. Saliva samples were analyzed for ^2H (D) enrichment by cavity
169 ringdown spectroscopy (L2130-i, Picarro Inc., Santa Clara, CA). Measurements were corrected
170 for machine drift and background enrichment, and the ^2H (D) isotopic enrichments for saliva
171 were converted to atom % excess using standard equations as reported previously (4, 6, 16, 17).

172 *Myofibrillar Extraction.* Snap-frozen muscle samples were homogenized using 5mm stainless
173 steel beads in a 2mL Eppendorf (2 x 40 s at 20 Hz, TissueLyser, Hilden, Germany) with 500µl
174 fresh, ice-cold homogenization buffer (25mM Tris buffer [Tris-HCl, Trizma Base, doubly
175 distilled H_2O (ddH_2O) pH 7.2], 1 PhosStop Tablet (Roche, Switzerland), 1 complete (Roche)
176 mini protease inhibitor tab, 100µl TritonX-100). Samples were then processed as described in
177 our previous work (4, 17).

178 *Integrated myofibrillar protein synthesis.* Ingestion of D_2O was used to label newly synthesized
179 myofibrillar proteins (18). Myofibrillar protein synthesis rates were determined using the
180 standard precursor-product method (19, 20). Total body water (saliva) deuterium (^2H)
181 enrichment (converted to its natural log) was used as a surrogate for plasma alanine labeling
182 (precursor). The change in ^2H enrichment (relative to ^1H) of muscle alanine (product) over time
183 was used to calculate the myofibrillar fractional synthesis rates (4, 17).

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184 *Creatinine Enrichment.* Samples were thawed at room temperature and had 250 µL of ice-cold
185 acetonitrile added, then were vortexed, mixed, and cooled on ice for 30 min. Samples were then
186 centrifuged at 17000g for 20 min. The supernatant was filtered through a 0.2 µm filter and
187 transferred to vials ready for mass spectrometry analysis as previously described (4, 6, 16, 17).
188 The estimated creatine pool size was divided by 4.3 g/kg, which reflects the average
189 concentration of creatine found in whole wet muscle tissue (21). Details of the analyses are
190 provided in our previous papers (4, 6, 16, 17).
191 *Plasma D_3-3-methyl-histidine.* We have previously described the methods used for this analysis
192 (4, 6, 16, 17). Briefly, plasma samples were defrosted and centrifuged at 1200g for 3 min. A 0–
193 10% D_3-3-methyl-histidine enrichment curve was prepared as a serial dilution. 100 µL of plasma
194 was de-proteinized using 1 mL of MeCN: MeOH (1:1). Samples were vortex mixed and
195 incubated at − 20°C for 1 h. Samples were centrifuged at 20,800g for 5 min at 4°C. The
196 supernatant was dried down in a TechneBlock at < 40°C using nitrogen gas. Samples were re-
197 suspended using 100 µL MeCN: ddH2O (65:35) and ready to be analyzed using High-
198 Performance Liquid Chromatography (HPLC; Dionex Ultimate3000, Thermo Scientific) mass
199 spectrometry (MS; Q-Exactive, Thermo Scientific) with a Sequant ZIC-HILIC column (150 mm
200 Å~ 2.1 Å~ 5um; Merck Millipore) using previously described methods (6, 16). The enrichment
201 ratios were log-transformed to determine the decay rates (k), representative of the rate of whole-
202 body MPB (22).
203 *Statistical Analysis.* Data were analyzed using SPSS (IBM Corp. Released 2023. IBM SPSS
204 Statistics for Windows, Version 29.0.2.0 Armonk, NY: IBM Corp) using a 2-way ANOVA with
205 repeated measures with OCP phase (Active or Inactive) and leg (exercise or control) as within-
206 subject factors; all factors nested within-subject. Significance was set at $P < 0.05$. Data are

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207 presented as means ± standard deviation (SD) in tables and as box and whisker plots showing
208 interquartile range, median, and upper and lower bounds in figures unless otherwise indicated.
209
210 **RESULTS**
211 *Participants.* Participants used daily monophasic pills (Alesse and Alysena) containing 0.02 mg
212 ethinylestradiol and 0.1 mg levonorgestrel (days 1-21), with a 7-day inactive week (days 22-28).
213 Participant characteristics are shown in Table 1.
214 *Hormones.* Serum E2, P4, and LH were assessed in both phases. Data are presented in Table 1.
215 There were no differences across any phase in any hormone measured (all $P > 0.4$).
216 *Myofibrillar Protein Synthesis.* The mean MPS in the Active pill phase were 1.44 ± 0.14 %•d^{-1} in
217 the control leg and 1.64 ± 0.15 %•d^{-1} in the exercise leg ($p < 0.001$). The Inactive phase MPS
218 rates were 1.49 ± 0.12 %•d-1 %/d in the control leg and 1.71 ± 0.16 %•d^{-1} in the exercise leg ($p <$
219 0.001). The two-way ANOVA showed a main effect of condition (leg; $p < 0.001$) but no
220 significant effects of phase ($p = 0.48$; effect size 0.16) or interaction between phases ($p = 0.63$;
221 effect size 0.12). The results are presented in Figure 2.
222 *Myofibrillar Protein Breakdown.* The mean rate (k) of whole-body myofibrillar proteolysis was
223 0.018 ± 0.01 in the Active phase and 0.018 ± 0.006 in the Inactive phase ($p = 0.55$). The results
224 are presented in Figure 3.
225
226 **DISCUSSION**
227 While there was a strong and consistent effect of RET on MPS in both phases, the
228 response did not differ between the active and inactive OCP phases (Figure 2). We also observed
229 no difference in whole-body myofibrillar proteolysis (Figure 3), which would include and may

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230 be predominantly skeletal muscle-derived (22). Our work shows that females taking OCP derive
231 no specific anabolic advantage, nor are they in a pro-catabolic state in any particular phase. We
232 also note that compared to our recent data (using an identical design) from naturally cycling
233 women (4), we observed no marked differences between resting or post-exercise anabolic and
234 catabolic rates. In fact, combining datasets and running statistical analyses, including a between
235 factor for OCP use and either phase of the MC, showed no pill-by-phase interaction and only
236 main effects for exercise (exercise > rest; data not shown). Thus, our data show, in accordance
237 with meta-analytic analyses of cross-sectional OCP effects (10) and phase-specific effects across
238 the MC (5, 23), that there are no specific differences in terms of muscle anabolism or catabolism
239 in response to RET related to OCP use.

240 As expected, endogenous ovarian hormones were downregulated, compared with
241 normally cycling females (4), in the OCP cohort across all time points. Our data indicate that a
242 week of inactive pills was insufficient to restore the endogenous hormone profile of a naturally
243 menstruating individual. As a result it is perhaps unsurprising that we did not see a change in the
244 muscle protein synthetic or whole-body myofibrillar proteolysis responses.

245 We acknowledge that our data are short-term (days) but propose that they offer new and
246 deeper insight than acute infusions of isotopes (hours) (7, 8). Longer-term trials and the use of
247 third- and fourth-generation OCP, as well as intrauterine progesterone-emitting contraception,
248 would be interesting avenues in which to apply similar methods. We note, however, that these
249 methods of contraception have been compared in vascular and cellular physiology and show
250 statistically significant but physiologically trivial differences (24). We hypothesize that since
251 third- and fourth-generation OCPs have even less androgenic forms of progesterone (25) than

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second-generation OCPs, it is unlikely there would be marked anabolic or catabolic effects of these OCPs compared to earlier generations (5).

Our results are largely in line with those of Hansen and colleagues, who assessed MPS in a group of naturally cycling participants compared to a group of habitual OCP users, reporting that MPS and MPB did not differ between groups (7). However, MPS was significantly lower in a sub-group (n=7) of third-generation but not second-generation (n=4) OCP users compared to the naturally cycling group (n=9). Further investigations into other forms of contraception, including intrauterine devices, would be an interesting avenue to pursue.

We conclude that second-generation OCP phase does not alter muscle anabolic capacity nor influence myofibrillar proteolysis in response to RET. Our results concur with reviews and meta-analyses showing no influence of OCP or MC-related changes in sex hormones on muscle propensity for anabolism (3, 5, 10, 23). Longer-term trials and studies of other contraceptive methods will be needed to confirm whether our findings of day-to-day protein turnover are generalizable and align with RET phenotypes.

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271

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279

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383 **Table 1. Participant characteristics and serum hormone levels**
384

Age (y)	20±2			
Height (cm)	164±2			
Body mass (kg)	61.9±8.3			
BMI (kg/m^2)	22.9±2.3			
Lean mass (kg)*	40.8±4.9			
Muscle mass (kg)**	22.3±1.2			
	Inactive Phase (OC Cycle Days 23-28)		Active Phase (OC Cycle Days 9-14)	
	Visit 1	Visit 6	Visit 1	Visit 6
E2 (pM)	97±26	153±47	108±50	101±29
P4 (nM)	9±3	7±2	8±3	7±3
LH (IU/L)	3±3	4±3	3±2	2±3

385 Values are means±SD. * Derived from DXA. ** Derived from D_3-creatine. E2 – estradiol; P4 –
386 progesterone; and LH – luteinizing hormone.
387
388
389
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391 Figure 1. Schematic depiction of the protocol that was repeated in each OCP phase. The
392 exercised limb was randomly selected and was switched in a counterbalanced manner, as was the
393 phase in which each participant began the protocol. RT – resistance training; Bx – muscle
394 biopsy; D_2O – oral dose of deuterated water; D_3Cr – oral dose of deuterated creatine; D_3-3-MH –
395 oral dose of deuterated 3-methylhistidine.
396
397 Figure 2. Integrated muscle protein synthesis in active and inactive phases. *Significant ($P <$
398 0.001) difference (main effect) between EX and CON. There was no significant effect of OCP
399 phase nor an interaction between phases and conditions (all $P > 0.5$).
400
401 Figure 3. Whole body myofibrillar protein breakdown rate (k) in active and inactive OCP phases.
402

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Day	1	2	3	4	5	6
RT	↑		↑			
Bx	↑					↑
Blood	↑					↑↑↑
Urine	↑			↑		
Saliva	↑	↑	↑	↑	↑	↑
D_2O	↑↑↑	↑	↑	↑	↑	
D_3-Cr	↑					
D_3-3-MH					↑	

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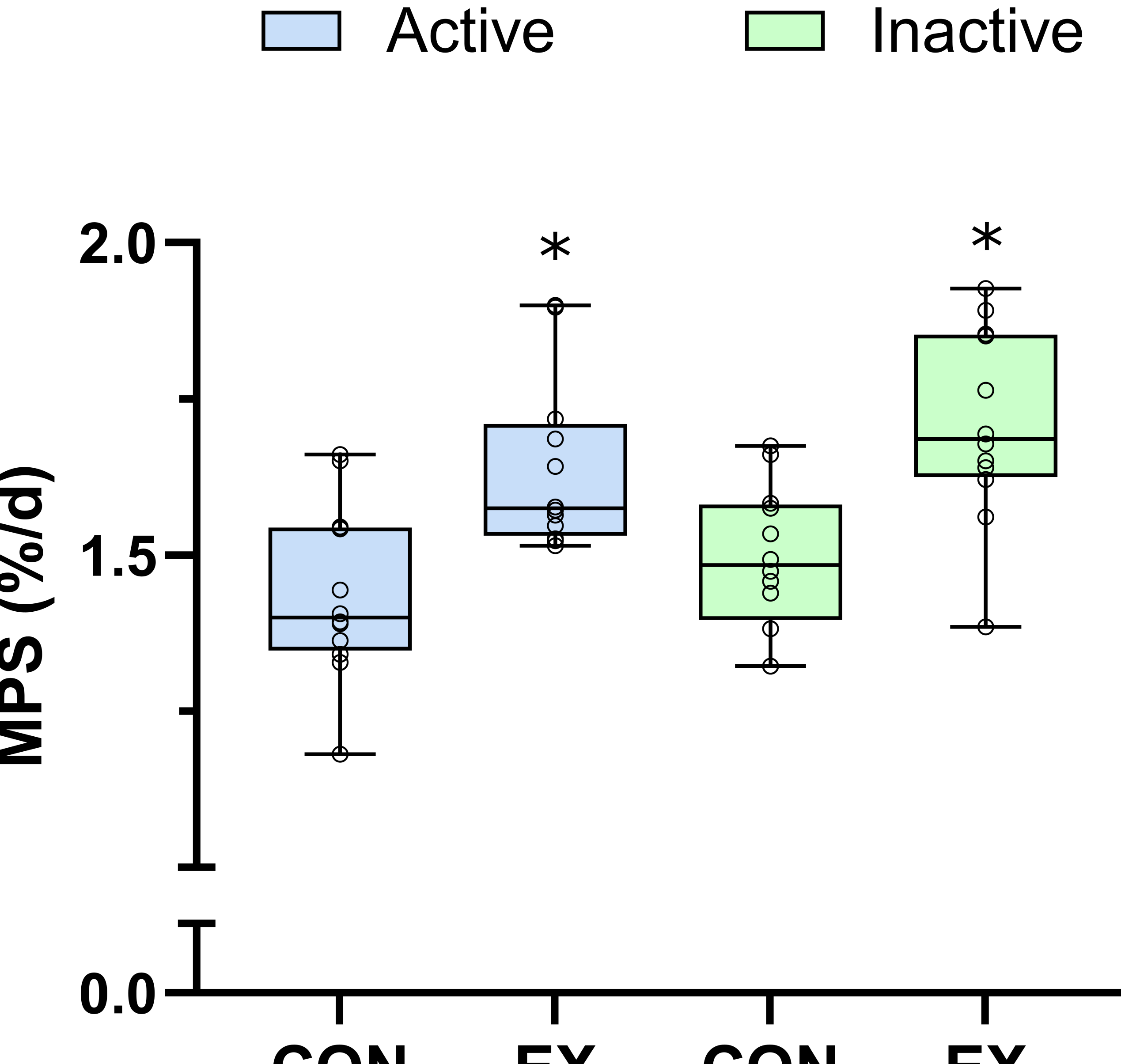

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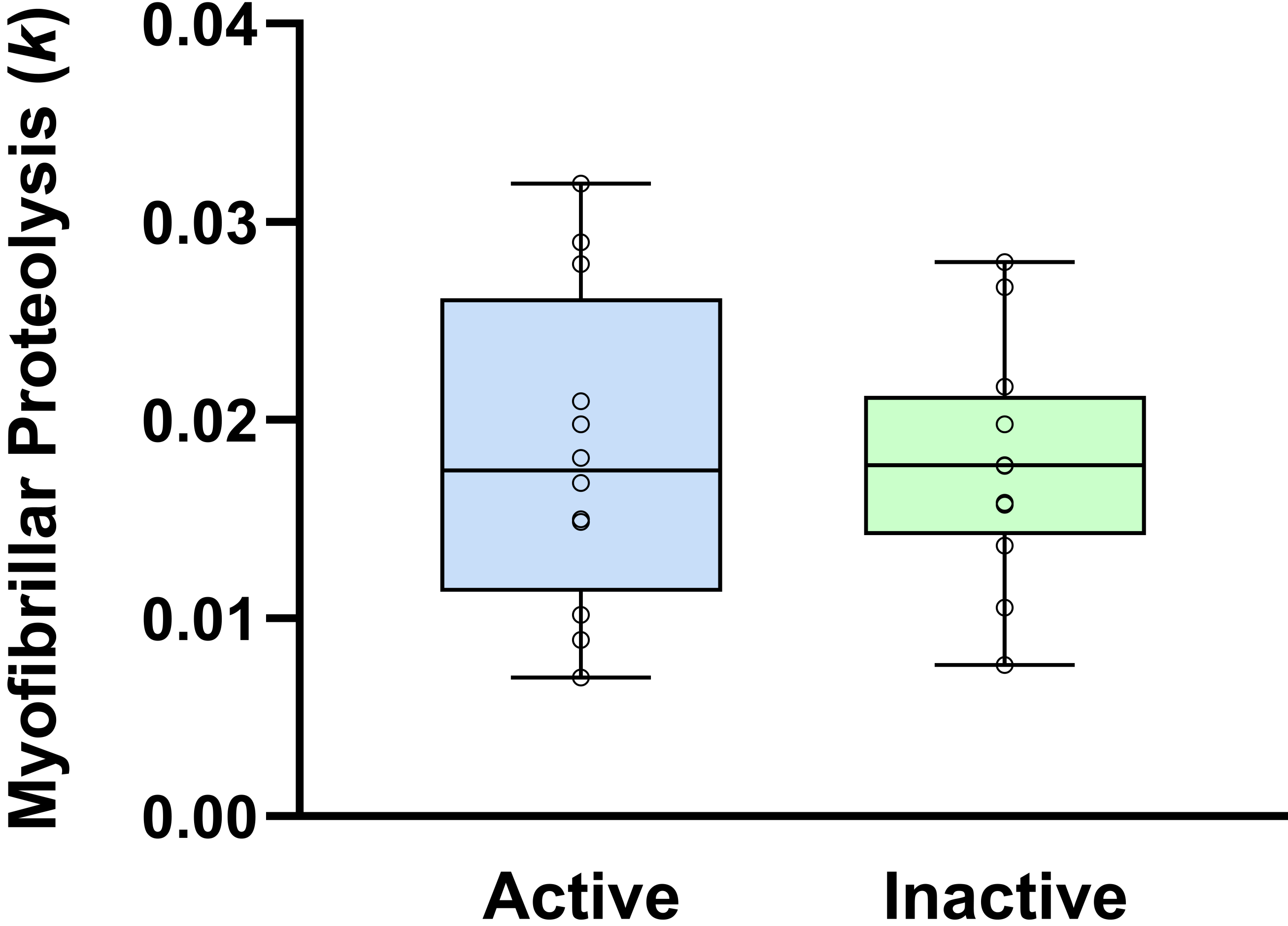

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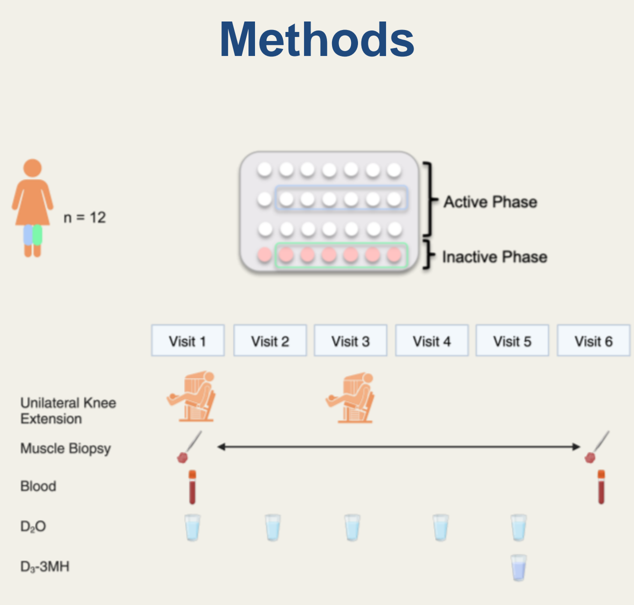
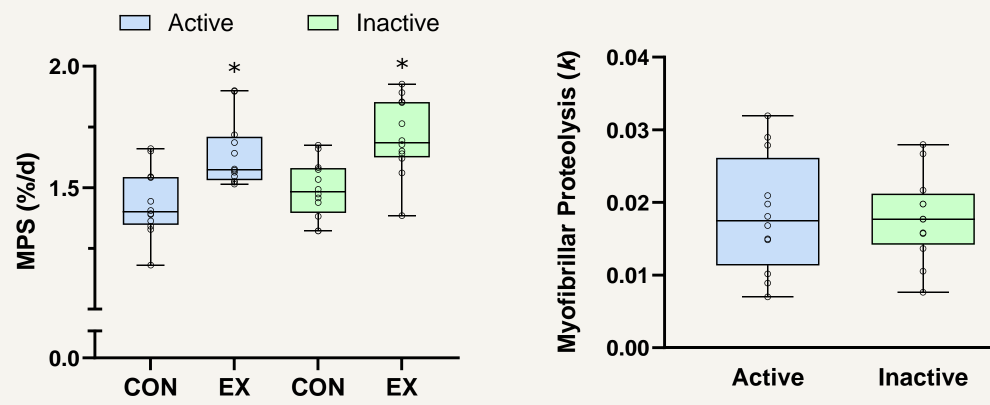
Muscle Protein Turnover is Unaffected by Oral Contraceptive Pill Phase

Methods



n = 12 females studied in Active and Inactive OCP phases. Unilateral resistance exercise in each phase: control (CON) and exercised (EX) legs.

No Effect of OCP phase on Muscle Protein Synthesis (MPS) or Whole-body Myofibrillar Protein Breakdown



Conclusion: Resistance exercise, but not OCP phase, increased MPS. Myofibrillar proteolysis was unaffected by OCP phase.

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