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PHYSIOLOGY AND NUTRITION

Non-targeted metabolomics analyses by mass spectrometry to explore metabolic stress after six training weeks in high level swimmers.

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ABSTRACT

The objective was to compare the metabolic responses of high-level national swimmers to threshold or polarised training. 22 swimmers (n = 12 males and 10 females) participated in a 28-week cross-over intervention study consisting of 2 × 6 period weeks of training. Swimmers were assigned randomly to either training group for the first period: polarised (POL) (81% in energetic zone 1: blood lactate [La] b ≤ 2 mmol.L^{-1}; 4% in zone 2: 2 mmol.L^{-1} <[La]b ≤ 4 mmol.L^{-1}; 15% in zone 3: [La]b > 4 mmol.L^{-1}) or threshold (THR) (65%/25%/10%). Before and after each training period, urine samples were collected for non-targeted metabolomics analysis. Mixed model analysis was performed on metabolomics data including fatigue class factors and/or training and/or interaction. Ion intensities of 6-keto-decanoylcarnitine (+31%), pregnanediol-3-glucuronide (+81%), P-cresol sulphate (+18%) were higher in the threshold group ($P < 0.05$) indicating higher glycogenic depletion and inflammation without alteration of the neuroendocrine stress axis. 4-phenylbutanic acid sulphate was 200% higher in less fatigued swimmers ($P < 0.01$) linking the anti-inflammatory activity at the cell membrane level to the subjective perception of fatigue. This research suggests the importance of replenishing glycogen stores and reducing inflammation during high thresholds training loads.

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KEYWORDS
Swimming; metabolomics; periodization

Introduction

Over the past decade, metabolomics has allowed researchers to better quantify the adaptations of biological organs and systems to training induced by acute and chronic exercise. Research using the metabolomics approach has detailed acute metabolic changes induced by intense and prolonged running, football, cycling and swimming exercise (Sakaguchi et al., 2019). The majority of studies has reported increased concentration of lipid-related metabolites following intensive exercise. In the field of diagnosing overreaching, non-targeted metabolic analyses show that soccer players with signs of fatigue after three days of competition (Ra et al., 2014) exhibited reduced carbohydrate metabolism offset by higher mobilisation of fatty acids and amino acids. Among 14 football players undertaking a maximum effort YoYo endurance running test, metabolic analyses performed on post-test saliva samples permits reliable, reproducible and highly correlated measurements to plasma blood concentrations (Agatonovic-Kustrin et al., 2019). Results from this study showed that the worst performances had lower concentrations of lactate and glutamate associated with higher concentrations of tyrosine, inositol, creatine and lysine. This pattern of response is indicative of wider mobilisation of β-oxidation and neoglucogenesis in energy production (Santone et al., 2014).

Over the past 15 years, several studies have shown that world-class endurance athletes (cyclists, runners, rowers, rowers, cross-country skiers, orienteers and triathletes) use a polarised model of training (Neal et al., 2013, Muñoz et al., 2014; Ingham et al., 2008; Sandbakk et al., 2013; Mujika, 2014). With this model, the volume of lower intensity training (defined as <2 mmol.L^{-1} blood lactate concentration) is high accounting for ~75-80% of the total volume, with 15–20% at or above the speed (or power) corresponding to 4 mmol.L^{-1}. Other studies have confirmed the effectiveness of this model, with performance improving by 4–8% and physiological capacity by 5–10% (V O_2max, second lactate threshold) after 6–52 weeks of training (Neal et al., 2013; Munoz et al., 2014; Stöggl & Sperlich, 2014). The two likely explanations for the greater effectiveness of the polarised model in comparison with threshold training are a greater increase in physiological capacity and a lower risk of fatigue. Only two studies (Neal et al., 2013; Pechlivanis et al., 2013) compared metabolic responses induced by different training intensity distributions.

While it appears a polarised model elicits favourable performance and physiological effects the underpinning metabolic adaptations are not well understood. Neal et al. (2013) used a metabolomics approach to compare two 6-week periods of endurance training in elite cyclists with different intensity distributions (threshold or polarised). Cyclists who followed threshold

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training showed greater creatinine excretion, greater degradation of active muscle ATP (greater urinary hypoxanthine excretion) and higher metabolic expenditure. Increased excretion of urinary 3-methylxanthin could be interpreted as greater oxidative stress after threshold training, while a higher concentration of urinary hippurate reflects a greater microbiome diversity, energy metabolism, and/or immune function. In swimming, a recent study (Pla et al., 2019) showed a modest improvement in performance and less fatigue in swimmers employing a polarised approach to training compared with a threshold-oriented distribution. These results require a deeper understanding of the metabolic processes induced by both types of distribution. The aim of the study was to compare metabolic responses between threshold and polarised training over 6 weeks in a cohort of highly trained swimmers. Based on evidence from previous scientific research we expected that threshold training induces higher metabolic stress than polarized training. Assessment of metabolomic profiles will provide new perspectives in understanding the differential demands of threshold and polarised training in swimmers.

Methods

Thirty high-level national swimmers participated in this study but only 22 completed the entire study and were included in the final analysis (n = 12 males 17 ± 3 years and n = 10 females 17 ± 3 years), training experience: 8 ± 2 years; mean ± SD). In the 6 months preceding the study, all participants trained from 15 to 18 hours per week involving 8 ± 2 swimming sessions per week and competed at the French national swimming championships. Swimmers were excluded if they had an injury or illness requiring medical treatment or had missed training for more than a week. The experimental study was conducted in accordance with the Helsinki Declaration. After detailed verbal explanations, all participants signed an informed consent form to participate. Parental consent was obtained for the swimmers under 18 years of age.

The study followed a crossover design with a total duration of 28 weeks (Figure 1). To minimise the delayed effects of prior training, a pre-experimental period was imposed, comprising 1 week without training, 1 week of moderate load training and 3 weeks of controlled training. Preliminary tests were carried out to familiarise swimmers with the measurement procedures. The study consisted of 2 six-week periods in which swimmers were allocated randomly. Initially, the size of the two groups was equal (15 participants for each group). However, given several dropouts (illness, schooling, stopping training), a final total of 13 participants were included in the threshold group (THR) and 9 in the polarised training group (POL) for the first 6-week intervention. The two groups were similar in age, level and gender. After the first study period, all the swimmers performed identical training under the direction of the same three coaches during 11 weeks (of washout training). The swimmers then crossed over to complete the other arm of the study design.

Categorisation of training

Blood lactate concentration $[La]_b$ was measured in swimmers using a 5 × 200 m incremental test in the period prior to the intervention study to establish a lactate-velocity profile (Mujika et al.,). A capillary blood sample (5 µl) for determination of the $[La]_b$ concentration was taken from a fingertip during the one-minute rest interval after each 200 m. We established three training intensity levels according to the results of this test: Zone (Z)1: <~2 mmol.L^{-1}, Z2: between 2 and 4 mmol.L^{-1} at the onset of blood lactate accumulation, Z3: >4 mmol.L^{-1}. All workouts over the period were timed for each swimmer and the training intensity categorised according to the three intensity levels. The intensity distribution was calculated as the percentage of the volume swum at each intensity over the total distance. Training out of the water (strength, conditioning, flexibility) – outside the prescribed POL or THR – was the same for all participants during each 6-week intervention. Metabolomics analyses were performed at the beginning and end of each training period with an early morning urine sample (15 mL). To assess changes in basal levels and avoid bias in the data due to very different trainings between the two groups the day preceding each test consisted of the same relative recovery for both groups.

Fatigue categorisation

The swimmers completed a short wellness questionnaire, as described by Noon et al. (2015) every morning before breakfast for the 6 weeks of training intervention. We collected seven self-reported items: motivation to train, quality of the previous

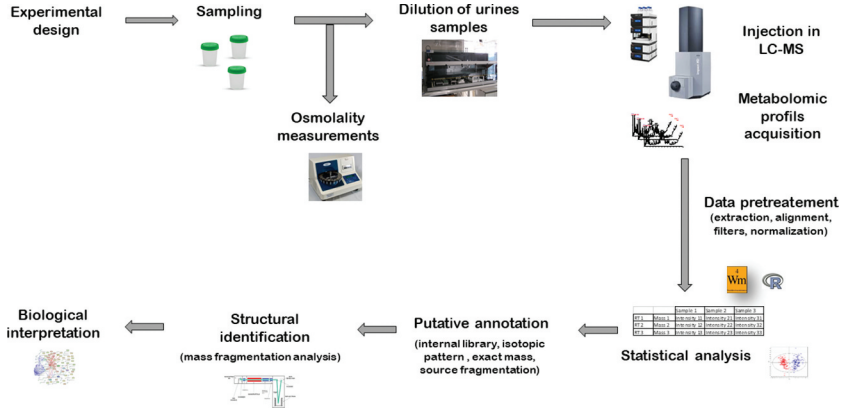

Figure 1. Workflow of untargeted metabolomic approach

night's sleep, perceived recovery, appetite, perceived fatigue, stress and muscle soreness. A cell phone application was used and swimmers asked to move the cursor on a visual analogue scale of 1 (worst) to 100 (best). A cluster analysis using the seven wellness items led to a classification of three groups of fatigue: high level of perceived fatigue, medium fatigue, and low fatigue.

Urine collection

Participants were instructed to collect urine upon awakening before any swimming training or testing. Urine was transferred initially to a 15 mL Falcon tube and finally into 1.5 mL eppendorf aliquots. Samples were stored at −80°C until further analysis. Urine osmolality representing w the total solute was expressed in mOsm/kg H_20 and measured with a 2020 Multi-Sample Micro Osmometer® (Advanced Instruments) using the freezing point measurement.

Mass spectrometry analysis

After urine samples were thawed at 4°C, they were centrifuged (Sigma 3–16PK, Fischer Bioblok Scientific) at 12,000 g at 4°C for 10 min. The supernatants were diluted 4 times with ultrapure water (18.2 MΩ.cm, total organic carbon <2ppb) using a robotic platform (Freedon EVO200, Tecan). A pool sample (Quality Control: QC) was prepared by mixing 10 µL from each of the diluted samples and injected 15 times during the batch analysis to monitor the drift of the mass spectrometer. Metabolic profiles were then acquired using ultra-performance liquid chromatography (U3000, Thermo Fisher Scientific) coupled with quadrupole-time-of-flight mass spectrometer (Impact HD II, Bruker). The spectrometer was equipped with an electrospray source and a lock-mass sprayer to ensure accuracy. Five µL were injected into a BEH Shield RP C18 1.7 µm 2.1 × 100 mm column at 20°C. Mobile phase components were A: ultrapure water with 0.1% formic acid and B: LC-MS grade acetonitrile with 0.1% formic acid. The column was eluted at a flow rate of 0.4 mL/min with a gradient of 0% of B for 2 min then increasing from 0 to 10% over 2–7 min, followed by an increase from 10 to 95% over 7–22 min. The mobile phase was then returned to 100% A at 22.1 min for 4 min re-equilibration.

Data were acquired in negative (NEG) and positive (POS) ion modes with a scan range from 50 to 1000 mass-to-charge ratio (m/z). Two separate injections were performed. The capillary voltage was set at 2500 V in positive mode and 4500 V in negative mode. The nebulizer pressure, drying gas flow and gas temperature were set to 41psig, 9 L/min, 200°C for positive and negative modes. Samples were randomised within the analytical sequence based on a Williams Latin Square strategy defined according to the main factors of the study: type of training (POL or THR), sex (male or female), and testing periods(two pre-tests, one washout and two post-tests periods).

Metabolomics data processing

Metabolomics data were processed with the Galaxy web-based platform Workflow4Metabolomics software (Giacomoni et al., 2015), first using XCMS (Tautenhahn et al., 2008) for data extraction, followed by quality checks and signal drift correction, according to the algorithm described by Van der Kloet et al. (2009). This process yielded a data matrix containing retention times, masses, and normalised peak intensities corrected for batch effects. To filter the background noise present in the data, ions whose variability in the QC pools was higher than those in the samples and/or 0.3 were removed from the dataset.

Following this data pretreatment, principal component analyses (PCA) were performed using SIMCA software (version 14.1, MKS Umetrics, 2015) on the matrix ion intensities. PCA is a descriptive (non-supervised) data analysis method well suited to metabolomic data since it tolerates collinearity between variables, and can be applied on a matrix having more variables than samples (Alonso-Gutierrez et al., 2015). PCA identifies outliers and provides a global view of the observations to detect particular effects in the data such as urine osmolality, which is a known confounding factor in metabolomics (Gagnebin et al., 2017). Given the large disparity of orders of magnitude and the variances of ion intensities, data were mean-centred and scaled to unit standard deviation. To observe an osmolality effect, the samples were figured according to their osmolality measurements. As an effect of osmolality on the data was observed, the optimal normalisation of the intensities by this variable was determined, by dividing by the osmolality the intensities of all ions, or only of ions moderately correlated to the osmolality (r-value>0.4 or 0.5). The effectiveness of these normalisations were also evaluated by PCA. Finally, the normalization of the intensities of ions moderately correlated for the POS mode dataset (r-value >0.4), and all the ions for the NEG mode dataset was kept.

Metabolomics data statistical analyses

Once the data were normalised, a similar methodology was used to identify co-factors for subsequent analyses: the samples were coloured according to their value or modality for each experimental variable in the metabolomic dataset. To determine the discriminating ions of the two types of training Partial Least Squares Discriminant Analyses were carried out using SIMCA, separately with the two normalised metabolomic datasets after mean-centring and scaling to unit standard deviation of the data. The response variable was the outcome variable (training) and the explanatory variables were the ions (variables obtained from metabolomics). The validity criterion of a model is the cumulative Q2 (Q2cum) for which the lower limit was set to 0.6 in order to have a highly discriminating model. This criterion is the fraction of the total variance of the matrix that can be predicted by all the components. It was estimated by repeated sevenfold cross-validation so that each part was predicted once, and computation of the ratio of predicted residual sum of square versus residual sum of square.

Given experimental constraints, blood or urine samples, or values of some variables needed to determine categories of fatigue or progression, were missing. Consequently, only 80 samples from 20 subjects were included in this study. Mixed model analyses with interaction were performed to test the effects of training and co-factors of interest (one by model) on the intensities at the post-test periods of each ion considered separately (variable "response"), taking into account the pairing

of the measurements for each subject. Training and co-factors were considered as fixed effects, and subjects as a random effect. The co-factors were fatigue, total progression, progression over the period. These mixed models were implemented under R (3.4.1) software (R Core Team, 2013) with the packages nlme, lmerTest and multtest. P-values were computed using the Satterthwaite's method for the denominator degrees of freedom. An adjustment of Benjamini-Hochberg (BH) for multiple tests was applied separately to the p-values obtained on the POS and NEG mode datasets to limit the number of randomness-related significant effects.

Metabolite annotation

Metabolites contributing to discrimination of the different groups were first screened using an in-house library containing the reference spectra of compounds analysed under the same analytical conditions. Database queries were performed with a mass error of 0.005 Da. The unknown compounds were identified based on an empirical formula determined by their exact masses compared with those registered in Metlin (http://metlin.scripps.edu) and the Human Metabolome Database (HMDB; http://www.hmdb.ca). Database results were confirmed using commercial standards where available with their isotopic patterns, and mass fragmentation analyses. Metabolite annotations were classified according to the levels of confidence in the identification process: identified (level 1, confirmed by standard), putatively annotated (level 2, based on physicochemical properties or spectral similarity with public/commercial spectral libraries), and level 3 for putatively characterised compound classes (Sumner et al., 2007). All standard compounds were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), except for pregnanediol-3-glucuronide and p-cresol sulphate, which were obtained, respectively, from Steraloids (Newport, USA) and Alsachim (Illkirch-Graffenstaden, France).

Results

Polarised versus threshold training

The total swimming volume in kilometres and the training load over the six-week training period were similar between POL and THR training (Table 1). The intensity distribution was 81/4/15 for POL and 65/25/10 for THR with substantially more training undertaken in zone I2 for the THR group.

Table 1. Details of the total training volume completed for polarized and threshold training groups (mean ± SD).

	Units	Baseline	POL	THR
Training Volume	kilometres per week	37 ± 3	42 ± 4	42 ± 4
Zone 1	% of training volume	70 ± 6	81 ± 3	66 ± 5*
Zone 2	% of training volume	20 ± 4	4 ± 1	25 ± 2*
Zone 3	% of training volume	10 ± 2	15 ± 2	9 ± 3*

Abbreviations: Baseline, 3 weeks period before training intervention; POL, polarised training; THR, threshold training. Intensity distribution was the same for both period of the study P1 and P2.
*Difference between POL and THR (P < 0,05).

Metabolomics analyses

After the extraction step, datasets containing 2673 and 1943 ions, respectively, were obtained from the positive and negative ion mode metabolomics analyses. Abnormal low intensities were detected in a sample in the positive ion mode, which we subsequently removed. After correcting the signal drift and background noise filtration, the intensity matrices contained 2207 ions for the positive mode and 1287 ions for the negative mode. PCA analyses showed the importance of urinary concentrations (according to their osmolality) on the first axis, which carries the higher variance (Figure 2(a,b): 30 and 31% of inertia). The intensities of the ions from the positive mode having a correlation coefficient with the osmolality >0.4 in absolute value (676 ions), and the intensities of all the ions in negative mode were divided by the osmolality measured in the urine sample. This normalisation allowed obtaining a PCA with the urinary osmolality linked to the 2nd axis, which represent respectively 7.8% and 14.4% of the total inertia of the datasets. The first major components of the two datasets were, respectively, 22 and 26% (Figure 3(a,b)) of their total inertia, and no longer related to the osmolality of the samples, confirming the efficacy of the correction.

PCA performed on normalised metabolomics data did not reveal any major factors that should have been considered or analysed in more detail in the context of the study. Consequently, no co-factor was added. The subsequent Partial Least Squares Discriminant Analyses failed to produce valid models. Mixed models performed on data of the last day of each period (post-tests 1 and 2), significant effects with BH adjustment of the fatigue class factors and/or training and/or interaction were identified for the intensities of 3 and 9 ions, respectively, in positive and negative ion modes (Tables 2 and 3).

Ion intensities of 6-keto-decanoylcarnitine (+31%), pregnanediol-3-glucuronide (+81%), P-cresol sulphate (+18%) were higher in the threshold group (P < 0.05) indicating higher glycogenic depletion and inflammation without alteration of the neuroendocrine stress axis.

Discussion

We compared metabolic responses using a non-targeted metabolomics analyses by mass spectrometry before and after six-weeks of threshold or polarised training in a cohort of high-level national swimmers. In this study, only four markers out of more than 1000 differentiated between threshold and polarized training groups, suggesting a moderate transformation of the metabolome according to the training modality. The ionic intensities of 6-keto-decanoylcarnitine, pregnanediol-3-glucuronide, and P-cresol sulphate were higher in the threshold group. 6-Keto-decanoylcarnitine is an organic compound and P-cresol sulphate a microbial metabolite which are involved in the metabolism of fatty acids (Heinonen, 1996) and tyrosine (Gryp et al., 2017) respectively. Pregnanediol-3-glucuronide is the main metabolite of progesterone which is a steroid sex hormone, close to oestrogens. The anti-inflammatory marker 4-phenylbutanic acid sulphate (Ozcan et al.,2006) was much higher in less fatigued swimmers.

The results point to higher substrate utilisation and inflammation with threshold-oriented training over six weeks in

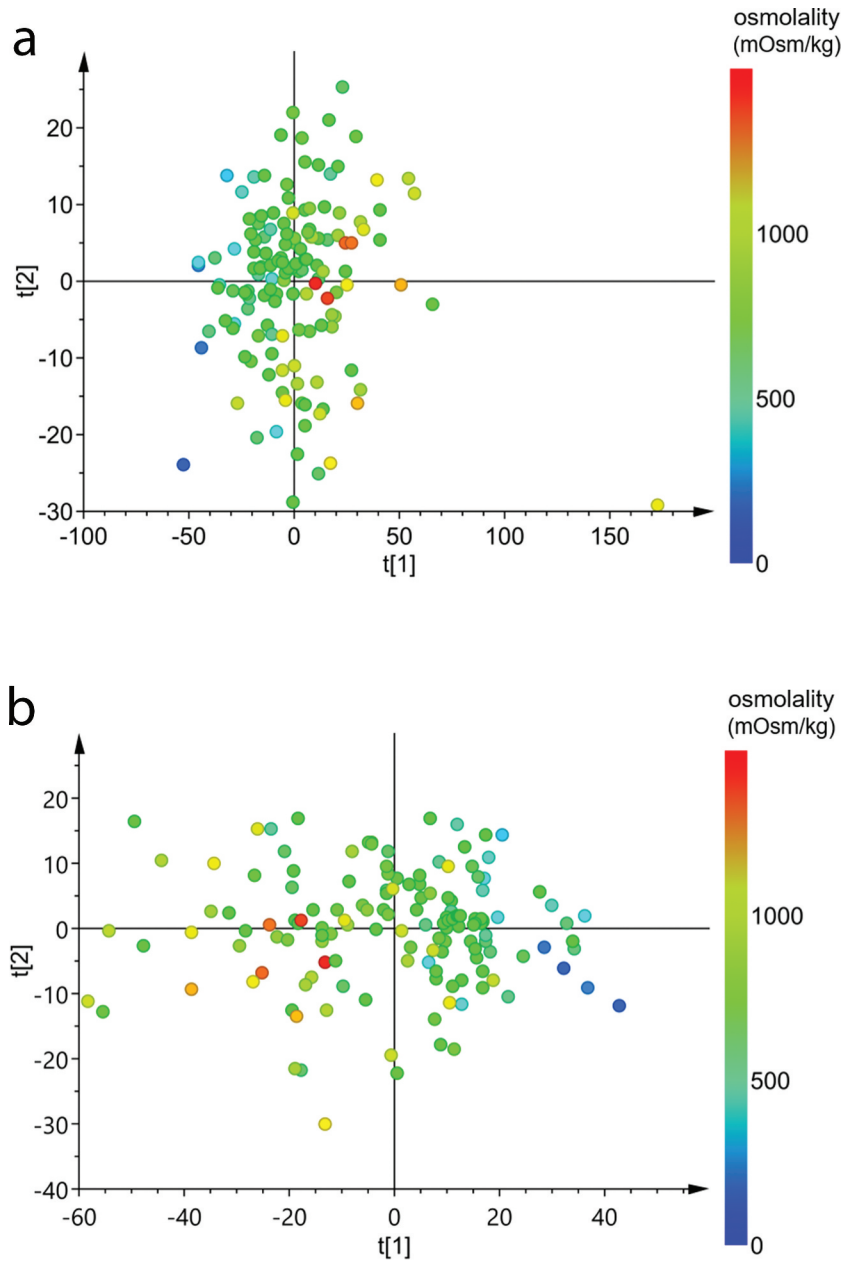

Figure 2. PCA based on samples analysed and processed without normalization

a cohort of highly trained swimmers. This information highlights the importance of managing the prescription of swimming training and recovery activities in swimmers.

The notion that polarised training is more effective and induces lower metabolic stress and fatigue than threshold training has been around for a decade (Seiler & Tønnessen,). Data in the literature report maximum fat oxidation in competitive athletes corresponding to 49% ± 15% $\dot{V}O_{2max}$ (Randell et al., 2017). In the polarized group, ~80% of the training was performed at speeds <2 mmol.L^{-1} (i.e. <65/70% of $\dot{V}O_2$max) likely inducing broader fat utilisation, thus amplifying the signal for aerobic oxidative enzymatic (Hansen et al., 2005) and mitochondrial (Holloszy, 2008) biogenesis. On the other hand, threshold training at a [BLa] of 2 to 4 mmol.L^{-1} representing an intensity of ~80 to 90% of $\dot{V}$O2 max likely induces high mental and metabolic stress associated with broader and longer sympathetic activation (Seiler, 2010). Chronic activation of sympathetic regulation could down-regulate α- and β-adrenergic receptor sensitivity (Fry et al., 2006), associated with a faster decrease in glycogen concentrations and ultimately severe fatigue (Bergström & Hultman, 1967). It appears that four ions relating to the metabolism of proteins, lipids, steroid hormones and moxocarboxylic 4-phenylbutyric can differentiate the effects of short term (6 weeks) polarised and threshold training in a cohort of elite swimmers.

The abundance of p-cresol sulphate was also higher in the threshold group. Urinary p-cresol sulphate is a microbial metabolite most likely derived from the secondary metabolism of p-cresol, which is itself a uremic toxin solution produced directly by the bacterial metabolism of tyrosine (Vanholder et al., 2003). In the context of renal failure P-cresol sulphate

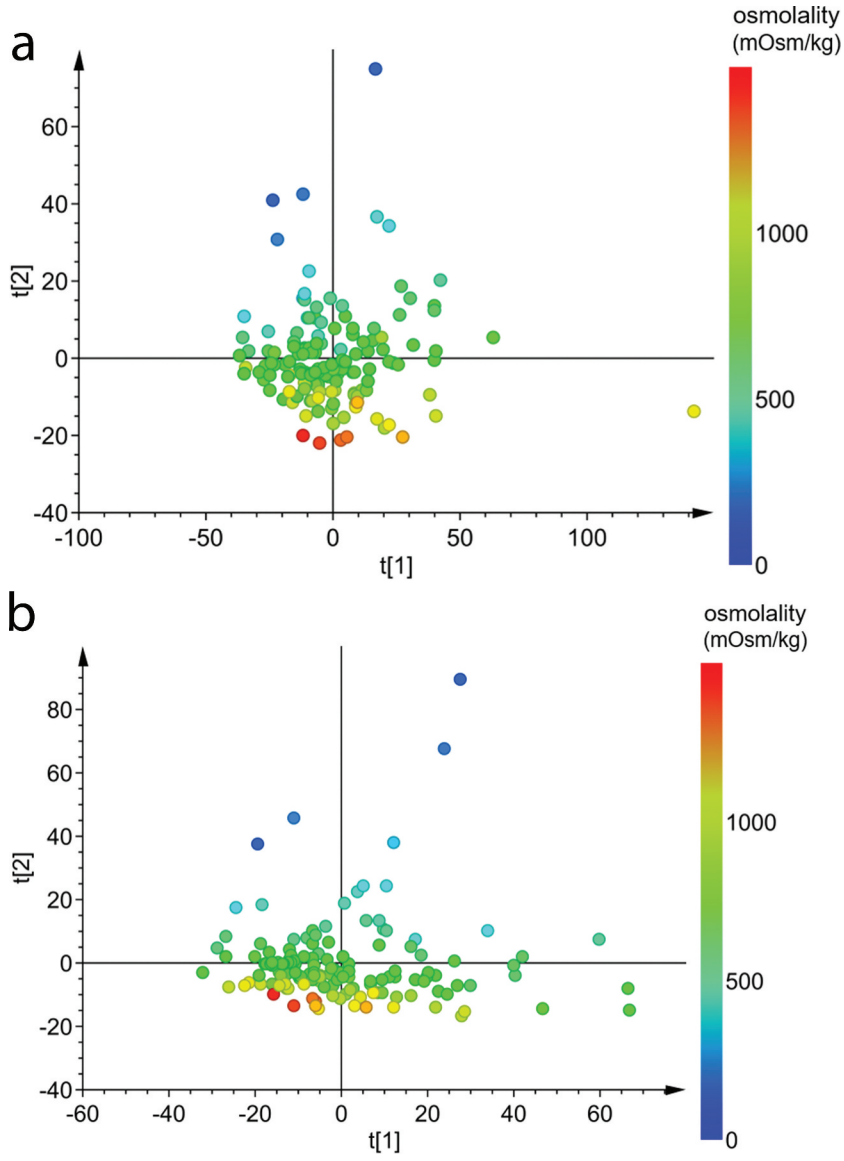

Figure 3. PCA based on sample analyses whose ions intensities wer enormalized with osmolarity values

Table 2. Metabolite signals after six weeks of training in elite swimmers obtained by mixed model analysis.

Annotation level*	Name	Variable label	Annotation	Measured mass (*m/z*)	Retention time (min)	Fatigue *p-value*	Training *p-value*	Interaction *p-value*
3	unkown	M275.16018T424	$C_{12}H_{23}N_2O_5$	275.1607	7.1	0.977	<0.001	0.002
2	6-keto-decanoylcarnitine	M330.22749T683	$[M + H]^+$	330.2273	11.4	0.001	<0.001	0.002
2	6-keto-decanoylcarnitine	M331.23084T683	[M + H]+13C1	331.2305	11.4	0.001	<0.001	0.002
2	4-phenylbutanic acid-O-sulphate	M163.07647T1079	$[M\text{-}H\text{-}SO_3]^-$	163.0764	18.0	0.003	0.144	0.376
2	4-phenylbutanic acid-O-sulphate	M243.03332T1079	$[M\text{-}H]^-$	243.0333	18.0	0.002	0.137	0.376
1	p-cresol sulphate	M187.00708T881	$[M\text{-}H]^-$	187.0070	14.7	0.002	0.017	0.105
3	unknown	M195.11382T761	$C_{10}H_{15}N_2O_2$	195.1135	12.7	0.002	0.009	0.050
3	unknown	M237.12453T760	$C_{12}H_{17}N_2O_3$	237.1242	12.7	0.002	0.009	0.050
3	unkown	M297.05521T716	$C_{12}H_{13}N_2O_5S$	297.0550	11.9	0.038	0.017	0.050
1	Pregnanediol-3-glucuronide	M495.2964T1065	$[M\text{-}H]^-$	495.2964	17.7	0.024	0.017	0.105
3	unkown	M194.08229T634	$C_{10}H_{12}NO_3$	194.0823	10.6	0.116	0.038	0.173
3	unkown	M274.03914T634	$C_{10}H_{12}NO_6S$	274.0391	10.5	0.104	0.017	0.154

Note: Fatigue = effects on perceived fatigue groups (high, medium and low fatigue); Training = effects on training mode (polarized or threshold training); Interaction = effects with both fatigue and training.

*Levels of confidence in the identification process: identified (level 1, confirmed by standard), putatively annotated (level 2, based on physicochemical properties or spectral similarity with public/commercial spectral libraries), and level 3 for putatively characterised compound classes (Sumner et al.,).

Table 3. Overview of significant metabolite modulations depending on fatigue class (FAT), training type (TRA) and their interaction (INT) after 6 weeks of swimming training.

Metabolites	FAT	TRA	INT		Low Fatigue	Mod Fatigue	High Fatigue	THR	POL
					n = 2	n = 24	n = 8	n = 34	n = 34
	p-values						*Mean ± SD*		
4-phenylbutanic acid-O-sulphate [M-H-SO_3]$^-$	**0.003**	0.144	0.376	NEG	36 ± 27	5 ± 5	8 ± 12	6 ± 6	8 ± 13
p-cresol sulphate	**0.002**	**0.017**	0.105	NEG	53 ± 28	57 ± 69	49 ± 35	63 ± 73	53 ± 33
unknown ($C_{10}H_{12}NO_3$)	0.116	**0.038**	0.173	NEG	14 ± 18	3 ± 3	6 ± 4	5 ± 6	3 ± 3
unknown ($C_{10}H_{15}N_2O_2$)	**0.002**	**0.009**	0.050	NEG	9 ± 3	21 ± 38	10 ± 3	19 ± 40	14 ± 12
unknown ($C_{12}H_{17}N_2O_3$)	**0.002**	**0.009**	0.050	NEG	15 ± 10	45 ± 93	20 ± 7	44 ± 99	28 ± 29
4-phenylbutanic acid-O-sulphate [M-H]$^-$	**0.002**	0.137	0.376	NEG	105 ± 77	13 ± 13	25 ± 34	16 ± 17	23 ± 39
unknown ($C_{10}H_{12}NO_6S$)	0.104	**0.017**	0.154	NEG	68 ± 88	13 ± 16	29 ± 23	25 ± 31	13 ± 14
unknown ($C_{12}H_{23}N_2O_5$)	0.977	**<0.001**	**0.002**	POS	2959 ± 1982	2682 ± 3417	2959 ± 1982	3418 ± 4320	2461 ± 2387
Unknown ($C_{12}H_{13}N_2O_5S$)	**0.038**	**0.017**	0.050	NEG	8 ± 9	2 ± 1	3 ± 2	3 ± 3	2 ± 2
6-keto-decanoylcarnitine [M + H]$^+$	**0.001**	**<0.001**	**0.002**	POS	66593 ± 71980	21732 ± 11363	21531 ± 8214	28737 ± 25611	21932 ± 11345
6-keto-decanoylcarnitine [M + H]+$13C_1$	**0.001**	**<0.001**	**0.002**	POS	12966 ± 13763	4148 ± 2127	4033 ± 1648	5518 ± 4848	4089 ± 2103
Pregnanediol-3-glucuronide	**0.024**	**0.017**	0.105	NEG	6 ± 2	18 ± 37	36 ± 44	52 ± 118	11 ± 11

Note: THR = threshold training; POL = polarized training. Significant differences are indicated in bold.

has been described as a potent uraemic toxin that can play a role in inflammation (Vanholder et al., 2003). These inflammatory effects may be cumulative when coupled with those induced by other inflammatory sources such as higher free radical production in athletes with high aerobic training loads (Kawamura & Muraoka, 2018). One interpretation of this result is that threshold training can increase intestinal tyrosine metabolism and upregulate inflammatory processes.

Another of our key results was a higher abundance for the metabolite 6-keto-decanoylcarnitine in the first morning urine for the threshold training group. 6-Keto-decanoylcarnitine is classified as a member of acyl-carnitines that play a role in the transport of fatty acids across mitochondrial membranes for oxidation of the three metabolic pathways (Heinonen, 1996). Metabolomic analyses indicate an increase in oxidation products of fatty acids such as acylcarnitine, especially after long-term maximum efforts in cycling (75 km at maximum speed, Nieman et al., 2014) or running (60 to 120 min~ 65–70% of $\dot{V}O_2$ Lehmann et al., 2010; 2.5 hours at 70% of $\dot{V}O_2$ for three days (Nieman et al., 2013). The higher the intensities (Hiatt et al., 1989) and longer the exercise duration (Decombaz et al., 1992), the lower the free carnitine concentrations, and the higher the concentrations of short-chain acylcarnitine in the muscle attesting to the oxidation rate of fatty acids in endurance efforts.

Abundance of 6-keto-decanoylcarnitine in the first morning urine in the threshold group may be related to an increased oxidation of fatty acids induced by a larger depletion of carbohydrate and lipid substrates. These changes give an indication of training stress where both glycogen and fat reserves are deployed. In the polarised swim training group, 80% of the training was performed <[Lab] 2 mmol.L^{-1}. At this intensity, the substrates consumed are mixed carbohydrates and lipids (Pelarigo et al., 2016). Swimmers who trained in the threshold mode achieved 25% of the total training volume at rates associated with [Lab] between 2 and 4 mmol.L^{-1} corresponding to ~75 and 90% of the $\dot{V}O2$ max by exclusively mobilising glycogenic sources (Seiler, 2010). Consistent with the above data the current study shows that the threshold training modality likely involves increased carbohydrate consumption leading to increased fat oxidation during night recovery periods.

Another important result of our study is that abundance of Pregnanediol-3-glucuronide were higher in the threshold training group and the most fatigued swimmers. Pregnanediol-3-glucuronide is the main metabolite of progesterone which is a steroid sex hormone, close to oestrogens, synthesised in women by the corpus luteum, or in the placenta, from pregnenolone. In humans, progesterone is synthesised by the testicles and adrenal glands under the action of the luteinizing hormone (LH). It appears that six weeks of threshold training performed by swimmers did not affect the functioning of the hypothalamic-pituitary axis and production of the LH gonadotropin regulating progesterone concentration.

The higher abundance of Pregnanediol-3-glucuronide in the morning urine of swimmers who trained in threshold mode are consistent with the results of the recent study by Al-Khelaifi et al. (2018). This team observed, based on a non-targeted mass spectrometry analysis, a marked increase (44% change, Bonferroni $p \leq 0.01$) in the abundance of pregnanediol-3-glucuronide involved in the synthesis of testosterone and progesterone in high-performance athletes compared to lower performance athletes. With regard to responses to acute physical exercise, other research has indicated that a significant increase (~19%) in progesterone production in response to intense exercise (Nakamura et al., 2011). In women, the effects of chronic training are different and the majority of studies report that urinary progesterone is lower in recreational runners (on average 20 miles per week) and women who exercise moderately or intensively (7.5 hours of intense physical activity per week) than sedentary women. In female athletes, research has shown a decrease in progesterone excretion in the luteal and follicular phases according to a dose-response relationship with both the volume and intensity of exercise (Nakamura et al., 2011).

In humans, progesterone is produced in the adrenal glands by the pituitary hormone ACTH, and in the testicles, by regulating LH. Among the few studies that measured progesterone production in male athletes Alén et al. (1988) reported less variation after chronic intensive training than female athletes. Changes in the relationships between 17-OH-progresterone were positively correlated with changes in maximum force indices. In our study, the higher concentrations in the most

fatigued group, and in the group that trained at the threshold, indicate that these swimmers were not prone to neuro-pituitary dysregulation resulting in lower LH and ACTH production and therefore progesterone. As higher concentrations of progesterone have been observed in the most fatigued swimmers, this production may be related to an anti-stress function, as previously suggested (Wirth, 2011).

One of the results that opens up prospects for future research is the significantly higher ionic concentration (~200%) of 4-phenylbutanic acid-O-sulphate in the two swimmers who reported the lowest levels of fatigue, the highest levels of motivation and the best sleep in the study. The moxocarboxylic 4-phenylbutyric acid is produced endogenously at the cell membrane level. This molecule has been identified as a chemical chaperone known to assist proteins in their maturation by improving, in particular, the folding capacity of the endoplasmic reticulum. They serve as a "quality control system", recognising, retaining and targeting misfolded proteins for degradation (Welch & Brown, 1996). Investigators have suggested the potential role of these chemical chaperones in the treatment of stress-related neurodegenerative diseases but also in diabetes (Lee et al., 2011). Sodium phenylbutyrate is the salt of 4-phenylbutyric acid and used in the treatment of diseases such as urea cycle disorders by ammonia uptake (Ozcan et al., 2006). Sodium phenylbutyrate can reduce oxidative stress has anti-inflammatory effects, and improves insulin sensitivity and promotes glucose metabolism in skeletal muscle (Ozcan et al., 2006; Lee et al., 2011). We speculate the higher concentrations of 4-phenylbutanic acid-O-sulphate in the two swimmers who reported less subjective fatigue, higher motivation and better sleep quality suggests a relationship between peripheral membrane chemical metabolism and behavioural indices. Understanding the mechanisms of this possible interaction requires future research.

This research confirms and extends the findings of Neal et al. (2013) who first identified greater metabolic/cellular energy stress induced by threshold compared to polarised training. In the study by Neal et al. (2013) cyclists who had performed threshold training were characterized by greater changes in water status, greater degradation of ATP (greater creatinine excretion) of the active muscle, and thus higher metabolic expenditure. By observing increased excretion of urinary 3-methylxanthin, the Neal et al. team also assumed greater oxidative stress was induced by threshold training. Our results broaden the clinical picture of metabolic responses to threshold training by showing this type of training may be related to increased lipid and tyrosine consumption, and accentuated inflammatory responses.

In the study by Neal et al. (2013) of the threshold training model, higher concentrations of the urinary metabolite hypoxanthine interpreted as a greater metabolic stress and lower concentrations of the metabolite 3-methylxanthin interpreted as a lesser degradation of purine nucleotides were not found in the present study. The two training modalities were more clearly differentiated in Neal's study 80/0/20% for the polarized model and 57/43/0% for the threshold model, compared to those in our study of 81/4/15% and 65/25/10%, respectively. On the other hand, cycling and swimming most likely involve different patterns of muscle recruitment.

One of the limitations of this study is that only one metabolomic analysis was performed at the beginning and end of two training periods. It is not possible to determine whether the metabolomics results reflect acute short-term adaptations or metabolic changes induced across all training cycles. Weekly analyses would have made it possible to monitor the dynamics and timecourse of metabolic transformations. As a perspective for future research, the effects of improved nutrition and recovery strategies to counterbalance the increase in metabolic stress induced by threshold training should be studied. Moreover, this study based on non-targeted metabolomics allows semi-quantitative measurements, representing relative ion intensities. Even if it is not absolute quantitation, there are linear relationships between ion intensities for each metabolite and its concentration levels in the samples. Moreover, even if level 2 annotation does not correspond to a formally identified compound given the absence of the corresponding standard, there is a high degree of confidence into the annotation (several independent analytical criteria: exact mass, isotope pattern, in-source fragmentation, RT, MS/MS fragmentation and similarity with spectra of identified compounds from the same class). Beyond this discovery step, the metabolites of interest need to be validated in large and diverse populations in order to study their robustness.

Conclusion

Metabolomic urine analysis revealed different metabolic responses between threshold and polarized training in high-level national swimmers over six weeks of swimming training. Glycogen depletion could be linked to higher levels of the metabolite 6-keto-decanoylcarnitine reflecting higher lipolytic activity for glycogen resynthesis as a metabolic priority. In the threshold group, this greater mobilisation of energy substrates was associated with higher production of the uraemic residue P-cresol sulphate. With threshold training, LH production by the hypothalamic-pituitary axis did not appear to be affected because the abundance of pregnanediol-3-glucuronide, the major metabolite of progesterone, was higherlall. The higher level of 4-phenylbutanic acid O-sulphate in the least fatigued and most motivated swimmers connect the anti-inflammatory and anti-degenerative activity at the cellular membrane level to behavioural outcomes.

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