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The Duration of the Repeated-Bout Effect After Eccentric Exercise

Anastasios A. Theodorou[1] | Nikos V. Margaritelis[2] | Anastasia V. Karyofyllidou[3] | Filippos Christodoulou[1] | Athanasios Z. Jamurtas[4] | Ioannis G. Fatouros[4] | Antonios Kyparos[2] | Michalis G. Nikolaidis[2] | Vassilis Paschalis[3]

[1]Department of Life Sciences, European University Cyprus, Nicosia, Cyprus | [2]Department of Physical Education and Sports Science at Serres, Aristotle University of Thessaloniki, Serres, Greece | [3]School of Physical Education and Sport Science, National and Kapodistrian University of Athens, Athens, Greece | [4]Department of Physical Education and Sport Science, University of Thessaly, Trikala, Greece

Correspondence: Anastasios A. Theodorou (a.theodorou@euc.ac.cy) | Vassilis Paschalis (vpaschalis@phed.uoa.gr)

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ABSTRACT

The duration of the repeated-bout effect following eccentric knee-extensor exercise remains undefined, despite its widespread use in muscle damage research and training. The present study examined how long this protective effect persists in the knee extensors. Forty-eight young men performed two identical bouts of maximal eccentric knee-extensor exercise (5×15 repetitions at 60°/s). Four independent groups performed the second bout 3, 6, 9, or 12 weeks after the first. Isometric torque, range of motion (ROM), delayed-onset muscle soreness (DOMS), muscle thickness, echo intensity, creatine kinase (CK), and high-sensitivity C-reactive protein (hs-CRP) were assessed before and 2 and 4 days after each bout. Baseline-adjusted linear mixed-effects models were used to compare responses between bouts, complemented by equivalence testing at 12 weeks. The repeated-bout effect was evident at 3 and 6 weeks across most outcomes and remained detectable at 9 weeks for selected markers. At 12 weeks, no significant differences between bouts were observed at either day 2 or day 4 for any outcome. Exploratory equivalence analyses further indicated that the effect was no longer meaningfully present at these time points for isometric torque, ROM, DOMS, muscle thickness, and echo intensity, whereas the findings for CK and hs-CRP remained inconclusive. Overall, the repeated-bout effect may persist for some outcomes for up to 9 weeks, whereas by 12 weeks no meaningful effect was detectable at the assessed post-exercise time points. This time course can guide the design of muscle damage studies and the scheduling of eccentric training sessions.

1 | Introduction

The repeated-bout effect is a robust phenomenon in exercise physiology, where a single bout of muscle-damaging exercise protects against subsequent muscle damage [1–3]. This protection is most prominent following eccentric contractions compared with other contraction modes, possibly because of unfamiliarity with eccentric contractions, which cause greater muscle damage [4, 5]. To date, only a few studies, mainly on the elbow flexors, have systematically examined the duration of the repeated-bout effect across varied intervals, and the persistence of this protection remains unclear.

In a study that employed the longest inter-bout interval reported to date, Nosaka et al. [6] showed that a single bout of maximal eccentric elbow-flexor exercise provided protection that was still evident 6 months later after the first bout. Some markers, such as the recovery of maximal isometric torque, persisted at month 9 but disappeared by month 12. This study serves as the principal reference in the field for framing the duration of the repeated-bout effect [2]. However, other studies indicate a substantially shorter duration of the repeated-bout effect. Byrnes et al. [7] found that the repeated-bout effect was present after downhill running at 3 and 6 weeks but had faded by 9 weeks. Nosaka et al. [8] found that protective adaptations in muscle damage markers

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partially persisted for up to 10 weeks after 70 maximal eccentric contractions of the elbow flexors. Consistent protection was found at week 6, suggesting that protection duration is not uniform across markers. Similarly, Nosaka et al. [9] showed that after 12 maximal elbow flexor contractions, a uniform protective response for all markers occurred at weeks 4 and 8, whereas by week 12, some markers remained attenuated. Overall, these findings support the consistent occurrence of the repeated-bout effect and indicate that the duration of protection differs across outcomes.

Although the repeated-bout effect has previously been demonstrated in the knee extensors [10, 11], these studies have generally examined a single, relatively short inter-bout interval rather than systematically characterizing the persistence of the protective effect across progressively longer intervals. Thus, the duration and progressive attenuation of the repeated-bout effect in the knee extensors remain insufficiently characterized. This represents an important gap because the knee extensors are extensively used in experimental models of exercise-induced muscle damage, while most studies specifically addressing the duration of the repeated-bout effect have employed the elbow flexors or downhill running. Moreover, elbow flexors and knee extensors exhibit different magnitudes and time courses of damage after eccentric exercise [12, 13]. These differences may reflect several factors, including muscle architecture, habitual loading, and muscle fiber composition [14]. Thus, the direct transferability of findings across these muscle groups is constrained, pointing to the need for a targeted study of the magnitude and time course of the repeated-bout effect in the knee extensors.

Therefore, we designed an experiment to systematically characterize the duration of the repeated-bout effect in the knee extensors across four different intervals. To our knowledge, this is the first study to examine this within the same knee-extensor eccentric exercise model using 3-, 6-, 9-, and 12-week intervals. Notably, independent groups were used for each interval to distinguish inter-bout intervals, ensuring that each participant completed only two eccentric exercise bouts. This design prevented the additional adaptive effects that could arise from exposing the same participants to multiple serial eccentric bouts and allowed the effect of the inter-bout interval to be examined independently. Defining the attenuation of commonly used markers of muscle damage by the repeated-bout effect is important both to understand the phenomenon itself and for improving the design of studies involving muscle damage. The present study was deliberately designed not as a mechanistic investigation of the repeated-bout effect, but as a practical and descriptive time-course study intended to guide researchers planning repeated muscle-damage protocols, including when the largest attenuations are expected and when a second eccentric bout can be performed with minimal concern for residual effects from the first bout. Collectively, the present study aimed to determine the duration of the repeated-bout effect after maximal eccentric exercise of the knee extensors using a comprehensive set of functional, perceptual, imaging-based, and biochemical markers.

2 | Materials and Methods

2.1 | Participants

In the present investigation, young males were enrolled (Table 1). Sample size was estimated a priori using G*Power 3.1 [15] for repeated-measures ANOVA with within-between interaction. The calculation was based on previous studies [6, 8, 9] examining the repeated-bout effect of eccentric exercise on muscle damage markers (e.g., isometric torque and DOMS). Assuming an effect size of 0.25, an alpha level of 0.05, a power of 0.90, a correlation of 0.50 among repeated measurements, and a nonsphericity correction of 1.0, the minimum required sample size was 36 participants. The target sample was set at 48 participants to ensure equal group sizes ($n = 12$ per group). Five participants completed the first bout and were allocated to an interval group but did not complete the second bout. A participant in the 3-week group had an ankle injury before the scheduled second bout; two participants in the 9-week group and two participants in the 12-week group declined further participation or were unable to complete all evaluations due to personal reasons. Recruitment and allocation therefore continued using the original randomization procedure among groups that had not yet reached the prespecified target, with groups removed from subsequent allocation once 12 participants had completed both exercise bouts. This process continued until the target of 12 completers per group was achieved (Figure S1). Allocation of subsequently recruited participants was based solely on which groups had not yet reached the prespecified target and was performed without reference to participant characteristics or study outcomes. The primary analyses were therefore based on participants who completed both eccentric exercise bouts and the required assessments. As the

TABLE 1 | Anthropometric characteristics and baseline peak isometric torque of the participants ($n = 12$ per group; mean ± SD).

Variable	Week 3	Week 6	Week 9	Week 12	*p*
Age (years)	22.3 ± 2.2	23.3 ± 3.5	24.3 ± 2.3	23.4 ± 3.4	0.420
Height (cm)	177.7 ± 6.1	176.3 ± 6.0	175.4 ± 5.4	174.2 ± 5.5	0.511
Body mass (kg)	74.0 ± 6.3	70.3 ± 4.3	69.6 ± 6.7	72.1 ± 5.6	0.263
BMI (kg/m^2)	23.4 ± 1.4	22.6 ± 1.2	22.6 ± 1.2	23.8 ± 1.5	0.087
Body fat (%)	15.6 ± 4.7	14.6 ± 4.5	15.5 ± 5.3	16.6 ± 4.3	0.774
Fat mass (kg)	11.7 ± 4.3	10.3 ± 3.4	11.0 ± 4.5	12.0 ± 3.4	0.726
Fat-free mass (kg)	62.3 ± 3.8	59.9 ± 3.7	58.6 ± 3.3	60.1 ± 5.1	0.179
Isometric torque (Nm)	214 ± 38	211 ± 45	229 ± 57	219 ± 45	0.810

primary analyses were performed using linear mixed-effects models, this calculation should be considered an approximate design-stage sample size justification.

Volunteers were excluded from the study if they had any muscle or metabolic disease or a prior musculoskeletal injury that could affect their performance. They had no prior experience with muscle-damaging exercise for at least 6 months before enrollment, nor were they taking any anti-inflammatory medication or nutritional supplement for 3 months before enrollment and throughout the study period. Participants were recreationally active young men and were instructed to maintain their usual daily physical activity throughout the study (e.g., dancing, jogging, swimming), but to avoid resistance training, unaccustomed vigorous exercise, and any exercise with a strong eccentric component during the interval between bouts. Additionally, they were instructed to refrain from exercise 2 days before each exercise bout and until completion of the assessments on day 4. Dietary intake was not standardized or systematically recorded during the study period. The study was approved by the Ethics Committee of the National and Kapodistrian University of Athens (Protocol No. 1858/03-10-2025). All participants provided written informed consent before participation, and all procedures were conducted in accordance with the Declaration of Helsinki.

2.2 | Study Design

The study design is depicted in Figure 1. Participants performed an initial bout of eccentric exercise with their dominant leg. Following this, the participants were randomly allocated to one of the four experimental groups, and each group repeated the same bout with the same leg at its designated interval at weeks 3, 6, 9, and 12 after the first bout. A range of physiological and biochemical evaluations were performed before and on days 2 and 4 after each exercise bout to assess muscle damage. More specifically, the physiological markers of muscle damage, function, and imaging-based muscle characteristics (isometric peak torque, ROM, DOMS, muscle thickness, and echo intensity), and the biochemical markers of muscle damage (CK) and inflammation (hs-CRP) were assessed.

2.3 | Anthropometric Characteristics

Body mass and height were measured using standard procedures. Body fat percentage was estimated from seven-site skinfold measurements using a Harpenden skinfold caliper, with body density calculated using the Jackson and Pollock equation and converted to body fat percentage using the Siri equation. Participant characteristics are presented in Table 1.

2.4 | Exercise Protocol

Both eccentric exercise bouts were performed with the knee extensors of the dominant leg on an isokinetic dynamometer (Biodex System 4 PRO, Biodex Medical Systems Inc., NY, USA). At each bout, participants performed a maximal eccentric protocol comprising 5 sets of 15 repetitions at an angular velocity of 60°/s (knee range: 0° at full extension to 90° of knee flexion). Participants performed the protocol in a seated position (120° hip angle) and were stabilized in the dynamometer chair according to the manufacturer's instructions. They were verbally encouraged to perform all repetitions with maximal effort of their knee extensors; as expected, performance declined gradually with fatigue. A 2-min rest period was interposed between the sets. Before the exercise protocol, participants completed an 8-min cycling warm-up, followed by lower-limb stretching. Before exercise, participants had a familiarization session with the isokinetic dynamometer. The familiarization involved only a small number of low-intensity contractions of the non-exercise leg and was therefore not expected to induce meaningful muscle damage or a contralateral protective effect. This session took place at least 2 weeks before the first session.

2.5 | Muscle Function and Damage Measurements

The isokinetic dynamometer was used to assess the peak isometric torque of the knee extensors at 60° of knee flexion. Peak isometric torque was selected because it is a widely used marker of exercise-induced muscle damage and repeated-bout effect, allowing comparison with previous studies. The average value of

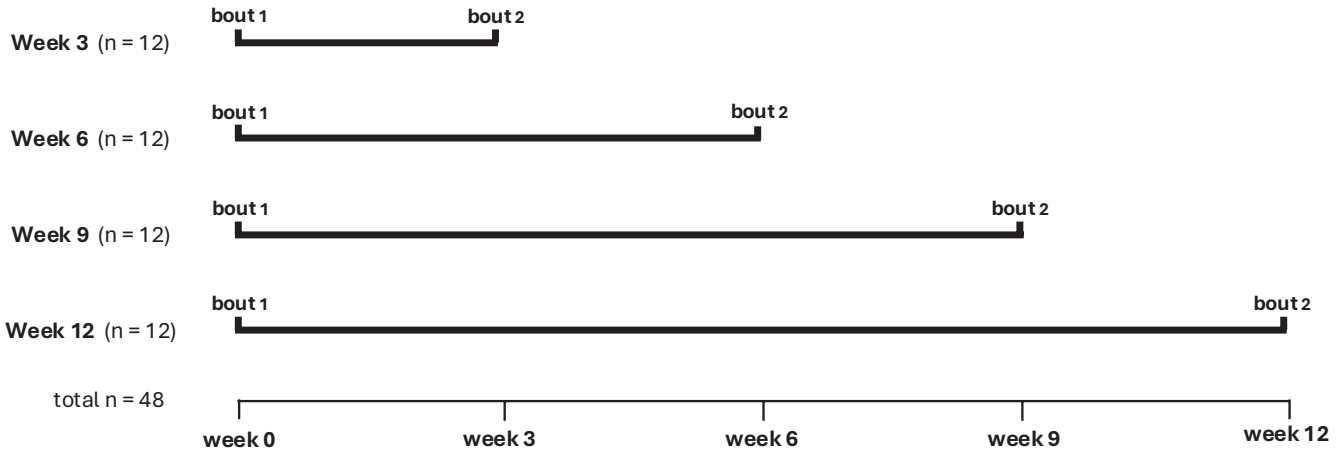


FIGURE 1 | Participants performed an initial bout of eccentric exercise using their dominant leg. Following completion of the initial bout, participants were randomly assigned to one of four experimental groups according to the interval between the first and second exercise bout. The identical eccentric exercise protocol was repeated with the same leg after 3, 6, 9, or 12 weeks. Physiological and biochemical assessments were conducted at baseline and at the designated follow-up time points following both exercise bouts to evaluate the persistence of the repeated-bout effect.

the torque of the best three maximal voluntary contractions was recorded. If the difference between the lowest and highest values exceeded 10%, the measurement was repeated. A 2-min rest was provided between maximal voluntary contraction attempts. Pain-free ROM was evaluated manually on the dynamometer, with the investigator moving the calf at a very low angular velocity starting from 0° knee extension to maximum knee flexion angle. The point where the participant first felt discomfort was recorded as the pain-free ROM. Test–retest reliability was assessed in a separate sample of 10 young men tested on two occasions under identical laboratory conditions, at the same time of day and by the same investigators, with sessions separated by 7 days. Reliability was quantified using a two-way mixed-effects, absolute-agreement, single-measure intraclass correlation coefficient (ICC). The ICC for isometric torque was 0.96 (95% CI: 0.83–0.99), and that for ROM was 0.88 (95% CI: 0.62–0.95). DOMS was assessed during participants' squat movements (90° knee flexion), and soreness was rated on a 10-point numerical rating scale from 1 (no muscle soreness) to 10 (very sore).

2.6 | Muscle Ultrasound Measurements

Muscle thickness and echo intensity of the vastus lateralis were assessed using a portable B-mode ultrasound system (ArtUs diagnostic ultrasound system, TELEMED, Vilnius, Lithuania) with a linear-array transducer. After 5 min in the supine position, participants were examined in the same position. Transverse images were acquired from the mid-thigh site corresponding to 50% of the distance between the greater trochanter and the lateral femoral epicondyle using a generous amount of transmission gel and minimal pressure to avoid compression of the tissue [16]. Ultrasound settings were kept constant for all measurements (frequency: 12 MHz; depth: 50 mm; dynamic range: 66 dB; power: −8 dB; gain: 45%). Images were exported as TIFF files and analyzed using ImageJ v.1.54 (National Institutes of Health, USA) [17, 18]. Muscle thickness was defined as the perpendicular distance between the superficial and deep aponeuroses of the vastus lateralis. A maximum-polygonal region of interest was selected within the muscle belly for echo-intensity analysis, avoiding the deep and superficial aponeuroses. Echo intensity was then calculated as the mean grayscale value of all pixels within this region, ranging from 0 (black) to 255 (white) [19]. Muscle thickness and echo intensity were quantified from the same ultrasound image, and the average of two images was used for statistical analysis. The corresponding ICCs were 0.93 (95% CI: 0.76–0.98) for muscle thickness and 0.89 (95% CI: 0.65–0.97) for echo intensity. The same examiner, blinded to bout, time point, and interval group, performed all muscle ultrasound image analyses.

2.7 | Blood Collection and Handling

Blood was collected into EDTA tubes, immediately centrifuged at 1370 g for 10 min, and plasma was collected. Samples were stored in aliquots at −80°C and thawed once before analysis. Plasma CK activity and hs-CRP concentrations were measured on a Cobas Integra 400 Plus analyzer (Roche Diagnostics, Mannheim, Germany). CK was determined using the Roche Creatine Kinase enzymatic UV assay (analytical range: 20–1300 U/L). Samples exceeding the upper analytical range for CK were diluted and reanalyzed. hs-CRP was determined using the Roche CRP Latex High Sensitive particle-enhanced immunoturbidimetric assay (analytical range: 0.3–350 mg/L; functional sensitivity: 0.3 mg/L). Intra- and inter-assay CVs were 3.2% and 4.4%, respectively, for CK, and 6.5% and 9.0%, respectively, for hs-CRP. All samples were analyzed in duplicate, and samples from the same participant were analyzed within the same batch.

2.8 | Statistical Analysis

A one-way analysis of variance was performed to compare baseline anthropometric characteristics and baseline isometric torque among the four groups (3, 6, 9, and 12 weeks). A two-way mixed analysis of variance was used to compare total work between the first and second eccentric exercise bouts across the four inter-bout interval groups, with group as the between-subject factor and bout as the within-subject factor.

The dependent variables were analyzed using baseline-adjusted linear mixed-effects models, with the pre-exercise value corresponding to each bout entered as a covariate. Model assumptions were evaluated by inspection of residual-versus-fitted and Q-Q plots, and models were checked for convergence, singular fits, influential observations, and substantial heteroscedasticity. In each model, the fixed factors were interval (3, 6, 9, or 12 weeks), bout (first vs. second), and day after exercise (2 and 4 days), together with all relevant interaction terms, whereas participant was included as a random factor (random intercept). No additional residual covariance structure was specified. The primary terms of interest were the interval × bout interaction, to examine whether the magnitude of the repeated-bout effect differed across the four recovery intervals, and the interval × bout × day interaction, to examine whether this interval-dependent repeated-bout effect varied according to the post-exercise assessment day. For variables with skewed distributions, namely CK and hs-CRP, a natural logarithmic transformation was applied, and results were interpreted on the transformed scale. Analyses were performed in *R* using the lme4, lmerTest, and emmeans packages [20]. Linear mixed-effects models were fitted using restricted maximum likelihood (REML). Omnibus tests for fixed effects were obtained using Type III *F*-tests with Satterthwaite-adjusted degrees of freedom. Post hoc second-versus-first bout contrasts were estimated using emmeans for each combination of inter-bout interval and post-exercise day. Holm adjustment was applied across the eight bout contrasts within each outcome to control for multiple comparisons. Data are presented as mean ± standard deviation (SD), and Figures 2–5 display both individual data points and mean ± SD. Statistical significance was set at $p < 0.05$.

The initial superiority analyses showed no statistically significant second-versus-first bout differences at the 12-week interval at either 2 or 4 days for all outcomes. However, non-significant superiority tests do not support a formal conclusion regarding the absence of an effect (i.e., absence of the repeated-bout effect) [21]. Therefore, to further evaluate whether the repeated-bout effect was indeed absent at the 12-week interval, equivalence testing was performed for all outcome variables using the two

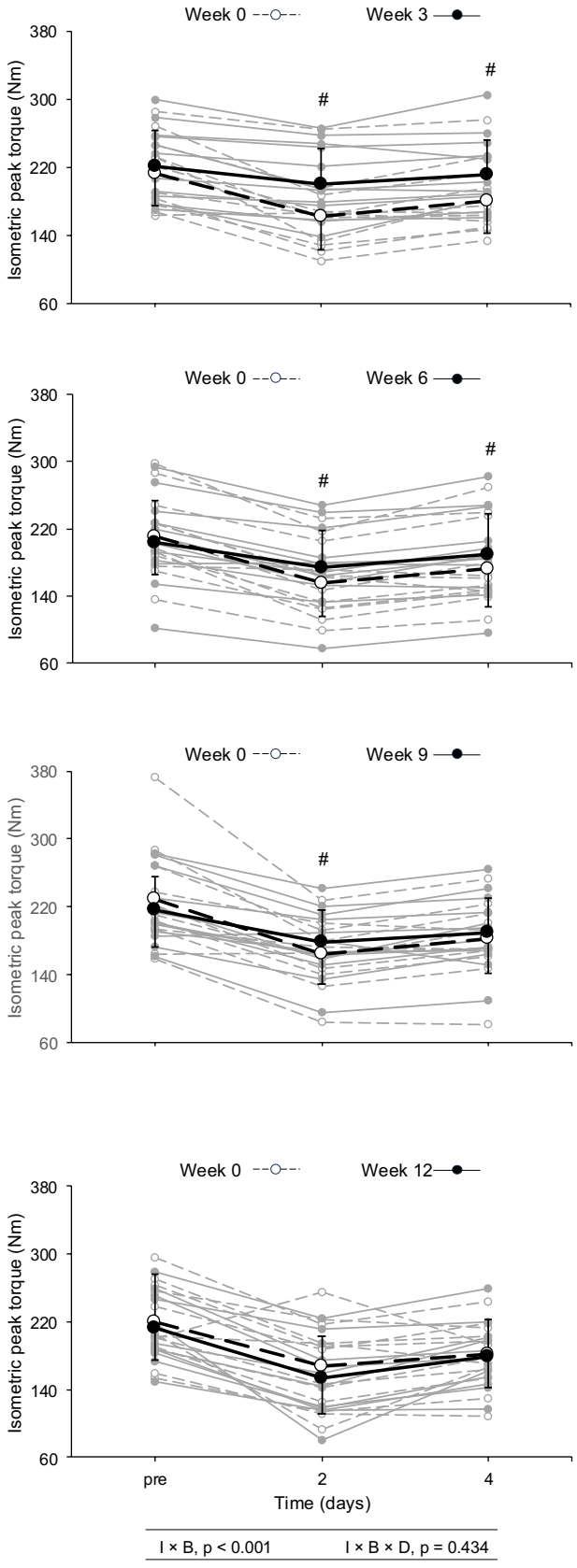


FIGURE 2 | Isometric peak torque at 60° knee flexion measured before, and 2 and 4 days after, the first and second eccentric exercise bouts. The second bout was performed at 3-, 6-, 9-, or 12-week intervals. $n = 12$ per group. I × B × D, interval × bout × day interaction; I × B, interval × bout interaction. #Significant difference between the two bouts at the specific time point.

one-sided tests (TOST) procedure [22]. In particular, equivalence testing was performed to determine whether the observed responses at 12 weeks were statistically compatible with the absence of a meaningful repeated-bout effect. These analyses were based on the baseline-adjusted contrasts comparing the second and first bout at each post-exercise time point (i.e., 2 and 4 days). As equivalence testing was introduced after inspection of the initial superiority analyses and the equivalence margins were not specified in a prospective statistical analysis plan, these analyses were considered exploratory. Outcome-specific equivalence margins were selected post hoc on the basis of available measurement-error, reliability, biological-variation, and clinical/practical-relevance data [18, 23–26], rather than on the magnitude of the repeated-bout effect observed in the present dataset. In particular, for isometric torque, DOMS, range of motion, muscle thickness, and echo intensity, equivalence was assessed on the difference scale using post hoc specified outcome-specific margins of ±25 Nm, ±2 points, ±7°, ±2 mm, and ±10 AU, respectively. For both CK and hs-CRP, which were analyzed on the log-transformed scale, equivalence was assessed using back-transformed ratio margins of 0.769–1.3. Equivalence was concluded when the 90% confidence interval of the estimated second-versus-first bout contrast fell entirely within the specified equivalence bounds, corresponding to a significant TOST result at an α level of 0.05. Equivalence analyses were performed in *R* using the TOSTER package, with model estimates and standard errors derived from the fitted baseline-adjusted models using lme4, lmerTest, and emmeans.

3 | Results

No significant baseline differences were observed among the four interval groups in anthropometric characteristics or baseline isometric torque ($p > 0.05$; Table 1). No significant main effects of Bout or Group, and no significant Group × Bout interaction was found for total work ($p > 0.05$; Table S1).

The linear mixed-effects models showed a significant interval × bout interaction for isometric torque ($p < 0.001$), DOMS ($p < 0.001$), range of motion ($p = 0.043$), muscle thickness ($p < 0.001$), echo intensity ($p = 0.002$), CK ($p = 0.010$), and hs-CRP ($p < 0.001$). The interval × bout × day interaction was not significant for any outcome variable ($p > 0.05$). For all outcomes, the values below represent model-estimated contrasts for the second bout relative to the first. For isometric torque, DOMS, range of motion, muscle thickness, and echo intensity, Δ represents the estimated difference between the second and first bout (i.e., 2nd minus 1st). Accordingly, positive Δ values for isometric torque and range of motion, and negative Δ values for DOMS, muscle thickness, and echo intensity indicate lower responses during the second bout due to the repeated-bout effect. For CK and hs-CRP, which were analyzed after logarithmic transformation, *R* is expressed as the ratio of the second bout to the first bout (i.e., 2nd/1st). Thus, for CK and hs-CRP, *R* ratios < 1 indicate lower responses during the second bout due to the repeated-bout effect (Table 2).

As the interval × bout × day interaction was not significant for any outcome, differences in statistical significance between the day-2 and day-4 simple contrasts should not be interpreted as

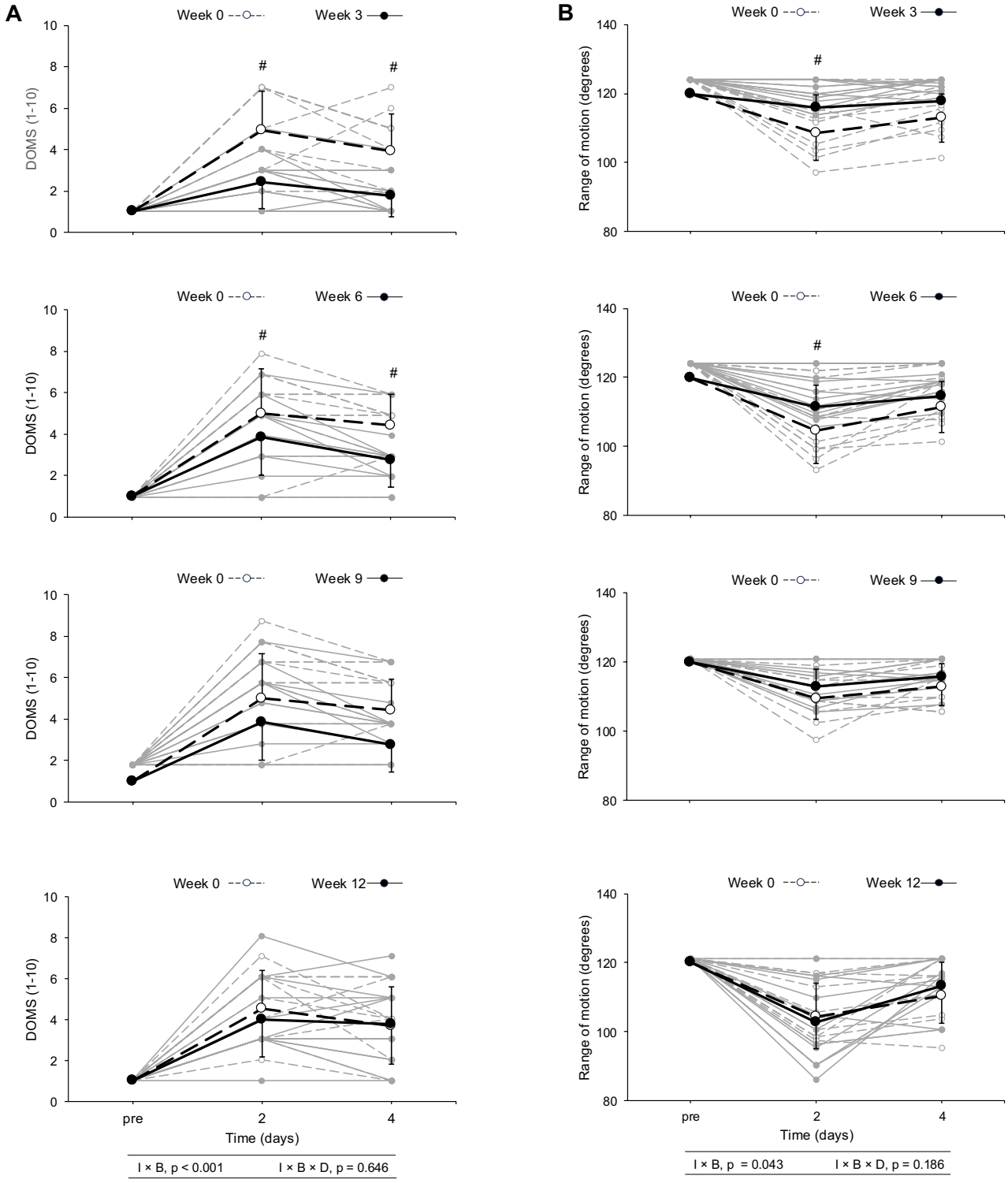


FIGURE 3 | (A) Delayed-onset muscle soreness and (B) range of motion measured before, and 2 and 4 days after, the first and second eccentric exercise bouts. The second bout was performed at 3-, 6-, 9-, or 12-week intervals. $n = 12$ per group. I × B × D, interval × bout × day interaction; I × B, interval × bout interaction. #Significant difference between the two bouts at the specific time point.

evidence that the magnitude of the repeated-bout effect differed between post-exercise days.

For isometric torque, the second bout resulted in significantly higher values than the first bout at both 2 and 4 days in the 3-week interval (Δ = 32.65 Nm and 24.48 Nm, respectively; $p < 0.001$ and $p = 0.003$) and in the 6-week interval (Δ = 21.99 Nm and 21.58 Nm, respectively; both $p = 0.007$). In the 9-week interval, a significant difference was observed at 2 days (Δ = 22.37 Nm, $p = 0.007$), whereas the difference at 4 days did not remain statistically significant after adjustment for multiple comparisons (Δ = 15.79 Nm, $p = 0.065$). At the 12-week interval, no significant second-versus-first bout differences were observed at either day 2 (Δ = −9.38 Nm, $p = 0.343$) or day 4 (Δ = 2.79 Nm, $p = 0.684$) (Figure 2).

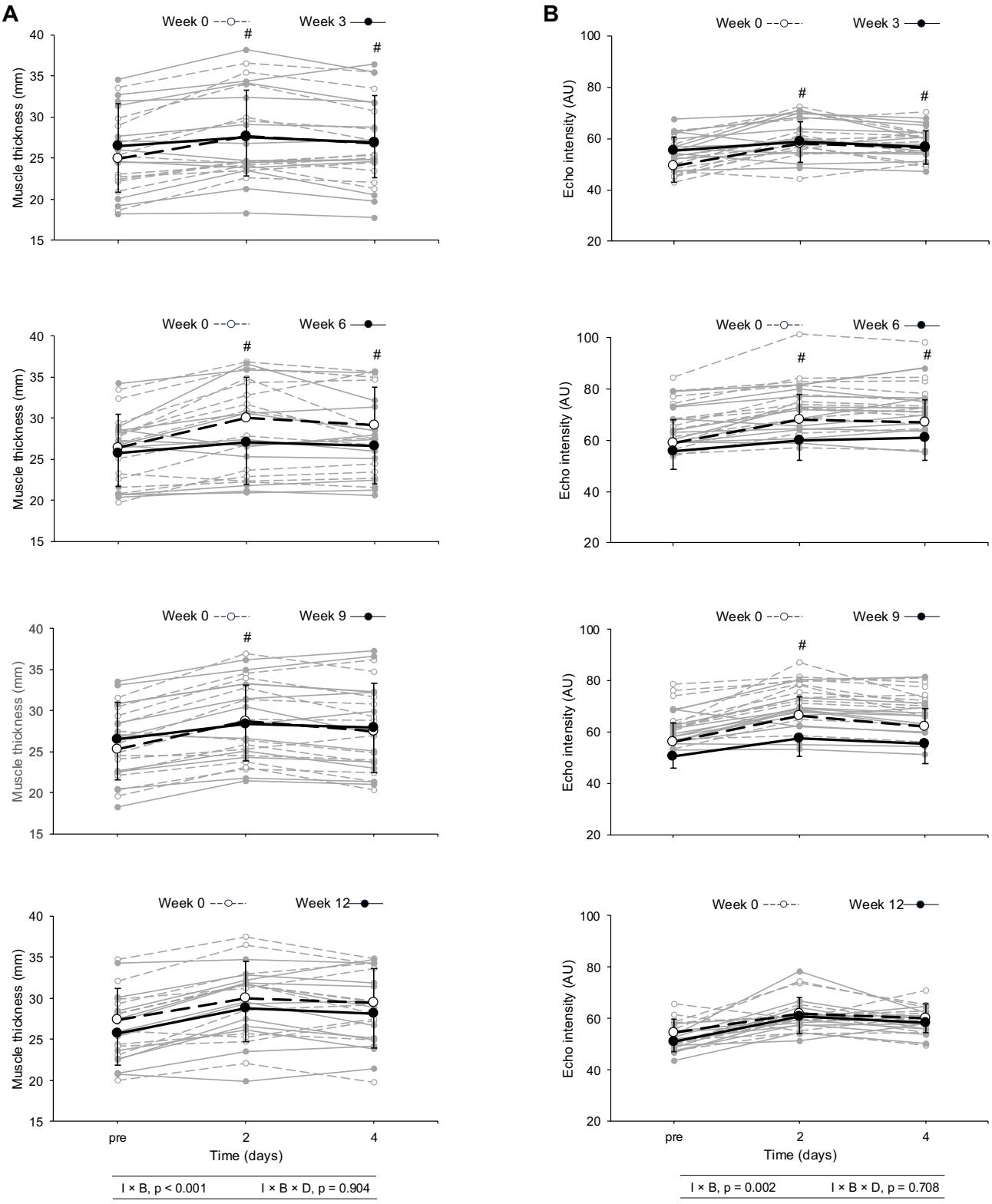


FIGURE 4 | (A) Muscle thickness and (B) echo intensity measured before, and 2 and 4 days after, the first and second eccentric exercise bouts. The second bout was performed at 3-, 6-, 9-, or 12-week intervals. $n = 12$ per group. I × B × D, interval × bout × day interaction; I × B, interval × bout interaction. #Significant difference between the two bouts at the specific time point.

For DOMS, the second bout was associated with significantly lower soreness at both 2 and 4 days in the 3-week interval ($\Delta = -2.50$ and -2.17 points, respectively; both $p < 0.001$). In the 6-week interval, soreness was significantly lower at 2 and 4 days ($\Delta = -1.17$ and $\Delta = -1.67$ points, respectively; $p = 0.015$ and $p = 0.007$). No significant differences were observed in the 9-week interval (2 days: $\Delta = -0.50$ points, $p = 0.990$; 4 days: $\Delta = -0.92$ points, $p = 0.297$) or in the 12-week interval (2 days: $\Delta = -0.50$ points, $p = 0.990$; 4 days: $\Delta = 0.08$ points, $p = 0.990$) (Figure 3A).

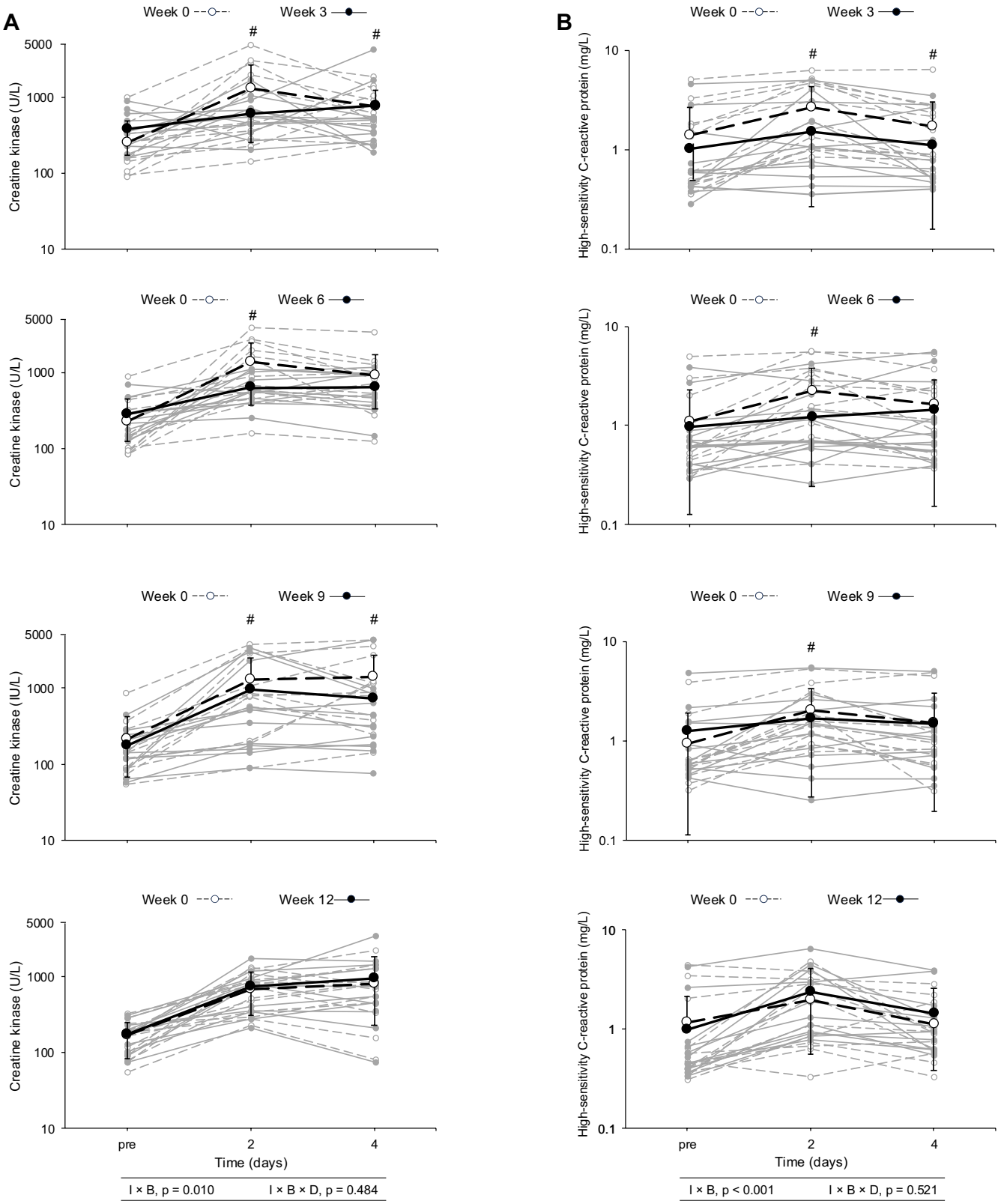


FIGURE 5 | (A) Plasma creatine kinase and (B) high-sensitivity C-reactive protein concentration measured before, and 2 and 4 days after, the first and second eccentric exercise bouts. The second bout was performed at 3-, 6-, 9-, or 12-week intervals. CK and hs-CRP are displayed on logarithmic *y*-axes. $n = 12$ per group. I × B × D, interval × bout × day interaction; I × B, interval × bout interaction. #Significant difference between the two bouts at the specific time point.

For ROM, the second bout resulted in significantly higher values than the first bout at 2 days in both the 3-week interval ($\Delta = 7.33°$, $p = 0.002$) and the 6-week interval ($\Delta = 7.00°$, $p = 0.003$), indicating a smaller exercise-induced reduction in ROM. The differences at 4 days did not remain statistically significant after Holm adjustment in either the 3-week interval

TABLE 2 | Model-estimated contrasts for second versus first bout responses across the four interval groups.

Outcome	*p* interval × bout	*p* interval × bout × day	3 weeks		6 weeks		9 weeks		12 weeks	
			2 days	4 days	2 days	4 days	2 days	4 days	2 days	4 days
Isometric torque (Nm)	**< 0.001**	0.434	**Δ: 32.65** **p < 0.001**	**Δ: 24.48** **p = 0.003**	**Δ: 21.99** **p = 0.007**	**Δ: 21.58** **p = 0.007**	**Δ: 22.37** **p = 0.007**	Δ: 15.79 p = 0.065	Δ: −9.38 p = 0.343	Δ: 2.79 p = 0.684
DOMS (1–10)	**< 0.001**	0.646	**Δ: −2.50** **p < 0.001**	**Δ: −2.17** **p < 0.001**	**Δ: −1.17** **p = 0.015**	**Δ: −1.67** **p = 0.007**	Δ: −0.50 p = 0.990	Δ: −0.92 p = 0.297	Δ: −0.50 p = 0.990	Δ: 0.08 p = 0.990
Range of motion (°)	**0.043**	0.186	**Δ: 7.33** **p = 0.002**	Δ: 4.67 p = 0.118	**Δ: 7.00** **p = 0.003**	Δ: 3.25 p = 0.439	Δ: 3.42 p = 0.439	Δ: 2.92 p = 0.439	Δ: −1.42 p = 0.479	Δ: 2.92 p = 0.439
Muscle thickness (mm)	**< 0.001**	0.904	**Δ: −1.83** **p = 0.013**	**Δ: −1.53** **p = 0.042**	**Δ: −2.34** **p < 0.001**	**Δ: −1.94** **p = 0.007**	**Δ: −1.58** **p = 0.040**	Δ: −0.88 p = 0.419	Δ: 0.58 p = 0.657	Δ: 0.41 p = 0.657
Echo intensity (AU)	**0.002**	0.708	**Δ: −4.06** **p = 0.013**	**Δ: −4.13** **p = 0.011**	**Δ: −5.75** **p = 0.004**	**Δ: −3.55** **p = 0.025**	**Δ: −4.55** **p = 0.005**	Δ: −2.43 p = 0.453	Δ: 1.49 p = 0.731	Δ: 0.59 p = 0.731
Creatine kinase (U/L)	**0.010**	0.484	***R*: 0.58** **p = 0.020**	***R*: 0.62** **p = 0.045**	***R*: 0.51** **p = 0.023**	*R*: 0.71 p = 0.406	***R*: 0.61** **p = 0.024**	***R*: 0.43** **p = 0.001**	*R*: 1.02 p = 1.000	*R*: 1.06 p = 1.000
hs-CRP (mg/L)	**< 0.001**	0.521	***R*: 0.65** **p = 0.002**	***R*: 0.73** **p = 0.010**	***R*: 0.54** **p = 0.003**	*R*: 0.81 p = 0.475	***R*: 0.59** **p = 0.016**	*R*: 0.83 p = 0.475	*R*: 1.36 p = 0.384	*R*: 1.30 p = 0.404

Note: For isometric torque, DOMS, ROM, muscle thickness, and echo intensity, Δ represents the estimated difference (2nd—1st). For hs-CRP and CK, *R* is presented as the ratio (2nd/1st), since these variables were log-transformed before analysis; accordingly, the results are dimensionless, and no units are reported. Pre-exercise values corresponding to each bout were included as covariates in the models. Thus, second-versus-first bout contrasts are presented only for post-exercise days 2 and 4. *p*-values for the second-versus-first bout contrasts were Holm-adjusted across the eight contrasts within each outcome. Bold indicates statistical significance ($p < 0.05$).
Abbreviations: CK, creatine kinase; DOMS, delayed onset muscle soreness; hs-CRP, high-sensitivity C-reactive protein.

(Δ = 4.67°, $p = 0.118$) or the 6-week interval (Δ = 3.25°, $p = 0.439$). No significant second-versus-first bout differences were observed in the 9-week interval (2 days: Δ = 3.42°, $p = 0.439$; 4 days: Δ = 2.92°, $p = 0.439$) or in the 12-week interval (2 days: Δ = −1.42°, $p = 0.479$; 4 days: Δ = 2.92°, $p = 0.439$) (Figure 3B).

For muscle thickness, the second bout resulted in significantly lower values than the first bout at both 2 and 4 days in the 3-week interval (Δ = −1.83 mm and −1.53 mm, respectively; $p = 0.013$ and $p = 0.042$) and in the 6-week interval (Δ = −2.34 mm and −1.94 mm, respectively; $p < 0.001$ and $p = 0.007$). In the 9-week interval, muscle thickness was significantly lower following the second bout at 2 days (Δ = −1.58 mm, $p = 0.040$), whereas no significant difference was observed at 4 days (Δ = −0.88 mm, $p = 0.419$). No significant second-versus-first bout differences were observed in the 12-week interval at either 2 days (Δ = 0.58 mm, $p = 0.657$) or 4 days (Δ = 0.41 mm, $p = 0.657$) (Figure 4A).

For echo intensity, significantly lower responses were observed during the second bout at both 2 and 4 days in the 3-week interval (Δ = −4.06 AU, $p = 0.013$; Δ = −4.13 AU, $p = 0.011$) and in the 6-week interval (Δ = −5.75 AU, $p = 0.004$; Δ = −3.55 AU, $p = 0.025$). In the 9-week interval, the effect was significant at 2 days only (Δ = −4.55 AU, $p = 0.005$), and no significant difference was observed at 4 days (Δ = −2.43 AU, $p = 0.453$). No significant second-versus-first bout differences were observed in the 12-week interval at either 2 days (Δ = 1.49 AU, $p = 0.731$) or 4 days (Δ = 0.59 AU, $p = 0.731$) (Figure 4B).

For CK, significantly lower responses were observed during the second bout at both 2 and 4 days in the 3-week interval ($R = 0.58$, $p = 0.020$; $R = 0.62$, $p = 0.045$) and in the 6-week interval at 2 days only ($R = 0.51$, $p = 0.023$), whereas the 4-day contrast was not significant ($R = 0.71$, $p = 0.406$). In the 9-week interval, significant differences were found at both 2 and 4 days ($R = 0.61$, $p = 0.024$; $R = 0.43$, $p = 0.001$). No significant differences were observed in the 12-week interval (2 days: $R = 1.02$; 4 days: $R = 1.06$, $p = 1.000$ for both) (Figure 5A).

For hs-CRP, significantly lower responses were observed during the second bout at both 2 and 4 days in the 3-week interval ($R = 0.65$, $p = 0.002$; $R = 0.73$, $p = 0.010$) and in the 6-week interval at 2 days only ($R = 0.54$, $p = 0.003$), whereas the 4-day contrast was not significant ($R = 0.81$, $p = 0.475$). Likewise, in the 9-week interval, a significantly lower response was observed at 2 days only ($R = 0.59$, $p = 0.016$), whereas the 4-day contrast was not significant ($R = 0.83$, $p = 0.475$). At the 12-week interval, hs-CRP responses were numerically higher following the second bout, although neither contrast was statistically significant after adjustment for multiple comparisons (2 days: $R = 1.36$, Holm-adjusted $p = 0.384$; 4 days: $R = 1.30$, Holm-adjusted $p = 0.404$) (Figure 5B). Exploratory equivalence testing also failed to establish equivalence at either time point.

Using the post hoc specified outcome-specific equivalence margins, exploratory equivalence was demonstrated for isometric torque at both 2 and 4 days (2 days: Δ = −9.38 Nm, 90% CI [−20.66, 1.90], $p = 0.011$; 4 days: Δ = 2.79 Nm, 90% CI [−8.49, 14.06], $p < 0.001$), for DOMS at both 2 and 4 days (2 days: Δ = −0.50 points, 90% CI [−1.34, 0.34], $p = 0.002$; 4 days: Δ = 0.08 points, 90% CI [−0.76, 0.93], $p < 0.001$), and for range of motion at both 2 and 4 days (2 days: Δ = −1.42°, 90% CI [−4.71, 1.88], $p < 0.001$; 4 days: Δ = 2.92°, 90% CI [−0.38, 6.21], $p = 0.032$). Equivalence was also demonstrated for muscle thickness at both time points (2 days: Δ = 0.58 mm, 90% CI [−0.40, 1.56], $p = 0.009$; 4 days: Δ = 0.41 mm, 90% CI [−0.57, 1.39], $p = 0.004$) and for echo intensity at both time points (2 days: Δ = 1.49 AU, 90% CI [−1.22, 4.21], $p < 0.001$; 4 days: Δ = 0.59 AU, 90% CI [−2.13, 3.30], $p < 0.001$) (Figure 6, upper panel). In contrast, equivalence was not established for CK at either post-exercise time point (2 days $R = 1.02$, 90% CI [0.706, 1.483], $p = 0.145$; 4 days $R = 1.06$, 90% CI [0.731, 1.535], $p = 0.182$) or for hs-CRP (2 days $R = 1.36$, 90% CI [1.021, 1.806], $p = 0.599$; 4 days $R = 1.30$, 90% CI [0.975, 1.724], $p = 0.493$) (Figure 6, lower panel). Overall, these exploratory findings indicate that at the 12-week interval, responses on assessed days 2 and 4 for isometric torque, DOMS, range of motion, muscle thickness, and echo intensity were statistically consistent with the absence of a practically meaningful repeated-bout effect, whereas the results for CK and hs-CRP remained inconclusive.

4 | Discussion

The present study was designed to determine the time course of the repeated-bout effect after maximal eccentric exercise of the knee extensors using an exercise protocol that has consistently induced prolonged muscle damage in previous studies from our group [27–29]. A key contribution of the present study is the systematic characterization of repeated-bout effect persistence across progressively longer inter-bout intervals using four independent groups that repeated the same exercise bout at 3, 6, 9, and 12 weeks. This design allowed the time course of the repeated-bout effect to be characterized without confounding effects from additional exercise bouts. The same eccentric exercise protocol was used in both bouts, and functional, perceptual, imaging-based, and biochemical markers of muscle damage and inflammation were assessed before exercise and on days 2 and 4 after exercise. This broad assessment battery was important because the apparent duration of the repeated-bout effect depended partly on the marker examined. The main finding was that the repeated-bout effect remained evident for selected markers up to 9 weeks after the first eccentric exercise bout, while exploratory equivalence analyses supported the absence of a practically meaningful repeated-bout effect at 12 weeks at days 2 and 4; for CK and hs-CRP, the evidence remained inconclusive at 12 weeks.

The marker-dependent pattern should be interpreted considering the fact that the overall muscle-damage response cannot be reduced to a single endpoint or to one biological level. Structural measures, including biopsy evidence of *Z*-line streaming and MRI-derived indices of tissue disruption, are valuable because they can capture tissue-level alterations associated with eccentric exercise-induced muscle injury [30, 31]. However, they do not fully represent the broader response to eccentric exercise, which also includes functional impairment, soreness, changes in range of motion, swelling, ultrasound-derived alterations, circulating muscle proteins, and inflammatory responses [32–34]. In this sense, exercise-induced muscle damage can be viewed as a partly latent variable, inferred from several observable indicators rather than

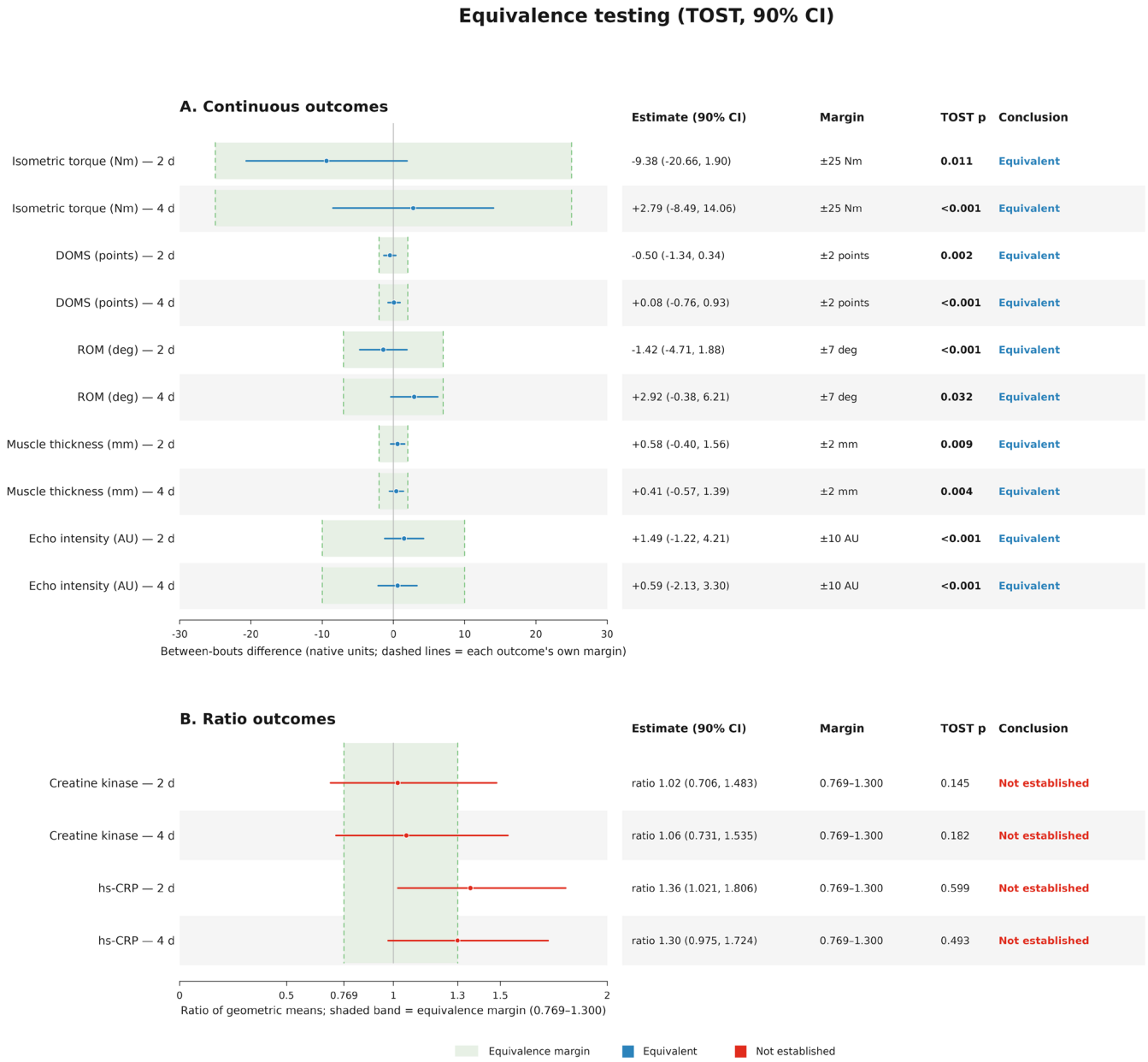


FIGURE 6 | Exploratory equivalence testing results at the 12-week interval. Points represent the baseline-adjusted estimates for the second versus the first bout, and horizontal lines represent the corresponding 90% confidence intervals. Green shaded areas indicate the equivalence bounds specified post hoc, and dashed vertical markers denote the lower and upper equivalence limits. Blue circles indicate outcomes for which equivalence was established by the two one-sided tests (TOST) procedure, whereas red circles indicate outcomes for which equivalence was not established. For isometric torque, DOMS, range of motion, muscle thickness, and echo intensity, estimates are expressed as differences (2nd—1st bout). For creatine kinase (CK) and high-sensitivity C-reactive protein (hs-CRP), estimates are expressed as back-transformed ratios (2nd/1st bout). $n = 12$.

measured completely by any single marker. This is particularly relevant for the repeated-bout effect, which is likely multifactorial and may include not only structural and cellular adaptations but also neural adaptations, as suggested by recent studies showing changes in motor-unit behavior across repeated bouts of eccentric or damaging resistance exercise [2, 35]. Accordingly, differences among markers are not necessarily contradictory but may indicate that different dimensions of the muscle-damage response and the repeated-bout effect persist for different durations.

The repeated-bout effect refers to the attenuated muscle-damage response after a second bout of eccentric exercise [36, 37]. The present findings extend previous work by highlighting a potentially important muscle-specific contrast in the persistence of the repeated-bout effect. Although week-scale studies of the elbow flexors have reported attenuation of selected markers at 10–12 weeks, in the present knee-extensor model no statistically significant second-versus-first bout differences were detected at days 2 and 4 at week 12, and equivalence was established within the selected margins for the functional, perceptual, and imaging outcomes examined. This contrast should be interpreted cautiously because the studies differ in eccentric exercise dose, marker selection, and post-exercise assessment schedules. The closest comparison regarding exercise dose to our study is Nosaka et al. [8], who used 70 maximal eccentric contractions

of the elbow flexors and reported uniform protection across markers at week 6, with attenuation of muscle soreness, relaxed and flexed elbow flexor angles, CK levels, and faster isometric strength recovery. Relaxed elbow flexor angle and CK remained attenuated at week 10. Byrnes et al. [7], using downhill running, reported attenuation of CK after the second bout at weeks 3 and 6, but not at week 9. Likewise, Nosaka et al. [9] employed a less demanding protocol of 12 maximal eccentric elbow flexor contractions and found attenuation after a second bout performed at weeks 4 and 8 for maximal isometric strength, relaxed and flexed elbow flexor angles, range of motion, arm circumference, muscle soreness, CK, and myoglobin. Extending the time frame to months, Nosaka et al. [6] reported that, after a first bout of 24 maximal eccentric elbow flexor contractions, the repeated-bout effect lasted for 6 months, persisted in some markers until 9 months, and disappeared at month 12. Collectively, the week-scale studies suggest that the repeated-bout effect becomes increasingly marker-dependent as the interval between bouts lengthens. The present data cannot determine whether an effect persisted at a time point that was not assessed post-exercise or for the biochemical markers CK and hs-CRP. For hs-CRP specifically, the 12-week estimates were numerically higher following the second bout, particularly on day 2. However, the contrasts were not statistically significant after Holm adjustment and equivalence was not established. Thus, the evidence for hs-CRP at 12 weeks remains inconclusive rather than supporting a clear persistence or reversal of the repeated-bout effect. The longer duration of the repeated-bout effect in the elbow flexor studies may reflect differences in the muscle group examined, the eccentric dose, the initial damage magnitude, the marker selection, and other muscle-specific characteristics.

Accordingly, our intention was not to explain why the repeated-bout effect occurs, but to define its usable time course in a knee extensor model commonly used in muscle-damage research. From this methodological perspective, the present findings have practical implications for experimental design. If the repeated-bout effect model is used to examine muscle responses and adaptations, among the intervals examined, the 3-week interval produced the most consistent protection across outcomes in the knee extensors. In contrast, when the aim of an investigation is to examine the effect of an intervention, such as a nutritional supplement or a recovery treatment, on muscle damage, residual protection from the first bout can confound a crossover design. Thus, in this model, a washout period of at least 12 weeks appears prudent to minimize residual protection at the assessed post-exercise time points. A long washout interval, however, reduces feasibility and makes it more difficult to control training status, physical activity, and diet, while also limiting some of the main advantages traditionally associated with crossover and unilateral exercise models, such as the reduced variability and improved statistical efficiency [38]. Although a contralateral-limb design has been proposed as an alternative option to minimize carryover effects and reduce interindividual variability [38], a moderate contralateral repeated-bout effect has been reported [39], especially with bout intervals of <6 weeks [40]. Therefore, a parallel-group design might be more appropriate for such studies. Additionally, participants' recent involvement in vigorous eccentric exercise should be assessed during screening for inclusion in studies with muscle damage.

Beyond experimental design, these findings may also have practical implications for sports training and competition. A single bout of unfamiliar or high-intensity eccentric exercise may transiently protect the knee extensors against subsequent muscle damage, which is relevant to activities such as running, jumping, landing, deceleration, and change of direction. Thus, eccentric exercise may be strategically included during preparatory phases, return-to-play progression, or before periods of highly competitive demand. However, because this protection was no longer meaningfully detectable at the assessed post-exercise time points by 12 weeks, periodic eccentric stimuli may be needed to maintain the adaptation while avoiding excessive muscle damage close to important competitions. These interpretations should be made cautiously, as the present study used a controlled maximal knee-extensor protocol in recreationally active young men rather than sport-specific training or competition tasks.

Several limitations should be considered when interpreting the present findings. Specifically, (i) the sample consisted only of young, recreationally active men; (ii) a unilateral maximal eccentric knee-extensor model was used, so the findings may not apply to other muscle groups or eccentric exercise models; (iii) participants' habitual physical activity between bouts was not objectively monitored, which may have increased individual variability, particularly at longer inter-bout intervals; (iv) dietary intake was not standardized or systematically recorded during the inter-bout period; (v) beyond the inclusion criteria, participants' training history and physical fitness levels were not systematically quantified; (vi) muscle damage outcomes were assessed only on days 2 and 4 after each eccentric exercise bout, which cannot exclude the possibility that a repeated-bout effect was present during the immediate post-exercise or day-1 periods, or at other unassessed post-exercise time points; and (vii) for the equivalence tests, the outcome-specific margins were selected post hoc, and these analyses should be considered exploratory rather than confirmatory. Moreover, conclusions regarding equivalence are conditional on the selected margins and, as a result, smaller effects falling within these margins cannot be ruled out.

In summary, after a single bout of maximal eccentric exercise of the knee extensors, the repeated-bout effect remained statistically detectable for selected outcomes at intervals of up to 9 weeks. At 12 weeks, equivalence analyses indicated that no meaningful repeated-bout effect was detectable at the assessed days 2 and 4 for the functional, perceptual, and imaging outcomes, whereas the evidence for CK and hs-CRP remained inconclusive.

5 | Perspective

These findings have practical implications for both research design and eccentric training. Short inter-bout intervals are appropriate when the repeated-bout effect itself is the primary outcome, whereas studies evaluating nutritional, pharmacological, or recovery interventions should incorporate longer washout periods to minimize residual protection from prior eccentric exercise. In the present knee-extensor model, the exploratory equivalence analyses suggest that a washout period

of approximately 12 weeks may be sufficient to minimize meaningful residual protection at days 2 and 4 post-exercise for functional, perceptual, and imaging outcomes, whereas the time course for biochemical markers remains uncertain. From a training perspective, a single eccentric exposure may provide protection for several weeks, but periodic eccentric loading may be required to maintain this adaptation over longer periods.

Author Contributions

Anastasios A. Theodorou, Vassilis Paschalis, and Michalis G. Nikolaidis conceived and designed the study. Anastasios A. Theodorou, Nikos V. Margaritelis, Anastasia V. Karyofyllidou, Antonios Kyparos and Filippos Christodoulou conducted the experimental procedures and collected the data. Anastasios A. Theodorou and Nikos V. Margaritelis analyzed and interpreted the data. Anastasios A. Theodorou drafted the manuscript in collaboration with Vassilis Paschalis. Michalis G. Nikolaidis, Nikos V. Margaritelis, Antonios Kyparos, Athanasios Z. Jamurtas, Ioannis G. Fatouros, Anastasia V. Karyofyllidou, and Filippos Christodoulou critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Ethics Statement

The study was approved by the Ethics Committee of the National and Kapodistrian University of Athens (Protocol No. 1858/03-10-2025) and was registered at ClinicalTrials.gov (NCT07503860).

Consent

Written informed consent was obtained from all participants before enrollment in the study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Flow diagram of participant recruitment, random allocation to the four inter-bout interval groups (3, 6, 9, and 12 weeks), participant withdrawals, and the final sample included in the statistical analyses. **Table S1:** Total work (kJ) performed during the first and second eccentric exercise bouts across the four inter-bout interval groups ($n = 12$ per group; mean ± SD).