Adaptation of exercise-induced stress in well-trained healthy young men

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New Findings
- **What is the central question of this study?**
  Exercise is known to induce stress-related physiological responses, such as changes in intestinal barrier function. Our aim was to determine the test–retest repeatability of these responses in well-trained individuals.
- **What is the main finding and its importance?**
  Responses to strenuous exercise, as indicated by stress-related markers such as intestinal integrity markers and myokines, showed high test–retest variation. Even in well-trained young men an adapted response is seen after a single repetition after 1 week. This finding has implications for the design of studies aimed at evaluating physiological responses to exercise.

Strenuous exercise induces different stress-related physiological changes, potentially including changes in intestinal barrier function. In the Protégé Study (ISRCTN14236739; www.isrctn.com), we determined the test–retest repeatability in responses to exercise in well-trained individuals. Eleven well-trained men (27 ± 4 years old) completed an exercise protocol that consisted of intensive cycling intervals, followed by an overnight fast and an additional 90 min cycling phase at 50% of maximal workload the next morning. The day before (rest), and immediately after the exercise protocol (exercise) a lactulose and rhamnose solution was ingested. Markers of energy metabolism, lactulose-to-rhamnose ratio, several cytokines and potential stress-related markers were measured at rest and during exercise. In addition, untargeted urine metabolite profiles were obtained. The complete procedure (Test) was repeated 1 week later (Retest) to assess repeatability. Metabolic effect parameters with regard to energy metabolism and urine metabolomics were similar for both the Test and Retest period, underlining comparable exercise load. Following exercise, intestinal permeability (1 h plasma lactulose-to-rhamnose ratio) and the serum interleukin-6, interleukin-10, fibroblast growth

factor-21 and muscle creatine kinase concentrations were significantly increased compared with rest only during the first test and not when the test was repeated. Responses to strenuous exercise in well-trained young men, as indicated by intestinal markers and myokines, show adaptation in Test–Retest outcome. This might be attributable to a carry-over effect of the defense mechanisms triggered during the Test. This finding has implications for the design of studies aimed at evaluating physiological responses to exercise.

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Introduction

The importance of a properly functioning intestinal barrier, serving as a gatekeeper that allows the absorption of nutrients and provides a line of defense against a variety of micro-organisms and harmful molecules, is generally supported. Nevertheless, our understanding of the nature, regulatory mechanisms involved and consequences of what is generally referred to as 'increased intestinal permeability' is far from complete (Bischoff *et al.* 2014; Quigley, 2016). As a consequence, there is a demand for methodology to measure and understand intestinal functioning in more detail. Exercise-induced changes in gut barrier function have been studied in several experimental settings (Øktedalen *et al.* 1992; Ryan *et al.* 1996; Pals *et al.* 1997; van Nieuwenhoven *et al.* 1999*a*; Marchbank *et al.* 2011; Van Wijck *et al.* 2011*a*). Some of these studies have found an increase in intestinal permeability following exercise, whereas others have not. The same holds true for other exercise-induced parameters, such as the novel fibroblast growth factor-21 (FGF21). This factor has been shown to increase (Kim *et al.* 2013) or remain unchanged (Cuevas-Ramos *et al.* 2012; Catoire & Kersten, 2015) following acute exercise. The same ambiguity is found for the effect of exercise training on resting levels of FGF21 (Yang *et al.* 2011; Cuevas-Ramos *et al.* 2012). Inconsistencies in finding significant outcomes for these parameters are at least partly attributable to large variations in study set-up, such as participant characteristics, type and number of exercise sessions, and exercise intensity. This makes it difficult to draw final conclusions on the effects of exercise. The differences in outcome suggest a requirement for more in-depth research on the relationship between subjects, stressors and repeated bouts of exercise. Exercise performance is known to be trainable (Bassett & Howley, 2000; Hubal *et al.* 2005), but it is not entirely clear whether metabolic stress or tissue damage responses, such as increased intestinal permeability, are reproducible with repeated bouts of exercise in well-trained individuals who are used to strenuous exercise.

Insight into the test–retest variation of stress responses that are elicited by exercise may not only help to explain inconsistencies observed between different study results, but is also relevant to optimize exercise strategies for athletes and individuals with gastrointestinal disorders.

In the present study, we examined the test–retest repeatability of well-trained individuals by exposing them to a strenuous exercise protocol for two consecutive weeks and studying markers of metabolic and cellular stresses and some immunological parameters. Hence, we applied a strenuous exercise protocol that comprised cycling in glycogen-depleted conditions, which has previously been shown to result in a significant immune response in trained individuals (Carol *et al.* 2011).

Methods

Ethical approval

This study was approved by the medical ethical committee of Wageningen University (METC-WU; ISRCTN14236739) and conducted in accordance with the *Declaration of Helsinki* (revised version, October 2008, Seoul). All subjects gave their written informed consent.

Subjects

Twelve well-trained healthy male cyclists were recruited via posters at the university and local sport centres and via social media shortly after the end of the cycling competition season. Subjects were included if they had at least 2 years of competitive cycling experience, trained at least two times a week during the competition season and had no known records of milk allergy, immune diseases or intestinal diseases. Exclusion criteria were smoking, use of drugs such as heroin, cocaine and ecstacy, use of non-steroidal anti-inflammatory drugs on a chronic basis, or use of any medication for gastric or intestinal complaints. None of the subjects donated blood during the last 6 weeks before the start of the study. Subjects had to fill out a training diary, which validated that they only performed low-intensity (~50% maximal aerobic capacity) endurance training sessions during the study

Table 1. Subject characteristics

Identity	Age (years)	Body mass index (kg m^{-2})	Fat mass (% of total mass)	Maximal workload (W)	Maximal aerobic capacity (ml kg^{-1} min^{-1})	Training volume (h week^{-1}) Cycling	Other
1	27	21.1	6.5	400	62.2	4	1
2	31	24.9	26.7	370	47.8	6	3
3	29	23.7	21.0	400	64.3	3	2
4	33	21.0	14.0	380	66.8	6	3
5	24	21.9	12.7	430	62.4	9	—
6	22	22.6	15.2	430	65.5	12	—
7	22	21.0	13.4	400	70.2	8	1.5
8	25	20.7	5.1	380	67.0	6	6
9	29	22.3	11.4	430	58.1	9	3
10	26	21.4	10.6	400	64.0	9	1.5
11	26	24.5	13.1	450	59.6	6	6
Mean	27	22.2	13.6	406	62.5	7.1	3.0
SD	4	1.5	6.1	25	6.0	2.6	1.9

Other endurance training consisted of running and swimming, among other training. Twelve subjects were recruited, but only 11 of those subjects were used for further analysis. One subject was excluded for non-compliance with the dietary guidelines.

period, but no intense physical activity, such as competing in a race or performing a vigorous training session, for 3 days prior to the test days and between the two testing periods. They were also instructed not to use alcohol for 4 days prior to and during the test days and not to use cannabis products, such as hashish and marijuana, for 2 weeks prior to and during the test days. None of the subjects used non-steroidal anti-inflammatory drugs for 2 days prior to and during both testing periods. Data on baseline characteristics and the training volume of each of the subjects are shown in Table 1.

Pre-study assessment

Pre-study testing was performed 1 week prior to the start of the experimental period and consisted of anthropometric measurements, including a dual-energy X-ray absorptiometry scan and a maximal aerobic capacity test. The maximal aerobic capacity test was performed using an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). After a short warm-up, the subjects started cycling at 100 W, and every minute a 20 W increase was applied. The subjects had to cycle until they were no longer able to maintain the workload (W_{max}). Oxygen consumption and CO_2 production were measured with an Oxycon Pro (Jaeger, Hoechberg, Germany) to define the maximal aerobic capacity.

Study design

This intervention study consisted of a repeated single arm, before-and-after intervention design, with each subject acting as their own control. The complete experimental protocol (Test) consisted of three phases: (i) rest; (ii)

exercise; and (iii) 24 h postexercise (Fig. 1). The subjects arrived fasted on the first day (Rest conditions), and a blood sample was taken (time $t = 0$). They ingested a non-digestible multisugar test solution (see 'Sugar intestinal permeability test'), shortly followed by a protein shake containing 40 g of casein protein (kind gift of Dutch Protein & Services BV, Tiel, The Netherlands) dissolved in 400 ml of tap water. Blood was sampled after 1 h, and subjects received lunch. The same testing was done after a dual exercise protocol described by Carol *et al.* (2011). In short, the subjects came back to the university in the evening of the first day, 2 h after having their dinner at home. They were asked to stick to their usual eating habits. They completed a glycogen depletion exercise consisting of 2 min blocks of cycling, alternating between 90 and 50% W_{max} at a pedal frequency of 90–100 r.p.m. When the subjects were no longer able to cycle at 90% W_{max} (<70 r.p.m), the intensity was reduced to 80% W_{max} (still alternating with 50% W_{max}) and, subsequently, 70% W_{max}. When alternating 70 and 50% W_{max} could not be maintained (<70 r.p.m), the exercise was stopped. The subjects then received one of two small high-fat snacks (snack 1, 164 kCal, with 53% energy as fat and 26% energy as carbohydrate, chosen by four participants; or snack 2, 260 kCal, with 61% energy as fat and 32% energy as carbohydrate, chosen by eight participants) to reduce feelings of hunger at night. The next day, the subjects came in fasted and received the same high-fat snack as before, after which they had to cycle for 90 min at 50% W_{max}. Directly after completing the second part of the exercise protocol, a blood sample (time $t = 0$) was taken. Next, the non-digestible multisugar test solution was ingested, shortly followed by the protein shake. Blood was sampled after 1 h, and the subjects received lunch. Finally,

subjects came back the next morning (24 h postexercise) to donate a single fasted blood sample before receiving breakfast. This 3 day experimental protocol was repeated 1 week later (Retest) to evaluate the test–retest variation in the metabolic and stress/damage responses provoked by the dual exercise protocol. From now on, these two intervention periods are referred to as Test and Retest. Subjects were not allowed to switch between the two types of high-fat snack between the Test and Retest.

Blood and urine sampling

During both rest and exercise, blood was sampled from the antecubital vein before (at $t = 0$) and 1 h after the intake of the sugar test solution and the casein protein shake. At 24 h postexercise, only a fasted blood sample was drawn. Blood was sampled in tubes (Vacutainer; Becton Dickinson, Breda, The Netherlands) containing either EDTA or NaF, which were directly centrifuged at $2000g$ for 10 min at 4°C, aliquoted and stored at −80°C within 20 min after sampling until further analysis. Blood was also sampled in serum separator tubes. It was left to clot in the dark for at least 30 min at room temperature, after which the tubes were centrifuged at $2000g$ for 10 min at room temperature to obtain serum, which was directly aliquoted and stored at −80°C until further analysis. During both rest and exercise, urine samples were collected before the intake of the sugar test solution and the casein protein shake, and supplemented with chlorhexidine to prevent bacterial growth. Total sample volumes were recorded, and aliquots were stored at −20°C until further analysis.

Markers of workload

Several markers were analysed to characterize the effect of the exercise protocol on energy metabolism. Analyses of plasma concentrations of glucose and lactate were performed by a medical laboratory (Hospital Gelderse Vallei, Ede, The Netherlands). Serum free fatty acid (FFA) concentrations were measured with a commercial assay (HR series NEFA-HR(2); Wako Chemicals GmbH, Neuss, Germany) according to the instructions provided by the manufacturer, with minor adaptations. In short, 5 μl serum was incubated with 200 μl of reagent R1 and subsequently with 100 μl of reagent R2 for 10 min at 37°C. Absorption values were measured at 546 nM, and concentrations were calculated from a standard curve. Serum β-hydroxybutyrate (BHB) concentrations, as an indicator of ketone body formation, were also measured with a commercial assay (Ab83390; Abcam, Cambridge, UK) according to the manufacturer's instructions.

Energetic responses to the exercise and casein protein bolus intake were analysed with indirect calorimetry. Before and at several time points after intake of the non-digestible sugar test solution and protein shake, during rest and exercise, O_2 consumption and CO_2 production were monitored every min for 10 min with the DeltaTrac II Metabolic Monitor (Datex Ohmeda, Helsinki, Finland). Before measurements were taken, subjects rested in a supine position for 5–10 min. The Deltatrac ventilated hood system consists of a flowmeter, a paramagnetic oxygen sensor and an infrared sensor for carbon dioxide measurements. This system was used in canopy mode with
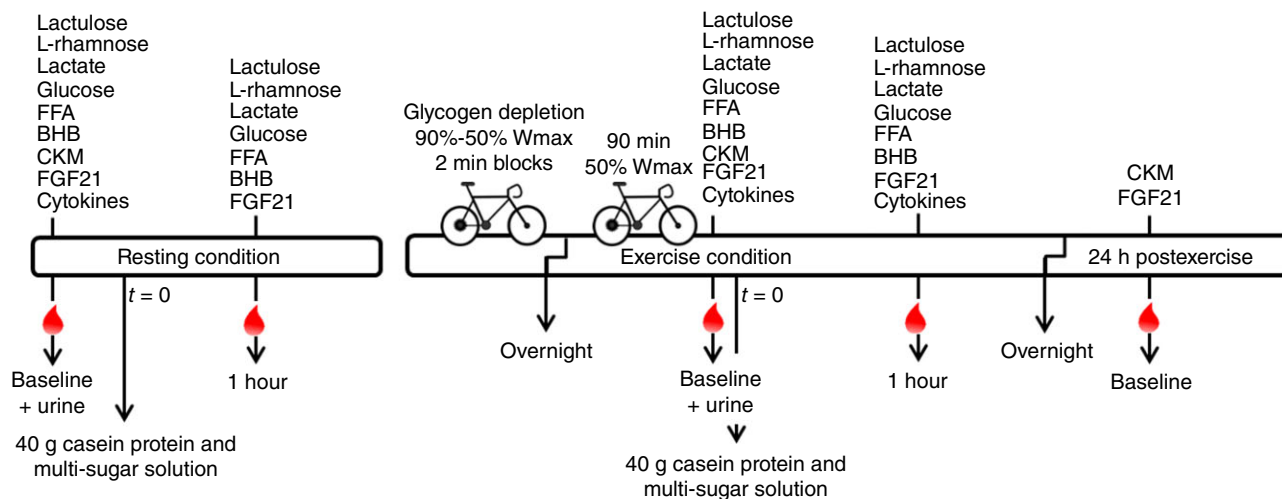

Figure 1. Schematic overview of blood sampling and analyses
This scheme shows the dual exercise protocol and blood sampling during rest, exercise and 24 h postexercise (Test). This complete experimental protocol was repeated in exactly the same way 1 week later (Retest). The blood analyses performed at the different sampling time points are listed. Abbreviations: BHB, β-hydroxybutyrate; CKM, creatine kinase, muscle-specific; FFA, free fatty acids; FGF21, fibroblast growth factor 21; and W_{max}, maximal workload.

a flow rate of 40 l min^{-1} and was calibrated daily using a mixture of oxygen and carbon dioxide (QuickCal^{TM}; Datex-Ohmeda). For practical reasons, only eight of the 12 subjects were measured and analysed for their respiratory exchange ratio (RER) and energy expenditure.

Untargeted urine metabolomics

Untargeted urine metabolomics was performed on samples collected before the intake of the multisugar test solution and protein shake at rest and following exercise. Urine samples were diluted with acetonitrile (1:1 ratio) and centrifuged. Hydrophilic interaction liquid chromatography-high resolution mass spectrometry (HILIC-HRMS) experiments were performed on a QExactive system (Thermo Electron Corporation, Waltham, MA, USA) consisting of a Dionex 3000 UltiMate RS autosampler, Dionex 3000 UltiMate UHPLC pump and a QExactive mass detector. The system is controlled

using Thermo Fisher Scientific Xcalibur^{TM} version 2.2 SP1.48 software. High resolution full MS detection was carried out using heated electrospray ionization (HESI) in both the positive and negative ionization modes using a scan range from m/z 100–1000 with the resolution set at 17,500. Prior to analysis of the study samples, the HRMS system was successfully calibrated in the positive and negative ionization mode to obtain high mass accuracy. Liquid chromatography–mass spectrometry (LC-MS) profiling was performed using hydrophilic interaction liquid chromatography and a 13.5 min gradient from 10 mM ammonium formate in 95% acetonitrile to 10 mM ammonium formate in 50% acetonitrile. Peak detection and matching across LC-MS profiles were performed using the R package XCMS version 1.30.3, based on the centWave method (Tautenhahn *et al.* 2008). The resulting peak intensity table was processed using MetaboAnalyst 3.0 (Xia *et al.* 2015). The number of features was reduced based on the interquartile range, and normalization to constant sum
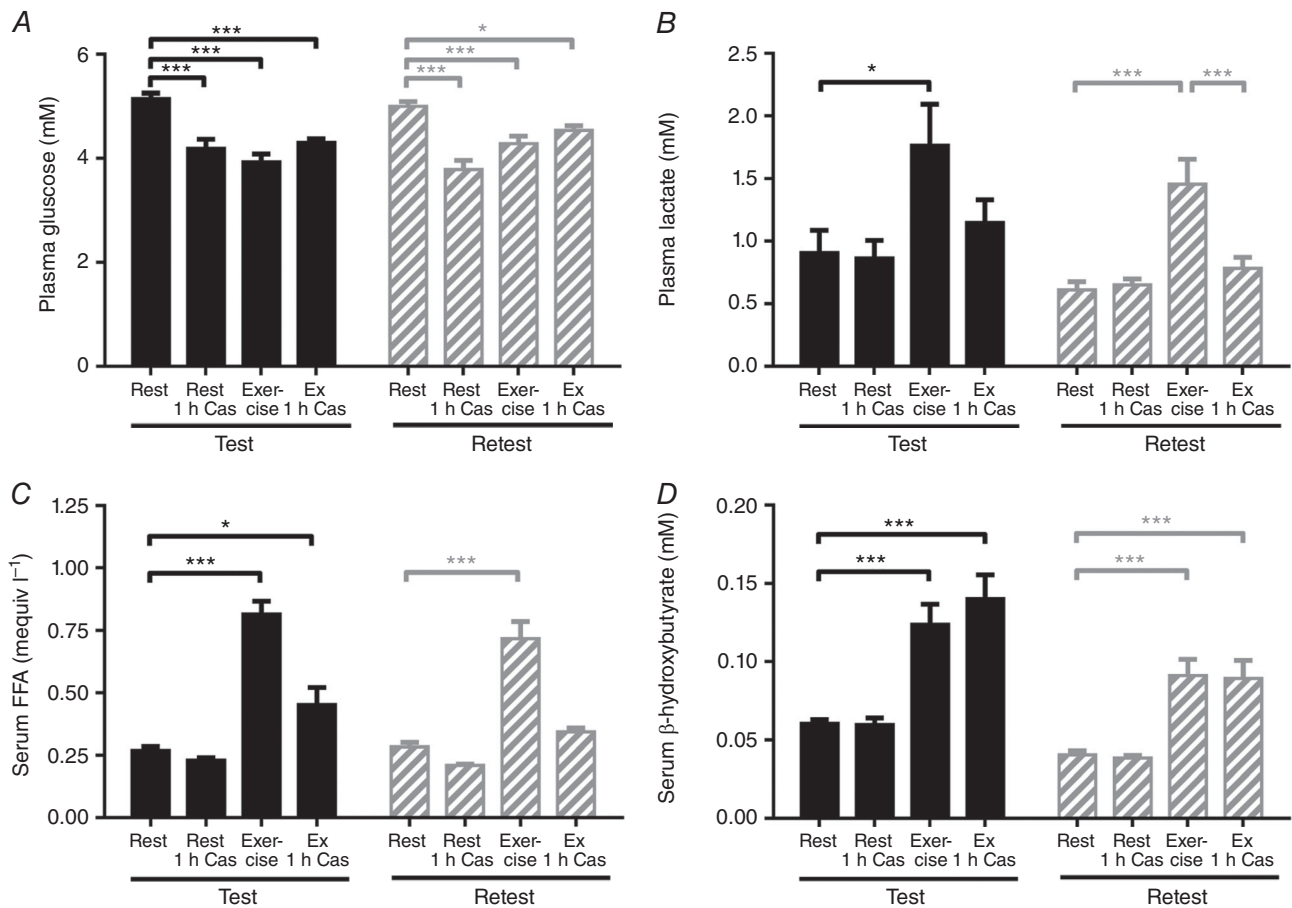

Figure 2. The effect of strenuous exercise on markers of energy metabolism
Plasma glucose (*A*), plasma lactate (*B*), serum free fatty acids (FFA; *C*) and serum β-hydroxybutyrate (BHB; *D*) are shown as mean values + SEM. Comparisons are made with the rest values during the Test (black bars, $n = 10$ for FFA and BHB) and Retest (grey hatched bars, $n = 11$) of the experimental protocol. Abbreviation: Cas, casein protein intake. *$P < 0.05$ and ***$P < 0.001$.

Table 2. Spearman correlation coefficients to Test–Retest correlation

Parameter	Rest and exercise values		Exercise values	
	Spearman's r correlation coefficient	*P*-Value	Spearman's r correlation coefficient	*P*-Value
Glucose	0.580	<0.0001	0.447	0.04
Lactate	0.584	<0.0001	0.809	<0.0001
Free fatty acids	0.811	<0.0001	0.726	0.0002
β-Hydroxybutyrate	0.779	<0.0001	0.583	0.006
Creatine kinase (muscle)	0.074	0.68	−0.332	0.13
Lactulose-to-rhamnose ratio	0.150	0.53	−0.149	0.68
Fibroblast growth factor-21	−0.069	0.67	0.096	0.68
Fractalkine (CX$_3$CL1)	0.596	0.0003	0.444	0.04
Interleukin-6	0.781	<0.0001	0.816	<0.0001
Interleukin-10	0.584	0.0004	0.422	0.05
Interferon-γ inducible protein-10	0.239	0.180	0.215	0.34

Spearman's r correlation coefficient was calculated because values were not normally distributed.

and auto scaling were applied. The resulting data table was used for principal component analysis.

Muscle-related parameters

Muscle-specific creatine kinase (CKM) released in response to exercise was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Ab185988; Abcam). Serum samples were diluted 200–8000 times with sample diluent in order to be able to interpolate the values from the standard curve.

Concentrations of FGF21 in serum and plasma in response to exercise were measured with a commercial sandwich ELISA assay kit (DF2100; R&D Systems, Wiesbaden, Germany) according to instructions provided by the manufacturer.

Sugar intestinal permeability test

The sugar solution for studying small intestinal permeability contained the following three inert, non-digestible sugar probes: 5 g of lactulose, 1 g of L-rhamnose and 0.5 g of D-xylose (BCM Specials, Nottingham, UK). These were dissolved in 100 ml of tap water. Concentrations of lactulose and L-rhamnose were measured to determine intestinal permeability (Van Nieuwenhoven *et al.* 1999*b*; Haase *et al.* 2000) in EDTA plasma sampled at $t = 0$ and 1 h after the intake of the sugar test solution and the protein shake during rest and exercise. To remove plasma proteins, EDTA plasma (125 μl) was transferred to Eppendorf tubes containing a 3000 Da cut-off filter (Amicon Ultra 0.5 ml 3 K; Millipore, Cork, Ireland). The tubes were centrifuged for 30 min at 11,000*g* and 4°C, and the clear plasma filtrate was inserted in the cooled sample processor (233 XL; Gilson, Middleton, WI, USA). Sugars were subsequently analysed by LC-MS

as described elsewhere (van Wijck *et al.* 2011*b*). Plasma lactulose-to-rhamnose (L/R) ratios were calculated from the 1 h plasma concentrations and corrected for the concentrations found at $t = 0$.

Immune markers

Plasma concentrations of fractalkine (also known as CX$_3$CL1), in response to exercise, were measured with a commercial sandwich ELISA assay kit (DCX310; R&D Systems) according to instructions provided by the manufacturer.

Immune inflammation-related proteins [interleukin (IL)-1α, IL-1β, IL-1 receptor antagonist (RA), IL-2, IL-5, IL-6, IL-10, IL-12, IL-13, IL-25, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IFN-γ- induced protein 10 (IP-10), tumor necrosis factor superfamily member 14 (LIGHT), thymic stromal lymphopoietin (TSLP) and granulocyte-colony stimulating factor (G-CSF)] were determined in serum samples by an in-house developed multiplex technology (xMAP; Luminex, Austin, TX, USA), according to the Luminex instructions, as previously described (de Jager *et al.* 2005). Non-specific heterophilic binding of immunoglobulins was prevented with HeteroBlock (Omega Biologicals, Bozeman, MT, USA), and no cross-reactivity was observed. Acquisition was performed with a Bio-Rad FlexMAP3D in combination with xPONENT software version 4.1 (Luminex). Data analysis was performed with Bioplex Manager 6.1.1 (Bio-Rad, Veenendaal, The Netherlands). For each of the cytokines, a standard curve was taken along on each of the sample plates. When sample levels measured were lower than the lower limit of quantification (LLOQ), the values were set at 10% of this cut-off value to enable statistical analyses.

Statistics

Linear mixed model analysis with Bonferroni multiple comparison tests were used to assess changes in the concentrations of all compounds analysed, except plasma L/R ratios (IBM SPSS Statistics 22, IBM Corporation, Armonk, NY, USA). Per protocol analyses were performed. Student's paired *t* tests were used to assess changes in plasma L/R ratios between the rest and exercise conditions (IBM SPSS Statistics 22). Two-way ANOVA with Bonferroni *post hoc* testing was used to assess changes in RER and energy expenditure over time and between the rest and exercise conditions (GraphPad Prism 6, GraphPad Software Inc., San Diego, CA, USA).

Student's paired *t* test with a statistical significance of one-tailed $P < 0.05$ was used to test whether the effect size of CKM, FGF21 and L/R ratio was decreased during the Retest compared with the Test (GraphPad Prism 6). In addition, Spearman's *r* correlation coefficients were calculated to determine the Test–Retest repeatability of the responses provoked by the dual exercise intervention. One subject was excluded because of non-compliance with the instructions with regard to dietary guidelines. Some individuals were excluded from analysis when there were missing blood samples. Therefore, the number of subjects included in each of the analyses is 11, unless stated otherwise in the figure legends. Statistical significance was defined as a two-tailed $P < 0.05$.
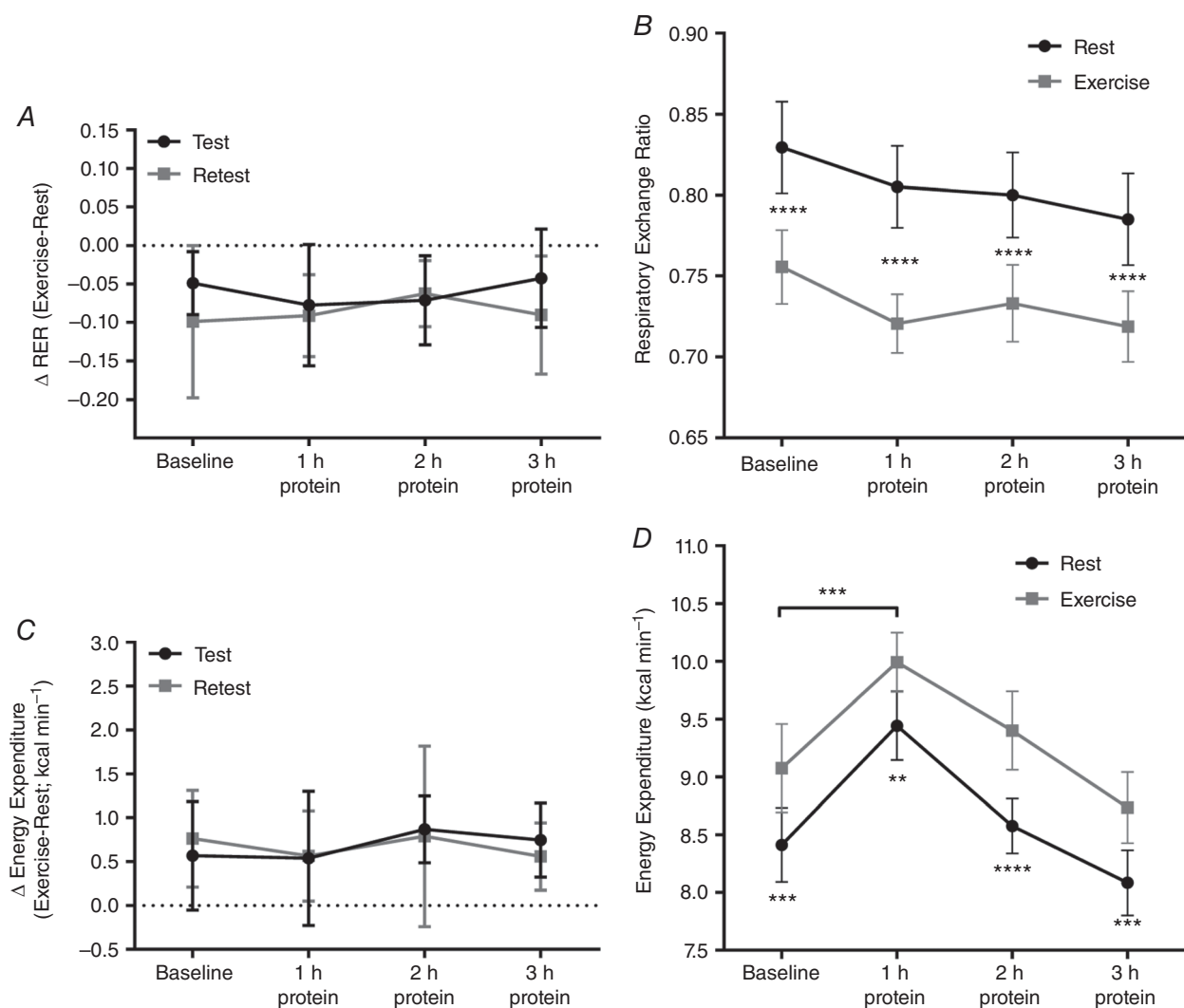

Figure 3. The effect of strenuous exercise and casein bolus intake on energy expenditure
The respiratory exchange ratio (RER; *A*) and energy expenditure (*C*) are shown as the difference (Δ) between exercise and rest values ± SD ($n = 8$) at time $t = 0$ and 1, 2 and 3 h after intake of the sugar test solution and protein shake. To compare rest with exercise, RER values (*B*) and energy expenditure levels (*D*) of Test and Retest are taken together ($n = 16$). **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

Results

Markers of workload

The impact of the exercise protocol on general metabolic markers, including glucose and lactate, was similar for both Test and Retest (see Fig. 2). This is supported by the similar workload during the glycogen depletion phase and endurance cycling phase of the intervention protocol between Test and Retest ($P > 0.05$, data not shown). Plasma glucose concentrations were reduced directly after exercise, which was still the case 1 h later. For both Test and Retest, plasma lactate concentrations increased slightly, but significantly, following exercise. These levels returned to resting values within 1 h. Also, serum FFA and BHB concentrations showed similar patterns during rest and exercise, with a significant increase in concentrations following exercise. In contrast to BHB, serum FFA concentrations declined 1 h following exercise, although they were still slightly higher than the rest levels during the Test period ($P = 0.03$). Each of the metabolic markers showed a significant correlation between the Test and Retest outcomes (Table 2).

There was no significant difference in RER between Test and Retest (Fig. 3*A*). Indirect calorimetry indicated a decrease in the RER after exercise, implying that the metabolism had shifted towards lipid oxidation, rather than carbohydrate oxidation, after completing the dual exercise protocol (Fig. 3*B*). There was also no significant difference between Test and Retest in energy expenditure (Fig. 3*C*). Energy expenditure patterns showed a significant increase in energy expenditure following exercise (Fig. 3*D*). Intake of casein induced a diet-induced thermogenic effect, as shown by a significant increase ($P < 0.001$) in the energy expenditure.

Urine metabolomics

Principal component analysis of the urine metabolic profiles revealed a clear separation between the urine metabolite profiles in the rest and exercise conditions (Fig. 4; light and dark blue *versus* red and green). These shifts were similar for both Test and Retest, and there was no clear difference in metabolite profiles between Test and Retest.
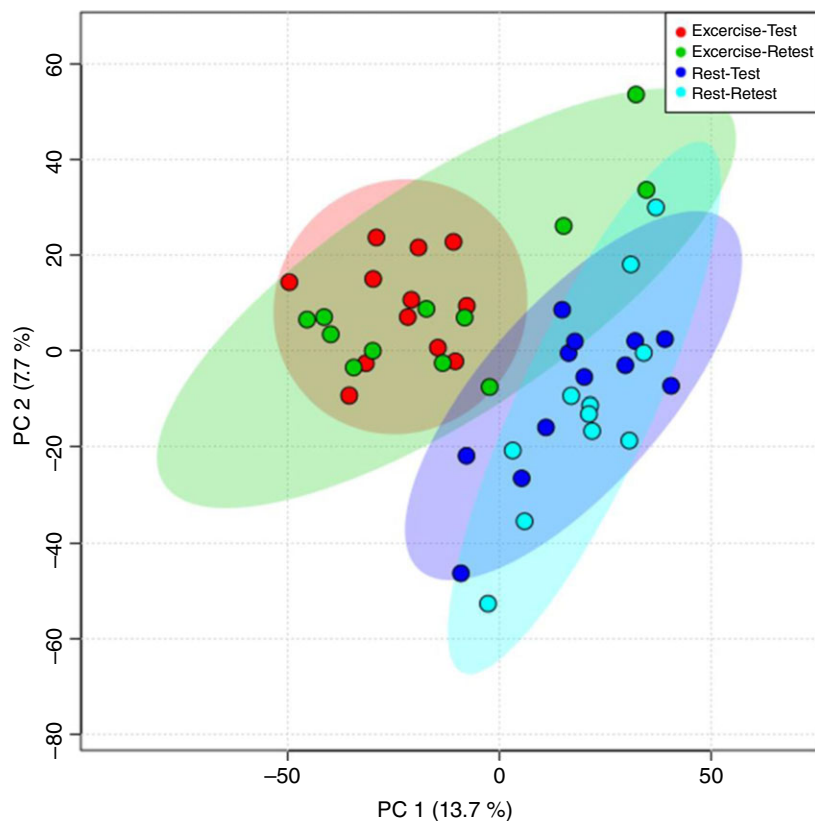

Figure 4. Principal component (PC) analysis plot of untargeted urine metabolomics
The principal component analysis of urine metabolomics is shown, comparing the rest and exercise conditions ($n = 11$ for each condition) for both Test and Retest.
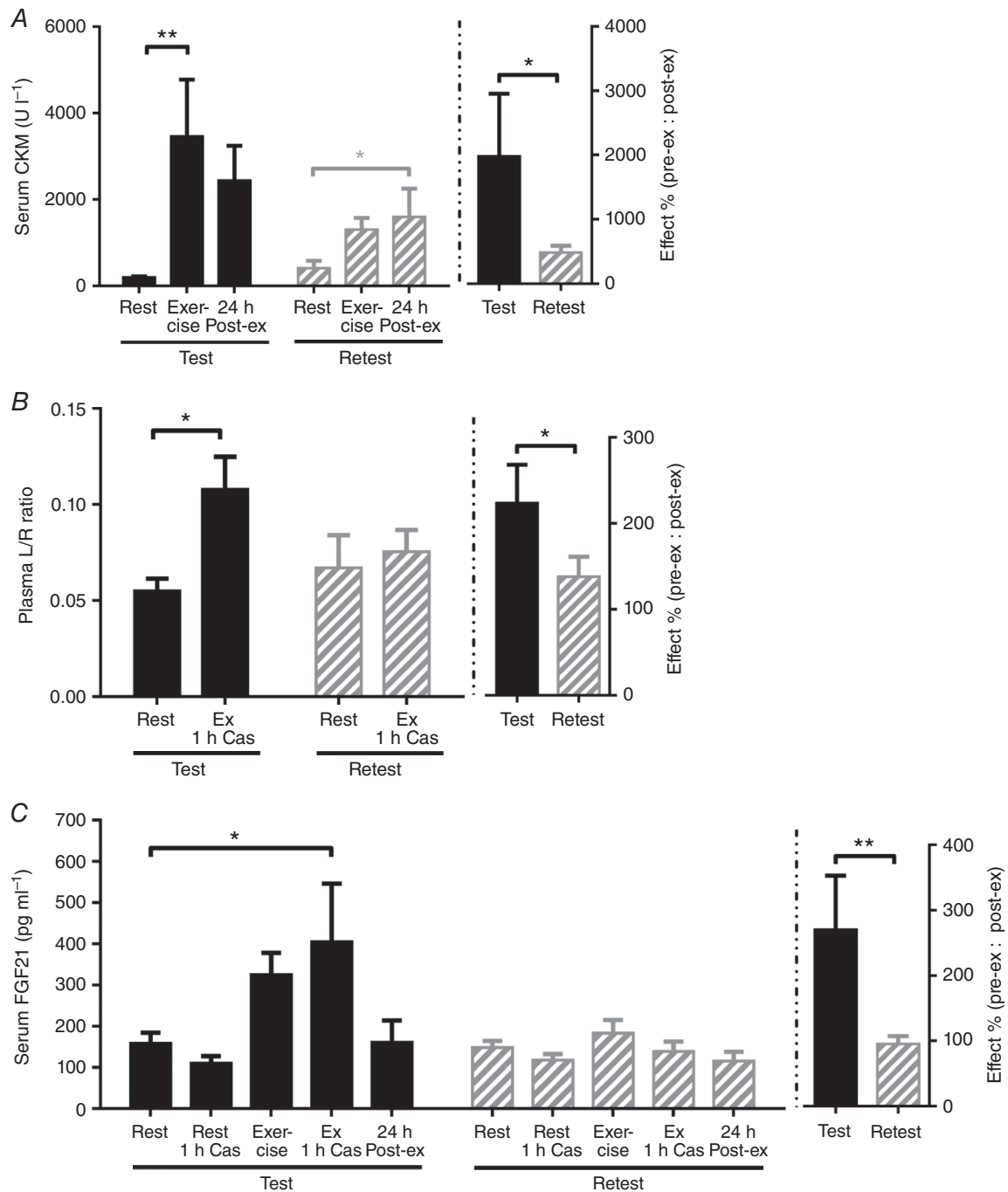
Figure 5. The effect of strenuous exercise on intestinal permeability and muscle-stress markers
Levels of serum muscle-specific creatine kinase (CKM; *A*), plasma lactulose-to-rhamnose (L/R) ratio (*B*) and serum fibroblast growth factor 21 (FGF21; *C*) are shown as mean values + SEM. Comparisons are made with the rest values during execution of the Test (black bars, $n = 10$) and Retest protocols (grey hatched bars, $n = 11$). The effect percentage is calculated for each individual. For serum CKM, the rest value is compared with the value postexercise (postex); for the plasma L/R ratio and the serum FGF21 concentrations, the rest value is compared with the value 1 h after exercise. Abbreviation: Cas, casein protein intake. $*P < 0.05$ and $**P < 0.01$.

Table 3. Exercise-induced cytokine responses during two protocol executions

Parameter	LLOQ (pg ml⁻¹)	Test						Retest					
		Rest		Exercise		Exercise 1 h Casein		Rest		Exercise		Exercise 1 h Casein	
		Average (pg ml⁻¹)	SD	Average (pg ml⁻¹)	SD	Average (pg ml⁻¹)	SD	Average (pg ml⁻¹)	SD	Average (pg ml⁻¹)	SD	Average (pg ml⁻¹)	SD
Fractalkine (CX₃CL1)	156.25	545.8	64.25	675.9*	133.3	812.1*	158.1	593.6	119.2	737.9*	147.9	799.6	225.7
IL-6	2.29	3.65	3.40	9.03*	6.47	8.40	7.94	6.93	10.85	5.68	4.40	5.16	4.10
IL-10	2.42	1.34	0.87	15.73	10.50	29.85*	38.15	2.12	1.49	4.96	3.30	5.78	5.08
IP-10 (CXCL10)	1.00	260.45	70.18	190.90*	37.97	190.52*	35.07	399.10	335.15	304.70	235.60	301.43	253.52
IL-1RA	29.34	92.32	250.77	126.67	235.08	498.12	884.95	101.02	212.96	106.64	233.29	119.28	211.07
IL-1α	1.47	11.14	25.27	9.47	21.29	11.74	22.71	10.46	20.30	10.64	21.64	9.00	18.38
IL-1β	1.14	0.49	1.15	0.54	0.97	0.56	1.01	0.38	0.66	0.40	0.71	0.29	0.63
IL-2	2.34	0.85	1.12	1.15	1.19	1.57	1.93	0.88	1.20	1.01	1.27	1.07	1.16
IL-5	1.64	25.62	60.37	26.08	55.63	28.51	57.62	24.22	46.90	24.27	50.64	21.51	45.92
IL-12	5.97	2.95	6.52	3.20	5.15	3.46	5.11	2.99	4.03	2.85	4.61	1.82	2.49
IL-13	1.23	74.26	172.12	72.35	152.13	81.31	159.81	67.32	133.39	72.27	143.83	65.24	132.15
IL-25	80.45	599.79	1028.86	565.05	894.23	678.97	1010.75	637.75	903.71	635.01	995.07	572.03	842.85
TNF-α	1.21	3.92	7.89	3.50	7.54	3.45	7.32	3.74	6.95	3.48	7.20	3.15	6.35
IFN-γ	3.11	17.13	39.38	18.35	37.86	18.99	37.99	17.22	34.42	17.03	34.62	15.38	31.27
LIGHT	30.96	120.16	212.39	117.01	202.54	140.12	215.02	124.46	208.57	116.83	208.11	108.52	186.24
TSLP	0.30	7.78	18.25	6.38	15.68	8.36	17.01	6.87	13.83	7.28	14.65	6.69	13.85
G-CSF	9.48	173.46	342.66	178.48	324.65	179.75	329.25	173.16	311.45	175.60	316.75	154.63	271.08

Comparisons are made with the values found in the rest condition (rest). *$P < 0.05$. The lower limit of quantification (LLOQ) was defined as the lowest measured level in the current assay. Abbreviations: IL, interleukin; IP-10, IFN-γ-induced protein 10; RA, receptor antagonist; TNF-α, tumor necrosis factor alpha; IFN-γ, interferron gamma; LIGHT, tumor necrosis factor superfamily member 14; TSLP, thymic stromal lymphopoietin; G-CSF, granulocyte-colony stimulating factor.

Exercise-induced stress-related markers

In contrast to the metabolic markers, parameters aimed at measuring exercise-induced stress or damage showed different patterns for Test and Retest. To assess exercise-induced effects on intestinal permeability, plasma concentrations of lactulose and L-rhamnose were determined and calculated as the L/R ratios. Figure 5 shows that during the Test period the plasma L/R ratio increased after exercise ($P = 0.03$). During the Retest period, such an increase was not observed. Serum concentrations of CKM increased significantly straight after completion of the exercise protocol during the Test period, whereas during the Retest period it took 24 h for the concentrations to increase significantly from resting levels. The recently discovered metabolic marker FGF21 also showed a significant increase in serum concentrations 1 h after exercise, but during the Test period only.

For each of the parameters of exercise-induced stress or damage, the effect size was found to be significantly larger during the Test period compared with the Retest period. In line with this finding, Test and Retest values were not significantly correlated with each other (Table 2).

Exercise-induced cytokine response

For most of the cytokines that were measured, no significant responses were observed (Table 3). Only four of the measured cytokines showed differences in their concentrations following exercise compared with rest. These cytokines are also shown in Fig. 6. Serum IL-6 and IL-10 concentrations increased after exercise during the Test period only. Serum IP-10 concentrations also only differed during the Test period, with a significant decrease after exercise. Fractalkine was the only exception and showed increased plasma concentrations following exercise compared with rest for both Test and Retest. In
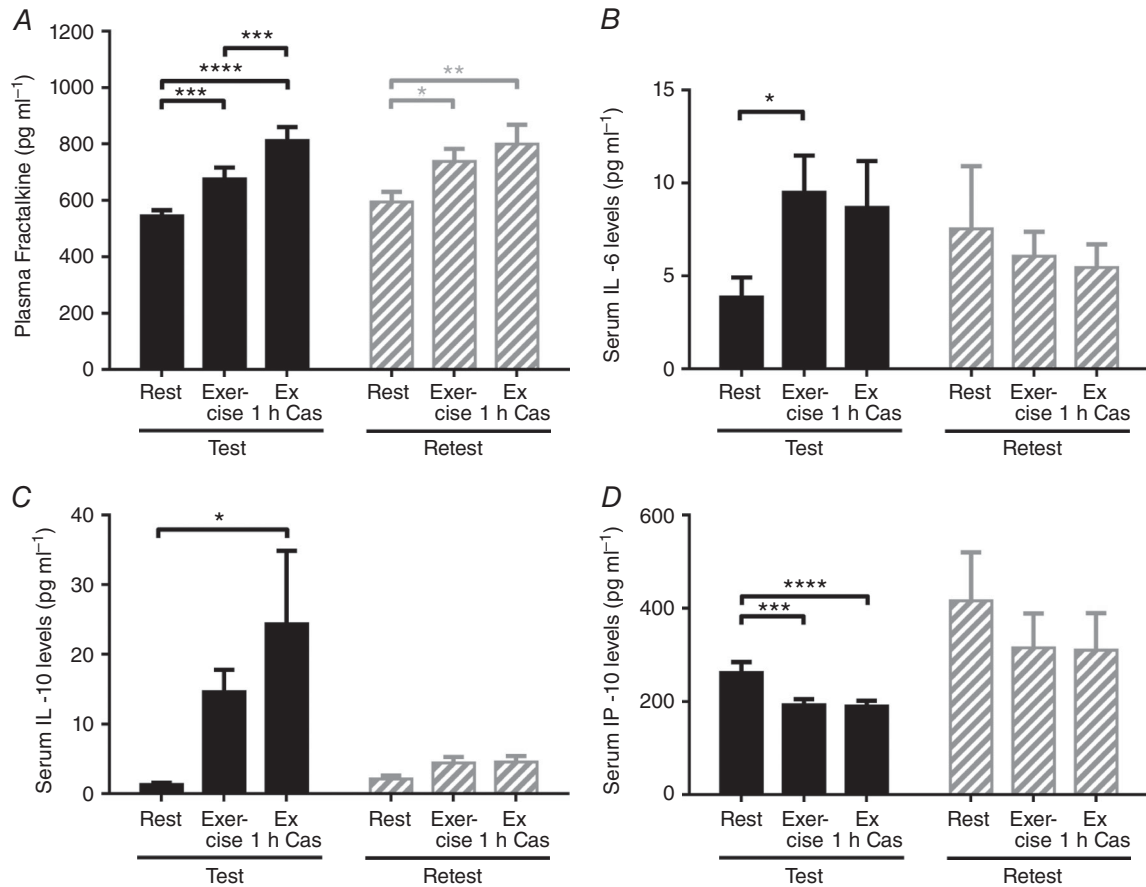

Figure 6. The effect of strenuous exercise on cytokine response
Plasma concentration of fractalkine (*A*), serum concentration of interleukin (IL)-6 (*B*), serum concentration of IL-10 (*C*) and serum IP-10 (*D*) are shown as mean values + SEM. Comparisons are made with the rest values during the Test (black bars, $n = 11$) and Retest (grey hatched bars, $n = 11$). Abbreviation: Cas, casein protein intake; IL, interleukin; IP-10, interferron gamma-induced protein 10. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

contrast to the exercise-induced muscle-related markers, the cytokine responses during Test were significantly correlated with those during the Retest (Table 2).

Discussion

The aim of the present study was to investigate test–retest repeatability of exercise-induced stress responses in well-trained subjects. Therefore, we measured a set of parameters for energy metabolism, markers of metabolic or cellular stress, and immunological factors.

The finding that parameters for general energy metabolism (glucose, lactate, FFA and BHB) and energy expenditure did not differ between the Test and Retest period indicates that the subjects delivered a similar workload. Remarkably, most immune markers were found not to be affected by exercise in these well-trained individuals. The effects of the exercise protocol on systemic concentrations of CKM and FGF21, the L/R ratio and concentration of some of the myokines, however, showed distinct patterns between the Test and Retest periods. The increase in concentrations of CKM, FGF21, IL-6 and IL-10 and the increase in the L/R ratio were diminished or even absent during the Retest period compared with the Test period. These results suggest adaptation when the exercise protocol is repeated, even in these well-trained individuals. The adaptation in intestinal permeability as measured with the L/R ratio suggests that the gut may be, at least to some extent, 'trainable', which could result in a reduction of the often reported gastrointestinal complaints by endurance athletes (de Oliveira *et al.* 2014). A clear distinction was observed between the urine metabolic profiles before and after exercise, but not between the Test and Retest. These results suggest that metabolites reflecting metabolic stress or tissue damage, although expected to be present, do not dominate the differences in metabolic profiles between rest and exercise.

When two bouts of exercise are performed within a short time period (e.g. within 24 h), a carry-over effect in exercise-induced responses is often seen (Ronsen *et al.* 2001; McFarlin *et al.* 2003; Robson-Ansley *et al.* 2009). In the present study, however, the opposite was seen with the responses during the Retest compared with the Test, with the exception of IL-6. One week of washout, as applied in the present study, is quite long. There might be, however, a carry-over-like effect of the defense mechanisms activated during the test period that are still active during the Retest, thereby attenuating the stress response (Shing *et al.* 2007). The considerable interindividual variation found in metabolic stress and tissue damage responses to the exercise protocol, especially during the Test period, may also, at least in part, be explained by this possible carry-over effect.

The response levels of the myokine fractalkine were similar for the Test and Retest periods. This finding

supports earlier results by Catoire & Kersten (2015), who designated fractalkine as an 'exercise factor' which, according to their definition, should not adapt to training. Interleukin-10, in contrast, seems to be responsive to adaptation to repeated exercise and could be considered an adaptive exercise factor. In contrast, the possible carry-over effect of the Test protocol to the Retest protocol on the concentrations of IL-6 suggests that excluding intense physical activity for 3 days prior to a study trial may not be long enough to prevent carry-over effects from prior exercise. It is important to distinguish the effects that are attributable to adaptation from those resulting from carry-over for functional interpretation of outcomes of exercise protocols.

For some exercise-induced stress markers, such as the newly discovered marker FGF21, effects of exercise protocols were inconsistent in earlier studies, leading to disagreement on the relationship between exercise and FGF21 (Yang *et al.* 2011; Cuevas-Ramos *et al.* 2012; Kim *et al.* 2013; Catoire & Kersten, 2015). This can, at least in part, be attributable to the large differences between the exercise protocols, ranging from a single bout of exercise to several weeks of exercise intervention. However, response adaptation, as shown in the present study, could also form part of the basis of these inconsistent findings. It is interesting to speculate on the functional role of a stress response and subsequent adaptation by, for example, FGF21 induction. Strenuous exercise is associated with endoplasmic reticulum stress, which can result in skeletal muscle damage at excessive levels (Ost *et al.* 2016). Secretion of FGF21 from skeletal muscle is induced in response to skeletal muscle endoplasmic reticulum stress leading to the activation of the integrated stress response, labelling FGF21 as a muscle-related stress marker rather than an exercise-induced myokine (Ost *et al.* 2016). A transient elevation in endoplasmic reticulum stress is thought to be necessary for adaptive improvement through mitohormesis (Ost *et al.* 2016). Repeated exercise could result in a higher resistance against skeletal muscle endoplasmic reticulum stress and, subsequently, also in an attenuated rescue signal of FGF21 (Ahn *et al.* 2013).

Conclusions

There seems to be an adaptation to exercise-induced stress or damage responses in well-trained healthy young men. Perhaps this is attributable to a carry-over effect of the defense mechanisms triggered during the Test. This finding may explain, in part, inconsistent outcomes in the literature concerning several factors of exercise-induced stress. It also has implications for the design of protocols to assess exercise-induced responses. More research is required to determine the washout period needed to prevent adaptation and carry-over effects of prior exercises. When exercise interventions

are not repeated within the study design, the duration of exercise restrictions prior to the start of the study may be an especially important factor to take into account.

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Additional information

Competing interests

None declared.

Author contributions

L.M.J., J.K., M.M., R.H.H.P., R.F.W., H.J.W. and K.v.N. were responsible for conception and design of the research.

L.M.J. and S.W.K. performed the experiments. L.M.J., K.L., S.W.K., R.B., L.R., M.C.M.V. and S.N. collected, assembled and analysed data. L.M.J., J.K., K.v.N., R.F.W. and H.J.W. interpreted the results of the experiments. L.M.J. drafted the paper. L.M.J., J.K., R.F.W., H.J.W. and K.v.N. edited the paper. M.M., R.H.H.P., K.L., R.B., S.W.K., L.R., M.C.M.V. and S.N. critically revised the paper. All authors have approved the final version of the paper and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.