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ESTIMATING STUDY SAMPLE SIZE AND ASSESSING RELIABILITY OF PEAK FAT OXIDATION (PFO) DATA (G/MIN) COLLECTED DURING FATMAX TESTS, WITH CONSIDERATION FOR MENSTRUAL CYCLE IN ENDURANCE-TRAINED WOMEN

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Abstract:
Background: Sample size estimations are completed to determine the number of participants needed to highlight a relevant treatment effect [1]. This is crucial to ensure that the results are reliable. Estimating sample size can also assist researchers by informing the financial outlay for a project and minimising any over spend. **Purpose:** In this study, different methods to estimate sample size using PFO data were utilised. PFO data collected using the test re-test method was employed to estimate sample size. PFO results from previous research were also applied to estimate sample size [2,3,5,6]. **Methods:** Subsequently, a group of endurance-trained women (n=5) aged 36 (+/- 2.4 years) were recruited from the main study. Menstrual cycle (MC) length was established and within the MC, the mid-follicular phase (MF) (low hormone phase) was determined [4]. Identifying the MF phase of the MC would allow for FATMAX test re-test's to be conducted when sex hormones were least likely to change or impose an effect on substrate utilisation. Tests were separated by 24 hours to allow participants to recover, to ensure hormonal change was minimal and to test the reliability of the results collected. **Results:** Results from previous research were statistically analysed individually and in group form to estimate sample size. When analysing PFO data from previous research, a mean sample estimation of 7 participants was suggested. Test re-test PFO results were

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assessed for reliability (ICC=0.82) before sample size estimations were conducted. Individual analysis from the test re-test method outlined a mean sample estimation of 14 participants, and group analysis from this method suggested a mean sample estimation of 14 participants. An overall mean sample from all estimations outlined a sample size of 12 participants.

Keywords: FATMAX, sample size estimation, peak fat oxidation, reliability

1. Introduction

PFO and the intensity that elicits PFO (FATMAX) are essential parameters in exercise physiology research. PFO is the highest rate of fat oxidation (g/min) during exercise, while FATMAX is the exercise intensity at which PFO occurs [2]. Estimating the sample size required for a study that involves PFO and FATMAX testing is crucial to ensure that the results are reliable. As mentioned above, it also informs budgeting around research projects, which minimises overspending and wastage. This article discusses how to estimate the study sample size with PFO data from previous research and with PFO data collected during FATMAX testing when participants are within the MF phase of their MC. New PFO data collected during FATMAX tests was also assessed for reliability.

1.1 Menstrual Cycle

The MC is a natural process that occurs in the female reproductive system. It involves a series of hormonal changes and physical events that prepare the body for pregnancy. It has been reported that this change in hormones can have a varying impact on substrate utilisation in eumenorrheic women [3,8]. Standardising testing in conjunction with specific time points within the MC is recommended [3,8]. The MC consists of 3 main phases: follicular phase, ovulation and luteal phase [16]. On average, MC last about 28 days, but can vary from woman to woman [17]. During menstruation, at the start of the follicular phase, the uterus coating is shed and expelled from the body [18]. Menstruation usually lasts about 3 to 7 days, which is part of the follicular phase. Following menstruation, which is typically referred to as the mid-follicular phase. The pituitary gland releases follicle stimulation hormone (FSH), which stimulates the ovaries to produce follicles. These follicles contain eggs and, as they grow, release oestrogen. Ovulation is the next phase of the MC. A mature egg is released from the ovary and travels down the fallopian tube to the uterus. This usually occurs around the 14th day of a 28-day cycle. Ovulation is triggered by an increase in luteinizing hormone (LH) from the pituitary gland. After ovulation, the luteal phase begins. The empty follicle from which the egg was released transforms into a structure called corpus luteum. The corpus luteum produces progesterone, which helps prepare the uterus for pregnancy. If fertilization is not achieved, the luteum membrane collapses, hormone levels decrease, and the menstrual cycle starts again. Both Oestrogen and Progesterone metabolites

(esterone-3-glucuronide and pregnanediol glucuronide) in urine can be easily measured using a Mira fertility analyser to pinpoint a phase within the females MC [21, 22].

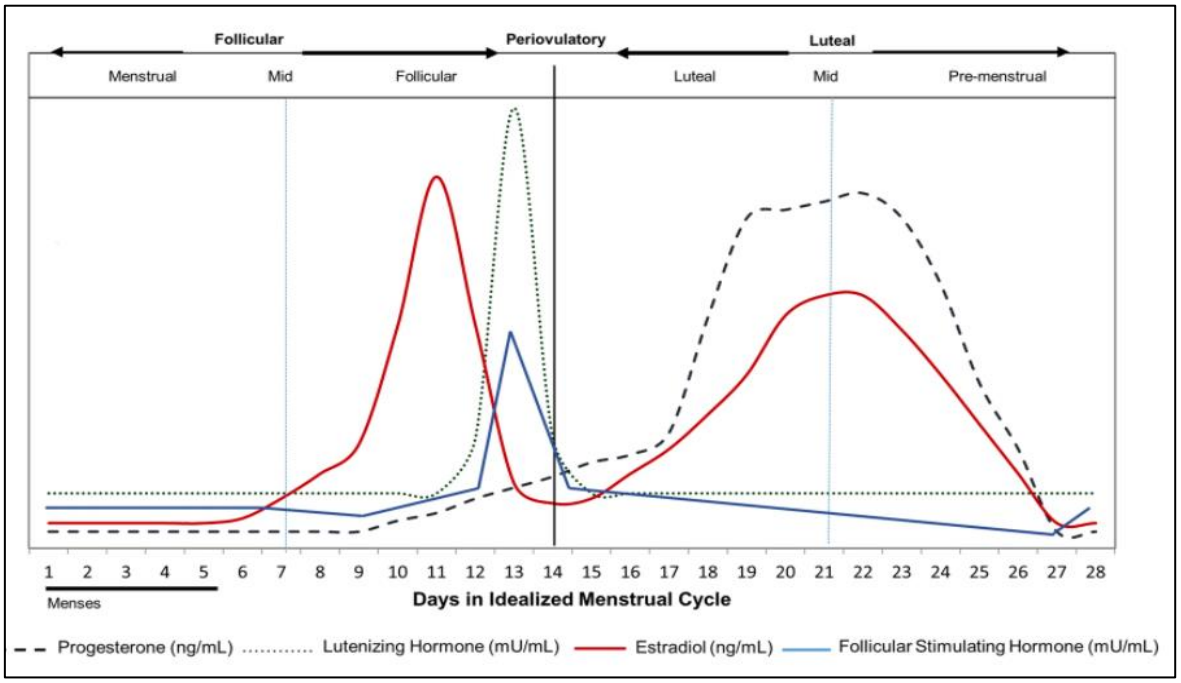

Figure 4.1: Menstrual Cycle Hormone Fluctuations Association with a Theoretical 28 day MC

1.2 Reliability of PFO and FATMAX

Previous research into the reliability of PFO and FATMAX is often limited to male-only or combined male/female studies [2,7]. Female-only studies are further limited by the complexity of accounting for sex hormones and the variability of their concentrations [29]. More recent studies have demonstrated the reliability of PFO and FATMAX in a large number of healthy men and women, with attempts to account for MC Phase and hormone concentrations in women [7]. The study estimated PFO and FATMAX Intraclass correlation coefficient (ICC) values were 0.78 and 0.81 respectively [7]. Generally, ICC values of 0.80 would indicate good reliability of the parameter, in this case PFO and FATMAX. However, the variability of the MC meant that MC was not always successfully accounted for, and participants were sometimes tested in the same phase and others in different phases. As outlined by Oosthuyse and Bosch, fluctuating sex hormones and their concentrations can have conflicting effects on fat oxidation and, therefore, potentially alter FATMAX results [8]. In a 2010 review, Oosthuyse and Bosch outline how different hormone ratios of Oestrogen and Progesterone during the mid-luteal phase can increase or decrease fat oxidation depending on the ratio of hormones at this time point. Also outlined in the aforementioned review the magnitude of change of oestrogen from mid follicular to mid luteal can impact fat oxidation at the mid luteal point.

From the above formula, PFO can be determined. Several other factors must then be considered when completing sample size estimation. These factors include the desired level of statistical power, the expected effect size, the level of significance, and data variability [11,12]. Effect size is a measurement of the magnitude of a difference between two groups and conditions [13]. In PFO and FATMAX tests, the effect size can be estimated based on previous studies or pilot data conducted. The power is the probability of rejecting the null hypothesis. The most common level of significance used in research generally is 0.05, meaning that there is a 5% probability of rejecting the null hypothesis when it is true. Data variability can be estimated based on previous studies and pilot data. Standard deviation (SD) is a measurement of data spread. Smaller SD means less data variability, which means that smaller sample sizes are required to detect significant differences. After estimating the effect size, significance level, and variability of data, it is possible to calculate sample sizes using statistical software or online calculators.

2. Methods

2.1 Calculating Sample Size

In order to estimate the size of the sample required for a study using PFO, the volume of oxygen (VO2) inspired and volume of carbon dioxide (VCO2) expired must be measured and then used to calculate the rate of fat oxidation in g/min [9,10]. The rate of fat oxidation can be calculated using the following equation [10]: Fat oxidation rate (g/min) = [(1.67 x VO2) - (1.67 x VCO2)] – 1.92n. Where VO2 is oxygen uptake in L/min, VCO2 is carbon dioxide production in L/min and n is estimated to be zero as protein catabolism is not contributing to energy production. The rate of fat oxidation can be calculated for each stage during the FATMAX test to determine the PFO rate.

2.2 Components of Breath-by-breath Results from FATMAX Testing

For the purposes of this project, FATMAX testing involved measuring the respiratory exchange ratio (RER) during incremental exercise until an RER of 1.00 is reached for one minute or 125 watts for 3 minutes [4]. The FATMAX test commenced with the recording of RER for 3 minutes at rest, then 3 minutes @ 35 watts, increasing by 15 watts every 3 minutes up to 125 watts. The components of interest from breath-by-breath results from FATMAX testing include RER, VO2 and VCO2 [15]. VO2 and VCO2 can then be used to calculate the rate of fat oxidation during exercise. As mentioned, each test was conducted during the MF phase within the MC, following an overnight fast and separated by 24 hours. The diet consumed by each participant was repeated throughout the day leading up to each test.

2.3 Identifying MC Phases and Verification

The MC phase of interest was the mid-follicular (MF) phase or low hormone phase. MC length was set at 100%, with time of ovulation at 50%. MF was then set at 25%, with LF

from 40 – 45 % and LF at 75%, as previously applied by Frandsen (3). Applying the below formula, the MF, LF and ML phases were identified;

- MF = MC length x 0.25,
- LF = MC length x 0.40,
- LF = MC length x 0.45,
- ML = MC length x 0.75.

MC length is the number of days from the start of menses, through one complete cycle and back to the start of menses (the theoretical model suggests 28 days +/- 5 days). Following phase identification using the above formula, the Mira fertility analyser was used to verify the pre-ovulation oestrogen spike over 3 days, which characterises the LF phase. Oestrogen concentrations can be determined (using the Mira fertility analyser and Max wand) from urinary analysis of the oestrogen metabolite esterone-3-glucuronide (E3G)[19,20]. Identification of the oestrogen spike would confirm the LF phase. Following confirmation of the LF oestrogen spike and verification of calendar dates, two FATMAX tests were scheduled 24 hours apart during the MF phase. Pre-FATMAX test hormones were also measured during the MF phase on both test days.

A. Method One: Calculating Sample Size Estimation with Data from Previous Research

1) Load clincalc.com [24]
2) Select Study Group Design - One Study Group V Population – one study group will be compared to a known value published in previous literature.
3) Select Primary Endpoint – Continuous (means) – the primary endpoint is an average.
4) Select Statistical parameters – enter the published mean and standard deviation.
5) Enter the study group mean.
6) Enter the Alpha Value – 0.05 used in medical literature – 0.05, meaning that there is a 5% probability of rejecting the null hypothesis when it is true.
7) Enter the Power Value – 90% used in medical literature – detecting a difference between groups when a difference exists.
8) Select calculate – the software tool computes a group and total sample size needed with sample calculations and formulas outlined.

B. Method Two: Calculating Sample Size Using PFO Data from FATMAX Test Re-test, Individually and in Group Format

On completion of FATMAX testing, analysis was conducted as follows;

1) Fat oxidation was calculated and PFO was determined for each participant, inputting VO2 and VCO2 data into the formulas stated.
2) Fat oxidation was calculated across the complete 24-minute FATMAX test, with PFO highlighted.
3) PFO data was analysed individually and in group format.

4) For PFO data, the same method was followed as outlined above. Inputting PFO and SD into the online calculator to estimate sample size based on individual and group results.

2.4 Intra Class Correlation (ICC)

ICC was calculated using the following method:

1) In Excel, a two-factor ANOVA was completed using PFO mean data from FATMAX Test one and two,
2) From the ANOVA results, the F value for subjects was identified. The F-value is the ratio of between-group variation and within-group variation,
3) Using an Excel calculator [23] an ICC of 0.82 was determined when there were 5 subjects (n=5), who completed 2 tests (k=2), f for subjects = 10.4 and the confidence interval = 90%.

2.5 Equipment

- Mira hand-held fertility analyser,
- Mira max wands for Oestrogen (E3G), Progesterone (PdG) and Luteinizing Hormone (LH),
- Keto-mojo hand-held glucose analyser,
- Keto-mojo glucose strips,
- Moxus Metabolic Cart,
- Lode Corival resistance simulating stationary bicycle.

3. Results

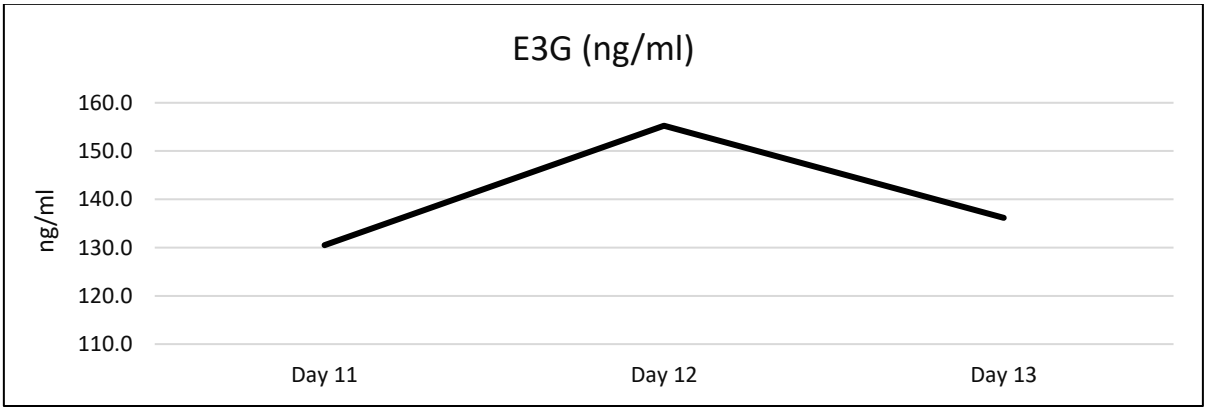

Figure 4.2: E3G Concentrations Across 3 Days of LF Phase Verification

Graph 1 displays mean E3G concentrations measured when verifying calendar predicted oestrogen spike of the LF phase. The oestrogen spike is suggested to occur around day 12 of the theoretical 28-day MC. Not all participants had a 28-day MC, so days 11, 12 and 13 outlined above are based on the theoretical model. Where a participant, for example, had a 30-day cycle (100%), the LF phase should occur across days 12,13,14,

as outlined previously. In this instance, days 12,13 and 14 are the equivalent of days 11,12 & 13 of the theoretical 28-day MC.

Table 4.1: Hormone Concentrations During MC Phase Verification of LF Oestrogen Spike with SD

Days (theoretical)	Day 11	SD	Day 12	SD	Day 13	SD
E3G (ng/ml)	130.5	39.9	155.3	47.1	136.2	55.1
PdG (ug/ml)	2.1	1.0	1.9	0.2	1.6	0.3
LH (IU/L)	3.1	1.8	3.4	1.4	3.7	1.2

Table 4.1 highlights E3G, PdG and LH concentrations measured across three days of verification of calendar-predicted dates.

Table 4.2: Glucose & Hormone Concentrations pre-FATMAX Test Day 1 and Day 2 During MF Phase with SD

	Mean Glucose (mmol/l)	Mean E3G (ng/ml)	Mean PdG (ug/ml)	Mean LH (IU/L)
Pre FATMAX Test Day 1	4.2	108.5	4.5	3.0
Pre FATMAX Test Day 2	4.1	101.3	3.8	2.9
Standard Deviation	0.22	3.63	0.38	0.04

Table 4.2 displays mean E3G, PdG, and LH concentrations measured approximately 90 minutes before FATMAX test day 1 and FATMAX test day 2. Glucose concentration is also displayed which was taken 3 minutes before the commencement of each test. Pre-test blood glucose (mmol/l) analysis was conducted to ensure participants were fasted (3.9 – 5.6mmol/L).

A. Method One

Table 4.3 below highlights the results obtained from sample size estimations using the data from the Frandsen et al. [1] study. Sample size estimations based on the minimum, maximum and SD figures attained during MF and LF points within the MC returned an estimate of 8 participants in both. Data attained during the ML point of the MC returned an estimation of 4, potentially due to the lesser SD.

Table 4.3: Power & Sample Size Calculation Summary Derived from Results Reported in Frandsen et al. [1]

Power Calc No	MC Point	Fat Ox (g/min) Min	Fat Ox (g/min) Max	Standard Deviation	Alpha	Power	Sample Size N =
1	MF	0.324	0.433	0.05	0.05	90%	8
2	LF	0.329	0.421	0.04	0.05	90%	8
3	ML	0.337	0.442	0.03	0.05	90%	4

B. Method Two

Table 4.4 below highlights the sample size estimation results obtained using the PFO data generated from FATMAX testing. As mentioned, the sample size was estimated based on the PFO data generated from each participant.

Table 4.4: Power & Sample Size Estimation Summary Generated from Peak Fat Oxidation (g/min) Rates Within Individual Reliability FATMAX Tests

Participant No	MC Point	PFO Test 1 (g/min)	PFO Test 2 (g/min)	Standard Deviation	Alpha	Power	Sample Size N =
1	MF	0.50	0.56	0.04	0.05	90%	18 (9&9)
2	MF	0.58	0.64	0.04	0.05	90%	18 (9&9)
3	MF	0.45	0.58	0.07	0.05	90%	12 (6&6)
4	MF	0.63	0.53	0.05	0.05	90%	10 (5&5)
5	MF	0.26	0.22	0.02	0.05	90%	10 (5&5)

Table 4.5: Power & Sample Size Estimation Summary Generated from Group Mean Peak Fat Oxidation (g/min) Rates Within Reliability FATMAX Tests

Participant No	MC Point	PFO Test 1 (g/min)	PFO Test 2 (g/min)	Standard Deviation	Alpha	Power	Sample Size N =
Group	MF	0.48	0.51	0.04	0.05	90%	14 (7&7)

Graph 2 displays PFO with standard deviation for each participant obtained from both FATMAX tests conducted during reliability testing.

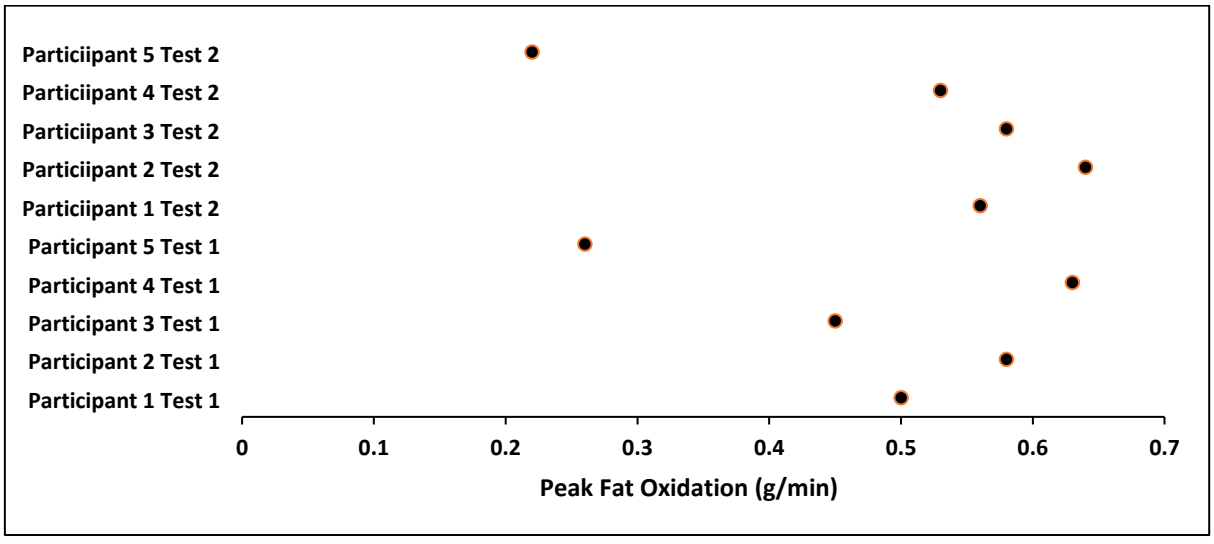

Figure 4.3: Peak Fat Oxidation Rate (g/min) for Five Participants, Two FATMAX Tests per Participant for Reliability Testing

Table 4.6 displays the mean sample size estimation collected from each sample size assessment. Sample estimation 1 is the mean of estimations retrieved from analysing

the Frandsen study [3]. Sample estimation 2 is the mean sample estimation calculated using each participant's PFO data (min, max & SD) gathered during reliability testing. Sample estimation 3 from group PFO data collected during reliability FATMAX tests.

Table 4.6: Mean Sample Size Estimation from Combined Power Analysis

Data	Mean Sample Size Estimation
Sample Estimation 1 - Frandsen et al [1] PFO data	7
Sample Estimation 2 - Reliability study PFO individual data	14
Sample Estimation 3 - Reliability study PFO group data	14
Overall	12

4. Discussion

Sample size estimation using data from published, peer-reviewed research is a method for estimating the number of participants needed to yield a relevant treatment effect [1,15]. The study selected for method one, estimation from peer-reviewed data, was conducted by Frandsen et al. [3]. The Frandsen et al. [3] study was selected due to the similarities in study design, which we also applied. Also, PFO was the preferred metric for analysis, with data being collected at three specific phases: MF, LF and ML within the MC. At the three phases of interest, minimum, maximum and standard deviation for PFO (g/min) were outlined (see Table 3 above). Inputting PFO data into the Clinical sample size calculator [24], a sample size with supporting calculations was generated. Sample estimations of n=8 at MF, n=8 at LF and n=4 at ML were outlined, with n=7 being the mean sample size estimation when all three results were reviewed.

Subsequently, PFO data gathered through a reliability study would allow further sample size estimations to be generated. Five endurance-trained women (36 +/- 2.4 years) were recruited. Following the completion of pre-test documentation (pre-screen & informed consent), MC data was collected, the date of ovulation was estimated applying the calendar method and then verified through urinary measurement of oestrogen (E3G) and progesterone (PdG). Having established ovulation, the MF phase or low hormone phase was identified as the point within the MC where hormones are most stable and would least affect substrate utilisation and FATMAX results [8]. Two FATMAX tests were conducted by each participant during the MF phase of their MC. Participants reported for testing between 9 – 9:30am following an overnight fast (last meal between 8 and 9pm allowing for an 11 – 13 hour overnight fast). Pre-trial and daily nutrition was replicated on both days as it has been previously reported that variations in nutritional intake pre-FATMAX test can have varying effects on FATMAX results [25,26]. Participants were asked to follow their normal daily routine over both days and refrain from any other exercise.

Following the collection of PFO data from FATMAX testing, this data was assessed for reliability. As mentioned, previous research investigating the reliability of PFO and FATMAX test results has suggested an ICC value of 0.78 and 0.81 [7]. An ICC value of >

0.75 and </= 0.90 would suggest good reliability [27,28]. When data from this study was assessed for reliability, it returned an ICC value of 0.82, which also suggests good reliability.

5. Conclusion

In conclusion, sample size estimation is a crucial aspect of research design that should not be overlooked. It ensures that the study has sufficient statistical power to detect meaningful effects and provides accurate and reliable results. By estimating the required sample size, researchers can confidently plan their studies. Furthermore, peak fat oxidation is an important physiologic parameter which can be utilised for sample size estimation. Test re-test reliability is another essential consideration in research design. By assessing the consistency of measurements over time, researchers can determine the reliability of their instruments or protocols. This information is vital for ensuring that the results obtained are not due to measurement error but reflect true changes or differences in the variables being studied.

In summary, PFO data from previous research using similar protocols suggested a mean sample size of n=7 when sample size suggestions from the different MC points were combined. PFO data gathered from FATMAX testing (ICC=0.82) using the test re-test method during the MF phase of the MC suggested a mean sample size estimation of n=14 from individual analysis and n=14 from group analysis. When all sample size suggestions were combined, a group mean sample size suggestion of n=12 is outlined.

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Conflict of Interest Statement
The authors declare that there are no conflicts of interest, financial or otherwise, that could compromise the integrity of this proposal.

About the Authors
Eoin Molloy PhD(c) began his studies at Waterford Institute of Technology, Waterford, Ireland, from which he holds a BSc (Hon 1:1) in Sports Coaching and Performance. Upon graduating from WIT, Eoin continued his studies and completed a Post graduate Diploma in Performance Nutrition (with Distinction) through the Institute of Performance Nutrition. Whilst completing his PgDip, Eoin decided to also set out on the PhD track investigating performance and health markers of fasted training on female

recreational endurance athletes. The aim of Eoin's research is to gain a better understanding of the effects of fasted training and the menstrual cycle phase on endurance performance and substrate utilisation in female athletes. The objective is to clearly demonstrate the importance of female-specific training programs derived from female-specific research.

Dr. Maria Murphy-Griffin is a full-time lecturer in the department's undergraduate programmes since 2002. She is interested in both exercise for health and in sport and performance and has a particular interest in exercise/sport physiology. Dr. Maria Murphy-Griffin has been awarded a BA in Physical Education and Mathematics (UL), an MSc in Sports Science (Loughborough University, Leics) and a PhD in Exercise and the Heart (UL).

Professor Michael Harrison has worked at WIT since 1999. He is the Head of the Department of Sport and Exercise Science and a former Dean of the School of Science and Computing. Michael's research, undertaken in conjunction with University Hospital Waterford and UPMC Whitfield, considers the impact of exercise on clinical populations, including age-related frailty, vascular disease and cancer. He has a specific interest in immune cell subsets that exert positive and negative influences on the processes that underlie ageing and vascular disease. Michael is Chair of the Register of Exercise Professionals in Ireland and sits on the Professional Standards Committee of the European Register of Exercise Professionals. He has been part of different national working groups and local initiatives to establish community-based exercise services for individuals with chronic health conditions and sits on the Advisory Board of the Exercise is Medicine Ireland National Centre. Michael holds a Fellowship from the American College of Sports Medicine.

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