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Comparison of Polarized *vs* Other Types of Endurance Training Intensity Distribution on Cardiorespiratory Function

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Resumo

Recentemente, o Treino Polarizado (POL) foi sugerido como superior a outras distribuições de intensidade de treino (TIDs) para melhorar a performance de *endurance*. O primeiro objetivo desta dissertação foi rever sistematicamente e meta-analisar as evidências que compararam o POL a outras TIDs em variáveis correlacionadas com a função cardiorrespiratória. PubMed, Scopus e Web of Science foram utilizadas para procurar estudos com a duração superior ou igual a quatro semanas que compararam o POL com outras TIDs e avaliaram o VO_2max/pico, contrarrelógio, tempo até a exaustão ou velocidade ou potência no segundo limiar ventilatório ou de lactato. Dezoito estudos preencheram os critérios de inclusão (n = 449). Os resultados sugerem que o POL é superior a outras TIDs para melhorar o VO_2max/pico. Essa superioridade, porém, ocorre apenas em intervenções de menor duração e em sujeitos moderadamente/bem treinados. As outras variáveis da função cardiorrespiratória foram afetadas de forma semelhante pelo POL em comparação com outras TIDs. O POL parece ser superior a outras TIDs para melhorar o VO_2max/pico, particularmente em intervenções de menor duração e no caso de sujeitos moderadamente/bem treinados, mas semelhante nas restantes variáveis da função cardiorrespiratória.

O segundo objetivo foi realizar um estudo experimental em ratos Wistar Han para comparar o treino POL *vs* Treino ao Limiar Anaeróbio (LIM), durante oito semanas, em relação ao VO_2max, capacidade de resistência, estrutura muscular do diafragma, respiração e marcadores da função e dinâmica mitocondrial no ventrículo esquerdo, diafragma, tibial anterior e sóleo. Não houve diferenças significativas no VO_2max relativo ou absoluto entre grupos nem na capacidade de resistência entre os grupos POL e LIM nas avaliações intermédias e finais. Não houve diferenças significativas na respiração mitocondrial nem nos marcadores da dinâmica mitocondrial em nenhuma das amostras de tecido analisado. Os resultados sugerem que o POL induz adaptações funcionais, estruturais e bioenergéticas semelhantes ao LIM.

PALAVRAS-CHAVE: TREINO POLARIZADO; DISTRIBUIÇÃO DE INTENSIDADE DE TREINO; VO_2MAX; RESPIRAÇÃO MITOCONDRIAL.

Abstract

Polarized Training (POL) has been recently suggested to be superior to other training intensity distributions (TIDs) regimens for improving endurance performance. The first aim of this thesis was to systematically review and meta-analyze the evidence comparing POL to other TIDs on surrogates of cardiorespiratory function. PubMed, Scopus, and Web of Science were searched for studies with more than or equal to four weeks duration comparing POL with other TID exercise interventions assessing either VO_2max/peak, time-trial, time to exhaustion or velocity or power at the second ventilatory or lactate threshold. Eighteen studies met the inclusion criteria (n = 449 subjects). The pooled effect estimates suggest that POL is superior to other TIDs for improving VO_2max/peak. This superiority, however, only occurs in shorter duration interventions (<12 weeks) and for the case of moderately/well trained subjects. All the remaining cardiorespiratory function surrogates analyzed were similarly affected by POL compared to other TIDs.

The second aim of this thesis was to perform an experimental study in Wistar Han rats to compare an eight-week POL *vs* Threshold training (LIM) protocol, regarding VO_2max, endurance capacity, diaphragm muscle structure, mitochondrial respiration and markers of mitochondrial function and dynamics in the left heart ventricle, diaphragm, tibialis anterior and soleus muscles. At the end of the training protocol, differences were not observed in relative or absolute VO_2max between groups and there were also no differences in endurance capacity between POL and LIM groups at the intermediate and final timepoint assessments. Differences were also not observed in mitochondrial respiration not in markers of mitochondrial dynamics between POL and LIM groups in any of the tissue samples analyzed. The results from our experimental study suggest that POL training induces similar, functional, structural and bioenergetic adaptations compared to LIM training.

KEYWORDS: POLARIZED TRAINING; TRAINING INTENSITY DISTRIBUTION; VO_2MAX; MITHOCONDRIAL RESPIRATION.

List of Abbreviations

All = Other Training Intensity Distribution.

AMPK = 5' AMP-activated protein kinase

BT = block training

CFE© = CrossFit endurance training ©

CG: Control Group.

ECOs = Objective Load Scale

ET = Endurance training

GRADE = Grading of Recommendations Assessment, Development, and Evaluation

h = hours

HIIT = high intensity interval training

HIT = high intensity training

HVLIT = high volume low intensity training

HVT = high volume training

I^2 (p): heterogeneity and p-value

$km.h^{-1}$ = kilometers per hour

LIT = low intensity training

LT = Lactate threshold

LTP = Lactate turn point

$m.s^{-1}$ = meters per second

MICT = moderate intensity continuous training

n = number of sessions

N-RCT = Non-Randomized Controlled Trial

NR = Not reported

P LT_2 = power at 2nd lactate threshold

P VT_2 = power at 2nd ventilatory threshold

PGC-1α = peroxisome proliferator-activated receptor-gamma coactivator-1 alpha

POL = polarized training

PPO = peak power output

PYR = pyramidal training

RCT = Randomized Controlled Trial

RCT = respiratory compensation threshold

s = seconds

SIT = sprint interval training

SMD = standardized mean difference

THR = threshold training

TID = training intensity distribution

TL = training load guided

TRIMP = training impulse

TT = time trial

TTE = time-to-exhaustion

V LT_2 = velocity at 2nd lactate threshold

V RCT = Velocity at respiratory compensation threshold

V VT_2 = velocity at 2nd ventilatory threshold

V/P at VT_2/LT_2 = velocity or power at 2nd ventilatory or lactate threshold

VO_2max = maximal oxygen uptake

VO_2peak = peak oxygen uptake

W = Watts

Z (p) = test for overall effect and p-value

Z1 = training zone 1

Z2 = training zone 2

Z3 = training zone 3

V VT_2 – velocity at 2nd ventilatory threshold

Mfn1= Mitofusin 1

Mfn2 = Mitofusin 2

SDS = Sodium Dodecyl Sulfate

SDS-PAGE = Sodium Dodecyl Sulfate - Polyacrylamide gel electrophoresis

TBS-T = Tris-Buffered Saline, 0.1% Tween 20

Tris-HCl = Tris hydrochloride

OROBOROS nomenclature

Ama = Antimycin A

As = Ascorbate

Azd = Sodium azide

c = Cyt. c - Cytochrome c

D = Adenosine diphosphate (ADP) + Mg2+

Dig = Digitonin

ETC = Electron Transport Chain

G = Glutamate

Gp = Glycerophosphate

LEAK = Non-phosphorylating electron transfer

M = Malate

Mir05 = Mitochondrial respiration medium

mt = Mitochondrial preparation

O = Octanoylcarnitine

OXPHOS = Mitochondrial Oxidative Phosphorylation System

P = Pyruvate

Rot = Rotenone

S = Succinate

SUIT = Substrate Uncoupler Inhibitor Titration protocols for High-Resolution Respirometry Assay = OROBOROS

Tm = TMPD - N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride

U = Uncoupler CCCP (Carbonyl Cyanide-m-Chlorophenylhydrazone)

General Introduction

Performance in endurance sports is highly dependent on training variables such as volume, frequency and load intensity. One key factor in determining the training stimulus is the training intensity and how it is distributed over time (Stöggl & Sperlich, 2015). This concept is well-known in scientific literature by training intensity distribution (TID) (Esteve-Lanao, Foster, Seiler, & Lucia, 2007; S. Seiler, 2010). Quantifying the TID can be performed through the definition of established parameters or physiological thresholds (e.g.: heart rate, blood levels of lactate, gas exchange, power output or velocity and perceived exertion) that delineate three (K. S. Seiler & Kjerland, 2006) or five intensity zones (Sylta, Tønnessen, & Seiler, 2014). The most frequently used model defines Zone 1 (Z1) as intensity below the first ventilatory or lactate threshold, Zone 3 (Z3) as above the second ventilatory or lactate threshold, and Zone 2 (Z2) between Z1 and Z3.

Of the various TIDs, the ones that are most frequently used in the preparation of endurance athletes are Polarized Training (POL), Threshold Training (THR), Pyramidal Training (PYR) and High Intensity Training (HIT) (K. S. Seiler & Kjerland, 2006; Stöggl & Sperlich, 2015). POL consists of performing high percentages of training time in Z1 (75-80%) combined with moderate percentages in Z3 (15-20%) and only a fraction that should not exceed 10% in Z2 (<10%) (Treff, Winkert, Sareban, Steinacker, & Sperlich, 2019). PYR also consists in high percentages of training time in Z1, with decreasing proportions of training time spent in Z2 and Z3 (e.g.: 70% Z1, 20% Z2, 10% Z3) (Stöggl & Sperlich, 2015). THR consists of devoting a high percentage of training time in Z2, never less than 35%, with the remaining time distributed between Z1 and Z3 (Treff et al., 2019). HIT mostly uses interval training and intermittent intervals with a great emphasis in Z3 training (e.g.: 20% Z1, 10% Z2, 70% Z3) (Stöggl & Sperlich, 2015). Of these TIDs, POL and THR seem to be associated with the best endurance performance achievements (Henritze, Weltman, Schurrer, & Barlow, 1985; Sjödin, Jacobs, & Svedenhag, 1982; Stöggl & Sperlich, 2015).

The THR model was, until recently, the predominant training model in endurance sports (Fiskerstrand & Seiler, 2004; Ingham, Fudge, & Pringle, 2012; Orie, Hofman, de Koning, & Foster, 2014; Yu, Chen, Zhu, & Cao, 2012). However, in the last decade, there has been a tendency towards the preferential

adoption of POL, due to mounting evidence suggesting its superiority (Hydren & Cohen, 2015). Several observational studies (for references see [(Stöggl & Sperlich, 2015)]) indicate that the majority of world-class endurance athletes currently use POL. The same tendency is verified in recreational athletes (Munoz et al., 2014). Several systematic reviews have tried to systematize the evidence regarding the TIDs adopted by endurance athletes, namely in middle distance and long distance running (Campos et al., 2022; A. Casado, González-Mohíno, González-Ravé, & Foster, 2022; Kenneally, Casado, & Santos-Concejero, 2018), swimming (González-Ravé, Hermosilla, González-Mohíno, Casado, & Pyne, 2021) and cycling (Galán-Rioja, Gonzalez-Ravé, González-Mohíno, & Seiler, 2023). These findings indicate that most athletes adopt POL and PYR TIDs, although THR is used by some of the best marathoners in the world.

The preferred adoption of the THR model until recently is due to the fact that Z2 training optimizes the recruitment of aerobic metabolism, allowing substantial volumes of training to be completed without excessive recruitment of the anaerobic metabolism and therefore without inducing significant homeostasis disruption (Arturo Casado, Foster, Bakken, & Tjelta, 2023) contributing thereby to the improvement of velocity at VT_2/LT_2 (Londeree, 1997). In opposition, the physiological justification behind the superiority of POL is based on the knowledge about the two main signaling pathways associated with mitochondrial biogenesis (Drake, Wilson, & Yan, 2016). One of these pathways stems from intracellular calcium signaling and is mainly potentiated by performing high training volumes at low intensity (Rose, Frøsig, Kiens, Wojtaszewski, & Richter, 2007). The other pathway is related to the energy depletion of the cell and the consequent activation of intracellular signaling dependent of 5' AMP-activated protein kinase (AMPK). This pathway being activated primarily by performing high-intensity efforts (Bartlett et al., 2012; Martin J. Gibala et al., 2009). The rationale of POL is based on the principle that using the appropriate combination of low (≈80% training time) and high (≈20% training time) training intensities allows optimizing the recruitment of these signaling pathways, enhancing thereby mitochondrial adaptations and, consequently, the improvement of the athlete's endurance performance (Arturo Casado et al., 2023).

Due to the growing interest on POL, several experimental studies exploring the effect of this TID on endurance athletes have been published in the

last five years. However, there are no studies that have aggregated, synthesized and meta-analyzed this new data and uncertainty remains as to the most appropriate TID for optimizing endurance performance. The first objective of this thesis was to review, synthesize and evaluate the degree of scientific evidence that supports the superiority of POL compared to other TIDs in improving cardiorespiratory fitness. To investigate this hypothesis, we selected a set of variables correlated with sports performance in endurance events, namely i) maximal oxygen uptake (VO_2max/peak); (ii) time-trial (TT); (iii) time to exhaustion (TTE) and (iv) velocity or power at 2nd ventilatory or lactate threshold (V/P at VT_2/LT_2) and determined the pooled effect size for each one of these variables from prospective studies comparing POL with others TIDs.

Nevertheless, the evidence supporting the superiority of POL compared to other TIDs has been questioned due to the inherent difficulty associated with rigorously controlling training variables such as volume, intensity and frequency and the different physiological metrics used in different studies for intensity prescription, such as physiological thresholds, heart rate zones and blood lactate values, which are not necessary interchangeable and do not delineate necessarily similar zones of intensity (Burnley, Bearden, & Jones, 2022). This limitation in the control of training variables could be surpassed by rigorously controlled experimental studies in animal models, since the laboratory environment allows controlling and equalizing training volume and intensity as well as food intake and environmental conditions. In this line, the second aim of this thesis was to compare the effect of an eight-week POL *vs* THR training protocol on key variables of endurance performance such as VO_2max and endurance capacity (EC) in Wistar Han rats. In addition, we also investigated potential mechanisms that could help to explain the superiority of each training model in the improvement of endurance performance. These physiological and biochemical mechanisms include the histological analysis of the *diaphragm*, evaluation of mitochondrial respiration of the *left heart ventricle*, *soleus, tibialis anterior and diaphragm muscles*, quantification of citrate synthase activity and expression of the key indicators of mitochondrial biogenesis peroxisome proliferator-activated receptor-gamma coactivator (PGC1α), transcription Factor A mitochondrial (TFAM), mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2) by Western Blot.

Chapter I

Systematic review with meta-analysis

Systematic Review with Meta-Analysis

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Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
View Submission View Reference Checking Results Correspondence Send E-mail	SPOA-D-23-00501	Comparison of Polarized vs Other Types of Endurance Training Intensity Distribution on Cardiorespiratory Function: A Systematic Review with Meta-Analysis	01 Jun 2023	30 Jun 2023	Under Review

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Sports Medicine

Comparison of Polarized vs Other Types of Endurance Training Intensity Distribution on Cardiorespiratory Function: A Systematic Review with Meta-Analysis

--Manuscript Draft--

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Comparison of Polarized *vs* Other Types of Endurance Training Intensity Distribution on Cardiorespiratory Function: A Systematic Review with Meta-Analysis

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Abstract

Background: Polarized Training intensity distribution (POL) has been recently suggested to be superior to other training intensity distribution (TID) regimens for improving endurance performance.

Aim: The aim was to systematically review and meta-analyse the evidence comparing POL to other TIDs on surrogates of cardiorespiratory function.

Methods: The PRISMA guidelines were followed. The protocol was registered in PROSPERO (CRD42022365117). PubMed, Scopus, and Web of Science were searched for studies with ≥4 weeks duration comparing POL with other TID exercise interventions assessing either VO_2max/peak, time-trial (TT), time to exhaustion (TTE) or speed or power at the 2nd ventilatory or lactate threshold (V/P at VT_2/LT_2).

Results: Eighteen studies met the inclusion criteria (n= 449 subjects). The pooled effect estimates suggest that POL is superior to other TIDs for improving VO_2max/peak (SMD=0.24 [95% CI 0.01, 0.48]; z = 2.02 (p = 0.040); n = 284; I^2 = 0%). This superiority, however, only occurs in shorter duration interventions (<12 weeks) (SMD=0.33; 95% CI 0.04, 0.63; z = 2.21; p = 0.03) and for the case of moderately/well trained subjects (SMD=0.37 [95% CI 0.01, 0.74]; z = 2.01 (p = 0.04); n = 121; I^2 = 0%). All the remaining cardiorespiratory function surrogates analyzed were similarly affected by POL compared to other TIDs, namely TT (SMD= -0.05 [95% CI -0.31, 0.21]; z= −0.36 (p= 0.72); n= 233; I^2= 0%), TTE (SMD= 0.30 [95% CI -0.20, 0.79]; z= 1.18 (p= 0.24); n= 66; I^2= 0%) and V/P VT_2/LT_2 (SMD= 0.04 [95% CI -0.21, 0.29]; z= 0.32 (p = 0.75); n = 253; I^2 = 0%).

Conclusions: POL seems to be superior to other TIDs for improving of VO_2max/peak, particularly in shorter duration interventions and for the case of moderately/well trained subjects. However, the effect of POL was similar to that of other TIDs on the remaining surrogates of cardiorespiratory function analysed.

Key points

- Polarized training is superior to other training intensity distribution models for the improvement of VO_2max/peak. There was no evidence of polarized training superiority for any of the remaining cardiorespiratory function surrogates investigated.
- Polarized training superiority was mostly evident for interventions lasting less than 12 weeks. When exercise interventions were longer than 12 weeks, VO_2max/peak was shown to increase similarly in those undergoing POL or other training intensity distribution models.
- Baseline cardiorespiratory fitness level was shown to influence the effect of Polarized training on VO_2max/peak improvement for the case of moderately/well trained subjects.

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no competing interests do disclose.

Data availability

Data could be made available for purposes found adequate by contact of the corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Code availability (software application or custom code)

Not applicable.

Authors' Contributions:

PO, GB e HF: study concept and design.

PO, GB e HF: data analyses.

GB: statistical analysis.

PO, HF: drafting of manuscript.

PO, GB, HF: revision of the manuscript.

All authors read and approved the final manuscript.

1. Introduction

Endurance performance is highly dependent on variables such as volume, frequency, intensity, and training intensity distribution (TID). Since high-level endurance athletes perform high training volumes, close to a maximum physiologically tolerable limit (Bourgois, Bourgois, & Boone, 2019), adequate manipulation of TID is fundamental for performance optimization (Esteve-Lanao et al., 2007; S. Seiler, 2010). TID can be characterized according to percentage of training volume spent on zones demarcated by established physiological thresholds. Three (K. S. Seiler & Kjerland, 2006) or five intensity zones (Sylta et al., 2014) are usually defined. The most used model defines Zone 1 (Z1) as intensity below the first ventilatory or lactate threshold, Zone 3 (Z3) as above the second ventilatory or lactate threshold, and Zone 2 (Z2) between Z1 and Z3.

Of the most frequently used TIDs (K. S. Seiler & Kjerland, 2006; Stöggl & Sperlich, 2015) Polarized Training (POL) and Threshold Training (THR) seem the most effective in endurance performance improvement (Henritze et al., 1985; Sjödin et al., 1982; T. Stoggl & Sperlich, 2014). POL consists on high training volumes in Z1 (75-80%), moderate volumes in Z3 (15-20%) and a small fraction in Z2 (<10%) (Treff et al., 2019). In THR training, most volume is spent on Z2 (>35%), with the remaining distributed between Z1 and Z3 (Treff et al., 2019). THR was, until recently, the predominant endurance training model (Fiskerstrand & Seiler, 2004; Ingham et al., 2012; Orie et al., 2014; Yu et al., 2012). However, recent evidence suggesting the superiority of POL has led to its preferential adoption (Hydren & Cohen, 2015) by high level (Stöggl & Sperlich, 2015) and recreational athletes (Munoz et al., 2014) alike. Preferred adoption of THR until recently was based on the argument that training mostly between ventilatory thresholds optimally recruited aerobic metabolism (Arturo Casado et al., 2023; Londeree, 1997). The rationale supporting POL superiority is based on the knowledge about the signaling pathways involved in mitochondrial biogenesis (Drake et al., 2016). The intracellular calcium signaling pathway is mainly potentiated by high training volumes at low intensity (Rose et al., 2007), while the 5' AMP-activated protein kinase (AMPK) pathway is optimally activated by depleting the cell ATP, particularly during high-intensity efforts (Bartlett et al., 2012; Martin J. Gibala et al., 2009). A combination of both low and high training

intensity would, therefore, optimally recruit these signaling pathways, enhancing endurance performance (Arturo Casado et al., 2023).

Despite these physiological arguments, studies comparing POL with other TIDs have provided conflicting results, with some showing superiority (Carnes & Mahoney, 2018; Hebisz, Hebisz, & Drelak, 2021; Neal et al., 2013; Schumann, Botella, Karavirta, & Hakkinen, 2017; T. Stoggl & Sperlich, 2014) while others not (Festa, Tarperi, Skroce, La Torre, & Schena, 2020; Rohrken, Held, & Donath, 2020; Treff et al., 2017). Conflicting findings might be due to the retrospective nature and reliance on training diaries on several studies (Stöggl & Sperlich, 2015), poor control of training variables (Burnley et al., 2022; Treff et al., 2019) and small sample size (Esteve-Lanao et al., 2007; Rohrken et al., 2020; Selles-Perez, Fernández-Sáez, & Cejuela, 2019; Treff et al., 2017) and low statistical power. A previous systematic review with meta-analysis has addressed this issue (Rosenblat, Perrotta, & Vicenzino, 2019), but the number of studies available at that time was still limited. Thus, uncertainty remains as to the most appropriate training model for optimizing cardiorespiratory function. Due to the growing interest on POL, several studies have been recently published. This increase justifies the need to perform a new systematic review with meta-analysis of the available evidence to address this issue.

The aim of this systematic review with meta-analysis is to determine if POL training is superior to other TIDs for the improvement of cardiorespiratory function. To test this hypothesis, a set of variables correlated with endurance performance were selected, namely (i) maximum oxygen consumption (VO_2max/peak), (ii) time-trial (TT), iii) time to exhaustion (TTE) and (iv) velocity or power at 2nd ventilatory or lactate threshold (V/P at VT_2/LT_2) (Stöggl & Sperlich, 2015).

2. Methods

This systematic review with meta-analysis followed the PRISMA 2020 guidelines (Appendix S1 of the Electronic Supplementary Material [ESM]) (Page et al., 2021). The protocol was defined and registered at PROSPERO (CRD42022365117) prior to the beginning of the data collection and analysis.

2.1. Eligibility criteria

Only studies published in scientific peer-reviewed journals in English were considered for the analysis. There were no restrictions regarding publication date. Eligibility criteria for study selection were set according to the PICOS (Participants, Intervention, Comparator, Outcome, Study design) framework:

(i) Participants: humans of both genders; age between 15-65 years; absence of comorbidities or physical limitations that could hinder exercise participation without restrictions at the onset of the intervention.

(ii) Intervention: endurance training interventions following the POL intensity distribution principle (Treff et al., 2019) with a frequency of ≥3 sessions/week and ≥4 weeks of intervention. There were no restrictions regarding the settings in which the intervention took place (e.g., elite, professional or recreational context).

(iii) Comparator: endurance training interventions following other TIDs principles such as, but not limited to, THR, Pyramidal Training (PYR), High Intensity Interval Training (HIIT) or Sprint Interval Training (SIT), High Volume Training (HVT) performed ≥3 sessions per week, ≥4 weeks of intervention.

(iv) Outcome: The outcomes of interest were surrogates of cardiorespiratory fitness. To be included in the analysis, studies should include at least one of the following outcomes: (i) VO_2max/peak; (ii) TT; (iii) TTE or (iv) V/P at VT_2/LT_2.

(v) Study design: Randomized controlled trials or non-randomized controlled trials with at least two groups, one experimental and one comparator and at least a baseline and a post-intervention measurement.

2.2. Information sources and search strategy

PubMed, Scopus and Web of Science databases were used to perform the searches that were carried out between the 10th and 20th october 2022. No filters were applied during searches. An example of the specific search strategy conducted in PubMed was as follow: (("polarized training") OR ("polarized endurance training")) AND ((((("training intensity distribution") OR ("endurance training")) OR ("pyramidal training")) OR ("threshold training")) OR ("high intensity training")) OR ("high volume low intensity training")). A preprint of the search strategies from PubMed, Web of Science and Scopus are presented [Appendix S2 of the ESM]. Reference lists of the included studies were also screened for potentially relevant studies (snowball technique). Whenever information regarding relevant variables was missing, the corresponding author was contacted by email and ResearchGate® to provide the missing information.

2.3. Study selection

After the initial database searches, references were downloaded to an EndNoteTM 20 for Mac (ClarivateTM) database for automated removal of duplicates followed by manual inspection and removal of remaining duplicates. Afterwards, titles were screened and studies that included polarized training interventions in humans were selected. After this stage, abstracts of the selected studies were reviewed and all of those potentially meeting the inclusion criteria were selected and full-text analysis was performed to ascertain inclusion of the study according to the pre-established criteria. Two researchers (PO and GB) independently conducted the literature search and study selection and then compared the results to ensure accuracy. Disagreements were resolved by consensus and included a third researcher (HF).

2.4. Data collection process

PO and GB independently collected the data items following a pre-defined data extraction sheet. Data collected was afterwards compared between researchers for consistency assessment. Cases of ambiguity regarding data collection were solved by consensus including a third researcher (HF). No automation tools were used for data extraction. Whenever the necessary data was not available in the text or tables, the WebPlotDigitizer tool was used to

extract the information from plots. Several studies included more than one intervention group of interest for the analysis. Whenever these cases were identified, results were included as separate reports in the analysis and identified with superscript letters indicating different sporting modalities (i.e. cycling, swimming, running) or different TIDs (THR, HIIT, HVT).

2.5. Data items

Variables relevant to assess the superiority or inferiority of POL compared to other TIDs for cardiorespiratory fitness improvement were collected and variables related with the study implementation context and exercise intervention characteristics were collected. The primary outcome was maximal (VO_2max), or peak (VO_2peak) oxygen uptake measured by indirect calorimetry. Secondary outcomes were (i) time to complete a pre-specified distance (TT); (ii) TTE in a pre-specified maximal exercise testing protocol; (iii) external load (velocity/power) at which the VT_2/LT_2 occurs. Due to the ambiguity regarding this concept in the literature (Poole, Rossiter, Brooks, & Gladden, 2021), we assumed as synonymous concepts lactate threshold (LT), lactate turn point (LTP) and respiratory compensation threshold (RCT). Additional variables assessed were:

(i) variables related with the description of the exercise intervention: weekly training volume, duration, distance, frequency, and weekly or total Training Impulse (TRIMP) (Lucia, Hoyos, Santalla, Earnest, & Chicharro, 2003).

(ii) variables related with the description of the participants: sport, competitive level, years of practice, number of subjects per group, age and gender.

(iii) Other variables: study design, study implementation location (e.g., country), funding sources. There was a need to convert time to exhaustion to percent variation change between post- and pre-intervention since this was the metric used in some studies.

2.6. Study risk of bias assessment

The Cochrane risk of bias tools for randomized controlled trials (RoB-2) and non-randomized studies of interventions (ROBINS-I) were used to assess the risk of bias of individual studies. Bias assessment that composes RoB-2 and ROBINS-I domains were rated as low risk, some concerns, or high risk. PO and

GB independently completed the risk of bias analysis which was later reviewed by a third author (HF). Where inconsistencies emerged, the original articles were re-analyzed until a consensus was reached.

2.7. Effect measures

For determining the superiority of POL in comparison to other TIDs for cardiorespiratory fitness improvement the effect size of individual studies was calculated as mean difference or standardized mean difference between intervention and comparator groups. The standardized mean difference was calculated as the difference between the mean of the POL group and comparator group divided by the pooled SD and was employed for analysis of TT and V/P at VT_2/LT_2 since these outcomes were reported by different measurement units in different studies. Mean difference was used for VO_2max or VO_2peak and TTE.

2.8. Synthesis methods

A qualitative synthesis of the included study's findings structured around the different exercise endurance training protocols e.g., pyramidal training (PYR), threshold training (THR), high volume low intensity training (HVLIT), high intensity training (HIT) in comparison with POL interventions was made. A random effects meta-analysis using the inverse variance method was also performed to compare the effect of POL with other TIDs regarding the primary and secondary outcome measures and results were displayed by forest plots. Fifteen of the 18 studies included in the systematic review integrated the meta-analysis. Sub-analyses were also performed to determine if the comparison between POL and other TIDs differed by gender (males *vs* females), intervention duration (interventions ≤ 12 weeks *vs* ≥ 12 weeks duration) or starting cardiorespiratory fitness level (high level *vs* moderate level/ well trained athletes *vs* recreational athletes *vs* sedentary). A sensitivity analysis was also performed by excluding one study at a time to determine the consistency of the results. To perform these analyses, the "meta" package in R software was used. The Z-test was used to assess overall effect and was considered statistically significant when $p<0.05$. The I^2 statistic was used to assess between-studies heterogeneity and was qualitatively characterized as: 0-40% not important, 30-60% moderate, 50-90% substantial and 75-100% considerable. A visual inspection of funnel plot and by Egger's

linear regression method test was used for three variables (VO_2max/peak, TT and V/P at VT_2/LT_2 with more than of included 10 studies. (Sterne et al., 2011)

2.9. Certainty of evidence assessment

We followed the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) tool to determine the certainty of the evidence of the findings regarding the study primary outcome (VO_2max/peak) (Malmivaara, 2015). Risk of bias, inconsistency, indirectness of the evidence, imprecision, and publication bias are the key domains used in the evaluation of the certainty of the evidence by GRADE.

3. Results

3.1. Study Selection

The flowchart details the studies included in the review. An initial search returned 275 results (35 PubMed, 203 Scopus and 37 Web of Science). After removing duplicates, 205 reports remained. The screening of titles and abstracts for eligibility criteria resulted in the exclusion of 163 reports, leaving 42 articles for full text analysis. Of these, 25 were excluded for not meeting the eligibility criteria: 7 were outside the research objective, 12 did not had the necessary intervention or comparators, 3 did not assessed any of the variables of interest, 2 were not written in English, and 1 was an *errata*. Snowballing revealed 1 additional potentially suitable article. After screening the abstract, a full text analysis was performed revealing that the article met the eligibility criteria. Eighteen studies were finally included in the systematic review. There were no discrepancies between raters in the selection of studies to be included in the final analysis (Fig. 1).

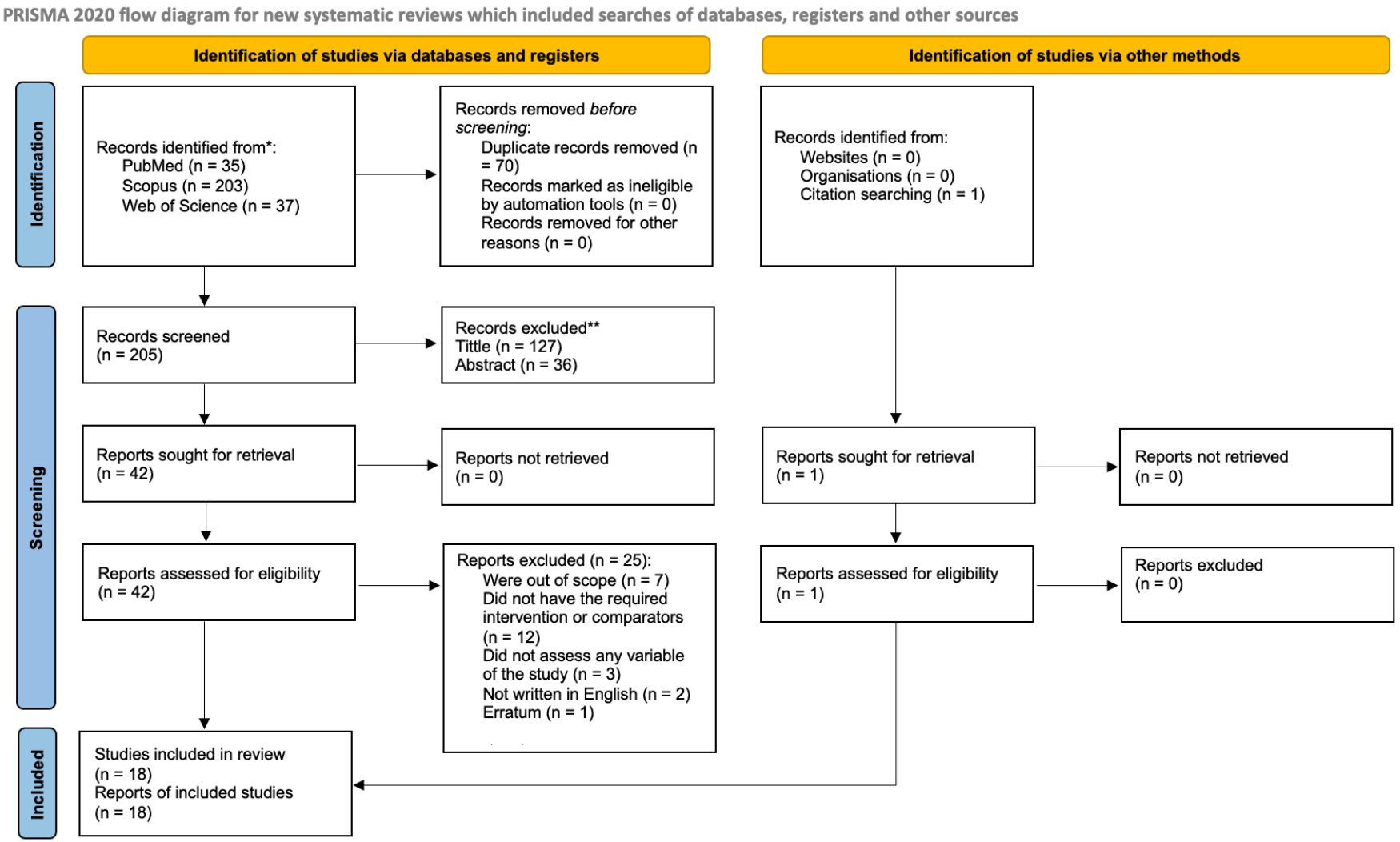


Fig. 1 PRISMA 2020 flow diagram

3.2. Characteristics of the studies included

Table 1 summarizes all the characteristics of the 18 studies included in this review that compared the effect of an intervention based on POL with other TIDs in improving cardiorespiratory fitness. The total number of participants was 449 (329 males and 89 females), of which 189 participated in POL interventions and 229 in other TIDs. One study (31 participants) did not discriminate the number of participants in each group (T. L. Stoggl & Bjorklund, 2017). Most studies (n=16) included only men or predominantly men. Only one study included only women or predominantly women (Zapata-Lamana et al., 2018) and only one study included a balanced sample of both genders (Pla, Le Meur, Aubry, Toussaint, & Hellard, 2019). All participants in the selected studies were adults or young adults, ranging from 17 ± 3 (Schneeweiss et al., 2022) to 44.2 ± 14.6 years (Carnes & Mahoney, 2018). The sample size in each study varied between n=11 (Neal et al., 2013) and n=56 participants (Filipas, Bonato, Gallo, & Codella, 2022) with a median of n=22 participants per study.

The studies selected for analysis included a wide variety of endurance sports, with running and cycling being the predominant. Seven studies evaluated endurance runners (Carnes & Mahoney, 2018; Esteve-Lanao et al., 2007; Festa et al., 2020; Filipas et al., 2022; Munoz et al., 2014; Schumann et al., 2017), one of these ultra-endurance runners (Perez et al., 2019). Four studies evaluated cyclists, one on road cyclists (Neal et al., 2013), one cross-country (Schneeweiss et al., 2022) and two mountain bikers (Hebisz & Hebisz, 2021; Hebisz et al., 2021). Six studies evaluated other sports, namely one study was performed with swimmers (Pla et al., 2019), one with rowers (Treff et al., 2017), two with triathletes (Rohrken et al., 2020; Selles-Perez et al., 2019) and two included multisport participants, namely cross-country skiers, cyclists, medium and long-distance triathletes and runners (T. Stoggl & Sperlich, 2014; T. L. Stoggl & Bjorklund, 2017). One study included previously untrained subjects (Zapata-Lamana et al., 2018).

Seven studies were performed on highly trained athletes, of which two included mountain bikers (Hebisz & Hebisz, 2021; Hebisz et al., 2021), one cross country cyclists (Schneeweiss et al., 2022), one swimmers (Pla et al., 2019), one rowers (Treff et al., 2017) and two a mixed sample of various disciplines (T. Stoggl

& Sperlich, 2014; T. L. Stoggl & Bjorklund, 2017). Three studies evaluated well trained athletes, of which two included runners (Esteve-Lanao et al., 2007; Filipas et al., 2022) and one road cyclists (Neal et al., 2013). Only one study, carried out in rowers, evaluated moderately trained athletes (Rohrken et al., 2020). Six studies evaluated recreational participants, of which four studies included runners (Carnes & Mahoney, 2018; Festa et al., 2020; Munoz et al., 2014; Schumann et al., 2017), one ultra-endurance runners (Perez et al., 2019) and one triathletes (Selles-Perez et al., 2019). One study included previously sedentary subjects (Zapata-Lamana et al., 2018).

The duration of training interventions ranged from four (Schneeweiss et al., 2022) to 24 weeks (Esteve-Lanao et al., 2007), with a median of 11 weeks. Three studies did not report the TID (Hebisz & Hebisz, 2021; Hebisz et al., 2021; Schumann et al., 2017). One study (Zapata-Lamana et al., 2018) reported the distribution of training intensity only in the POL group.

In two studies (Hebisz & Hebisz, 2021; Hebisz et al., 2021) the training duration and the number of sessions dedicated to high and low intensity training were reported without mentioning the percentage of the total training time distribution attributed to each intensity. Eight studies reported the weekly TRIMP, with studies from Carnes et al., (Carnes & Mahoney, 2018) and Röhrken et al., (Rohrken et al., 2020) showing the lowest (POL: 389 ± 101; CFE: 222 ± 68) and highest (POL: 882.0 ± 155; THR: 739.0 ± 162) weekly value. The minimum weekly training frequency was three sessions (Carnes & Mahoney, 2018) and the maximum was 10 sessions (Schneeweiss et al., 2022).

VO_2max or VO_2peak was assessed in 11 studies (Carnes & Mahoney, 2018; Esteve-Lanao et al., 2007; Festa et al., 2020; Filipas et al., 2022; Hebisz & Hebisz, 2021; Hebisz et al., 2021; Perez et al., 2019; Pla et al., 2019; Schumann et al., 2017; Selles-Perez et al., 2019; T. Stoggl & Sperlich, 2014; Treff et al., 2017; Zapata-Lamana et al., 2018), TT in 10 studies (Carnes & Mahoney, 2018; Esteve-Lanao et al., 2007; Festa et al., 2020; Filipas et al., 2022; Munoz et al., 2014; Neal et al., 2013; Pla et al., 2019; Schneeweiss et al., 2022; Schumann et al., 2017; Treff et al., 2017), TTE in four studies (Neal et al., 2013; Perez et al., 2019; Schumann et al., 2017; T. Stoggl & Sperlich, 2014) and V/P at VT_2/LT_2 in 12 studies (Festa et al., 2020; Filipas et al., 2022; Hebisz et al., 2021; Neal et al., 2013; Perez et al., 2019; Pla et al., 2019; Rohrken et al., 2020; Schneeweiss et

al., 2022; Selles-Perez et al., 2019; T. Stoggl & Sperlich, 2014; T. L. Stoggl & Bjorklund, 2017; Treff et al., 2017).

Table 1 Summary of the selected studies

Study	Sample characteristics			Competitive level	TID % (Z1/Z2/Z3)	Training Load	Training Characteristics	Outcomes used	Main findings
	Gender	Age	VO_2max baseline						
Hebisz et al. (2021)a RCT (8 weeks)	POL: 10, 7♂ BT: 10, 7 ♂	POL: 18.5.0 ± 1.9 BT: 18.4 ± 1.6	POL: 57.2 ± 5.8 BT: 60.0 ± 4.8	National-level Mountain bike cyclists	NR	NR	Total training sessions (n): POL: 12x SIT, 12x HIIT, 16x LIT BT: 11x SIT, 11x HIIT, 18x LIT	VO_2max ($mL \cdot min^{-1} \cdot kg^{-1}$) P VT_2 [W]	VO_2max: ↑ P VT_2: ←→
Munoz et al. (2014) RCT (10-weeks)	POL: 16 ♂ THR: 16 ♂	POL: 34 ± 9 THR: 34 ± 7	POL: 61.0 ± 8.4 THR: 64.1 ± 7.3	Recreational endurance runners	POL: 75/5/20 THR: 46/35/19	TRIMP/week: POL: 330 ± 67 THR: 370 ± 98 Total TRIMP: POL: 3299 ± 670 THR: 3691 ± 982	Total running time (h): POL: 39.1 ± 7.9 THR: 36.3 ± 8.1 Weekly running distance averaged (km): 50 Weekly training frequency (n): 5-6	10km TT (min)	TT: ←→
Hebisz and Hebisz (2021)b RCT (9 weeks)	POL: 14 ♂ CG: 12 ♂	POL: 21.7 ± 7.7 CON: 20.5 ± 5.5	POL: 62.3 ± 6.4 CON: 59.6 ± 8.4	Mountain bike cyclists	NR	NR	Total training sessions (n): POL: 2 × SIT, 1 × HIIT, and 2 × ET CG: 2 × HIIT, 3 × ET	VO_2max ($mL \cdot min^{-1} \cdot kg^{-1}$)	VO_2max: ↑

Perez et al. (2019) RCT (12 weeks)	POL: 11 ♂ THR: 9 ♂	POL: 40,6 ± 9,7 THR: 36,8 ± 9,2	POL: 55.8 ± 4.9 THR 57.1 ± 5.2	Recreational ultra-endurance runners	POL: 79.8 ± 2.1/ 3.9 ± 1.9/ 16.4 ± 1.5 THR: 67.2 ± 4.6/33.8 ± 4.6/0	Total TRIMP: POL: 5906.0 ± 708.8 THR: 6061.2 ± 1726.2	Weekly training time (h): POL: 6.0 ± 0.8 THR: 6.5 ± 1.4 Weekly training frequency (n): 5	VO_2max ($mL \cdot min^{-1} \cdot kg^{-1}$) TTE (s)	VO_2max: ←→ TTE: ↑
Schneeweiss et al. (2022) RCT (4 weeks)	POL: 10, 8 ♂ LIT: 8, 6 ♂	POL: 18.4 ± 4.7 LIT: 17.4 ± 1.9	NR	Competitive XCO athletes	POL: 86.6/0/13.4 LIT: 100/0/0	NR	Total training time (h): POL: 25 LIT: 40	P LT_2 (W) TT (300s)	P LT_2: ←→ TT: ←→
Filipas et al. (2022) RCT (16 weeks)	POL: 14 ♂ PYR: 14 ♂	POL: 38 ± 5 PYR: 35 ± 6	POL: 69 ± 3 PYR: 68 ± 4	Well-trained male runners	POL: 78-81/4-7/14-15 PYR: 77-78/ 15-17/6-7	TRIMP/week: Week 1-8: PYR: 463 ± 77 POL: 464 ± 81 Week 9-18: PYR: 462 ± 78 POL: 465 ± 79	Weekly training time (min): Week 1-8: PYR: 358 ± 63 POL: 348 ± 57 Week 9-18: PYR: 358 ± 63 POL: 348 ± 56	VO_2max ($mL \cdot min^{-1} \cdot kg^{-1}$) 5-km TT (s) V LT_2 ($km \cdot h^{-1)}$	VO_2max: ←→ TT: ←→ V LT_2: ←→
Pla et al. (2019) RCT (6 weeks)	POL: 9 THR: 13 12 ♂, 10 ♀	POL: 17 ± 3 THR: 17 ± 3	POL: 56.0 ± 11.3 THR: 56.4 ± 12.4	Elite junior swimmers	POL: 81/4/15 THR: 25/65/10	NR	Weekly training distance (km) POL: 42 ± 4 THR: 42 ± 4	100m TT (s) V LT_2 (m/s)	TT: ↑ V LT_2 ↓
Festa et al. (2020) RCT	POL: 19, 15 ♂	POL: 43.2 ± 8.4 THR: 39.4 ± 8.5	POL 53.0 ± 5.9 THR: 53.7 ± 1.9	Recreational runners	POL: 77/3/20	TRIMP/week: POL: 308 ± 47.46	Total running time (h): POL: 29.9 ± 3.1	VO_2max ($mL \cdot min^{-1} \cdot kg^{-1}$)	VO_2max: ←→

(8 weeks)	THR: 19, 16 ♂				THR: 40/50/10	THR: 319.8 ± 28.1 Total TRIMP: POL: 2464 ± 124 THR: 2558.2 ± 10.9	THR: 24.8 ± 2.0	vRCT (km h^{-1}) 2km TT (s)	V RCT: ←→ TT: ←→
Zapata-Lamana et al. (2018) RCT (12 weeks)	POL: 14 ♀ MICT: 14 ♀ HIIT: 14 ♀ CG: 10 ♀	POL: 21.8 ± 1.9 MICT: 21.3 ± 1.4 HIIT: 21.2 ± 1.4 CG: 22.7 ± 3.2	POL: 24.5 ± 2.5 MICT: 22.7 ± 3.1 HIIT: 25.3 ± 2.6 CG: 25.0 ± 4.0	Untrained	POL: 70-80/0/ 20-30 NR	NR	Total number of cycling sessions (n): 36 Weekly number of cycling sessions (n): 3 Weekly cycling time (min): POL: 120 MICT: 135-150 HIIT: 156	VO_2peak (mL·min^{-1}·kg^{-1})	VO_2peak: ↑
Treff et al. (2017) N-RCT (11 weeks)	POL: 7 ♂ PYR: 7 ♂	POL: 21 ± 2 PYR: 19 ± 1	POL: 68 ± 7 PYR: 64 ± 3	National elite male rowers	POL: 93/2,1 ± 1/6 ± 3 PYR: 94 ± 3/3 ± 2/2 ± 1	NR	Total rowing distance (km): POL: 1334 ± 67 PYR: 1255 ± 264 Total rowing time (min): POL: 5953 ± 315 PYR: 5919 ± 1216 Total number of sessions (n): POL: 80 ± 4 PYR: 84 ± 13	VO_2max (mL·min^{-1}·kg^{-1}) 2000m TT (s) P LT_2 (W)	VO_2max: ←→ TT: ←→ P LT_2 ↓

T. L. Stoggl and Bjorklund (2017) RCT (9 weeks)	31	31 ± 6	61.9 ± 8.0 (range 54–75)	Competitive endurance athletes (cross-country skiing, cycling, triathlon, middle- or long-distance running)	POL: 68 ± 12/ 6 ± 7/26 ± 7 HIIT: 43 ± 1/0/ 57 ± 1 HVLIT: 64 ± 20/35 ± 21/ 1 ± 1	NR	Total training time (h): POL: 104 ± 21 HIIT: 66 ± 1 HVLIT: 93 ± 13 Total number of sessions (n): POL: 54 ± 7 HIIT: 47 ± 1 HVLT: 54 ± 8	V/P VT_2/LT_2 ($km \cdot h^{-1}$ or W)	V/P at VT_2/LT_2: ←→
Selles-Perez et al. (2019) N-RCT (13 weeks)	POL: 6 ♂ PYR: 7 ♂	POL: 28.5 ± 7.7 PYR 29.23 ± 6.8	Run POL: 52.8 ± 4.1 Run PYR: 58.1 ± 3.9 Bike POL: 50.5 ±2.9 Bike PYR: 54.1 ± 5.1	Recreational-level triathletes	POL: 84.5/4.2/11.3 PYR: 77.9/18.8/ 3.3	ECOs/week: POL: 785.2 ± 244.9 PYR: 751.6 ± 234.9	Total training time (h): 155 Weekly average training time (h): POL: 11.9 ± 3.5 PYR: 11.9 ± 3.6 Total training sessions (n): PYR: 106 (28 for swimming, 34 for cycling and 44 for running). POL: (28 for swimming, 34 for cycling and 45 for running).	VO_2max running ($mL \cdot min^{-1} \cdot kg^{-1}$) VO_2max cycling ($mL \cdot min^{-1} \cdot kg^{-1}$) V/P VT_2 (Running, Cycling and Swimming)	VO_2max running: ↑ VO_2max cycling: ←→ VT_2 Running: ↑ VT_2 Cycling: ←→ VT_2 Swimming: ↓
T. Stoggl and Sperlich (2014) RCT (9 weeks)	POL: 12 ♂ HIIT: 10 ♂ HVT: 11 ♂ THR: 8 ♂	31 ± 6	62.6 ± 7.1 (range: 52–75)	Competitive endurance athletes (cross-country skiing, cycling, triathlon,	POL: 68 ± 12/ 6 ± 8/26 ± 7 HIIT: 43 ± 1/ 0/ 57 ± 1,	NR	Total training time (h): POL: 104 ± 20 HIIT: 66 ± 1 THR: 84 ± 7 HVT: 102 ± 11	VO_2peak ($mL \cdot min^{-1} \cdot kg^{-1}$) TTE (s)	VO_2peak: ↑ TTE: ↑ V/P LT_2: ↑

				middle or long-distance running)	THR: 46 ± 7.54/7/0 HVT: 83 ±6 / 16 ± 6/1 ± 1		Total number of sessions (n): POL: 54 ± 3 HIIT: 47 ± 1 THR: 49 ± 3 HVT: 58 ± 3	V/P LT_2 ($km \cdot h^{-1}$ or W)	
Carnes and Mahoney (2018) RCT (12 weeks)	POL: 9, 8 ♂ CFE: 12, 7 ♂	POL: 44.2 ± 14.6 CFE: 41 ± 12.9	POL: 45.9 ± 7.1 CFE: 46.5 ± 6.9	Recreational distance runners Weekly training time (hours): <5	POL: 85/5/10 CFE: 48/8/44	TRIMP/week: POL: 389 ± 101 CFE: 222 ± 68 Total TRIMP: POL: 4610 ± 620 CFE: 2641 ± 270	Weekly average training time (min): POL: 283 ± 75.9 CFE: 117 ± 32.2 Weekly average distance (km): POL: 47.3 ± 11.6 CFE: 19.3 ± 7.17	VO_2max ($mL \cdot min^{-1} \cdot kg^{-1}$) 5km run TT (min)	VO_2max: ↑ 5km run TT: ←→
Neal et al. (2013) RCT (6 weeks)	POL: 11♂ THR: 11 ♂	37.6	NR	Well-trained male cyclists	POL: 80/0/20 THR: 57/43/0	TRIMP/week: POL: 517 ± 90 THR: 633 ± 119	Weekly training time (min): POL: 381 ± 85 THR: 458 ± 120	40km cycling TT(s) TTE 95% PPO (s) P LT_2 (W)	TT: ←→ TTE: ↑ P LT_2: ↑
Rohrken et al. (2020) RCT (6 weeks)	POL: 7, 5 ♂ THR: 8, 6 ♂	POL: 29.1 ± 7.6 THR: 30.3 ± 6.1	NR	Moderately trained triathletes	POL: 75.2 ± 14.4/11.1 ± 10.9/13.7 ± 4.1 THR: 77.8 11.9/20.3 ± 10.8/2.0 ± 1.5	TRIMP/week: POL:882.0 ± 155 THR:739.0 ± 162	Weekly training time (h): POL: 10.8 ± 2.4 THR: 10.0 ± 2.7	V LT_2 ($km \cdot h^{-1}$) P LT_2 (W)	V LT_2: ←→ P LT_2: ←→

Schumann et al. (2017) N-RCT (12 weeks)	POL: 14 ♂ TL: 10 ♂	POL: 34 ± 7 TL: 34 ± 7	POL: 46.4 ± 5.9 TL: 47.3 ± 3.5	Recreationally endurance-trained	NR	TRIMP/week: Week 0-4: POL: 301 ± 35 TL: 285 ± 28 Week 5-8: POL: 315 ± 41 TL: 306 ± 42 Week 9-12: POL: 358 ± 52 TL: 269 ± 85	Weekly training time (h): POL: 10.8 ± 2.4 CON: 10.0 ± 2.7	VO_2max ($mL \cdot min^{-1} \cdot kg^{-1}$) 1000m TT (min) TTE (min)	VO_2max: ↑ TT: ←→ TTE: ←→
Esteve-Lanao et al. (2007) RCT (24 weeks)	POL: 6 ♂ THR: 6 ♂	27 ± 2	POL: 68.6 ± 2.4 THR: 70.3 ± 2.6	Well-trained runners	POL: 80/10/10 THR: 65/25/10	TRIMP/week: POL: 452 ± 23 THR: 460 ± 26 Total TRIMP: POL: 8134 ± 408 THR: 8277 ± 463	Total training time in Z1 (min): POL: 5246 ± 396 THR: 3830 ± 215 Total Time in Z2 (min): POL: 779 ± 116 THR: 1411 ± 95 Total Time in Z3 (min): POL: 502 ± 78 THR: 485 ± 65	10.4 km run TT (min)	TT: ↑

Abbreviations: BT = block training; CFE© = CrossFit endurance training ©; CG = Control Group; ECOs = Objective Load Equivalents; ET = Endurance training; h = hours; HIIT = high intensity interval training; HVLIT = high volume low intensity training; $km.h^{-1}$ = kilometers per hour; LIT = low intensity training; $m.s^{-1}$ = meters per second; MICT = moderate intensity continuous training; n = number of sessions; N-RCT = Non Randomized Controlled Trial; NR = Not reported; P LT_2 - power at 2nd lactate threshold; P VT_2 – power at 2nd ventilatory threshold; POL = polarized training; PPO = peak power output; PYR = pyramidal training; RCT = Randomized Controlled Trial; RCT = respiratory compensation threshold; s = seconds; SIT = sprint interval training; THR = threshold training; TID = training intensity distribution; TL = training load guided; TRIMP = training impulse; TT = time trial; TTE = time-to-exhaustion; V LT_2 – velocity at 2nd lactate threshold; V RCT = Velocity at respiratory compensation threshold; V VT2 – velocity at 2nd ventilatory threshold; V/P at VT_2/LT_2 = velocity or power at 2nd ventilatory or lactate threshold; VO_2max = maximal oxygen uptake; VO_2peak = peak oxygen uptake; W= Watts; Z1 = training zone 1; Z2 = training zone 2; Z3 = training zone 3.

Note: Age is expressed as means ± SD.

Symbols: Female (♀), male (♂), decreased (↓), increased (↑) and equal (←→) regarding POL compared to other TIDs

3.3. Qualitative synthesis of findings

For VO_2max/VO_2peak, differences between groups after the intervention were not observed in six studies (Festa et al., 2020; Filipas et al., 2022; Perez et al., 2019; Selles-Perez et al., 2019; Treff et al., 2017; Zapata-Lamana et al., 2018). Five studies (Carnes & Mahoney, 2018; Hebisz & Hebisz, 2021; Hebisz et al., 2021; Schumann et al., 2017; T. Stoggl & Sperlich, 2014) concluded that POL was superior compared to other TIDs and no studies suggested that POL was inferior. For the TT, eight studies (Carnes & Mahoney, 2018; Festa et al., 2020; Filipas et al., 2022; Munoz et al., 2014; Neal et al., 2013; Schneeweiss et al., 2022; Schumann et al., 2017; Treff et al., 2017) did not observed differences between interventions and two studies (Esteve-Lanao et al., 2007; Pla et al., 2019) showed favorable results for POL. Of the four studies analyzing TTE, three reported superiority of POL (Neal et al., 2013; Perez et al., 2019; T. Stoggl & Sperlich, 2014) with one study (Schumann et al., 2017) not reporting differences between interventions. Regarding V/P at VT_2/LT_2, eight studies did not revealed differences between interventions (Festa et al., 2020; Filipas et al., 2022; Hebisz et al., 2021; Perez et al., 2019; Rohrken et al., 2020; Schneeweiss et al., 2022; Selles-Perez et al., 2019; T. L. Stoggl & Bjorklund, 2017), two studies favored POL (Neal et al., 2013; T. Stoggl & Sperlich, 2014) and three studies (Pla et al., 2019; Selles-Perez et al., 2019; Treff et al., 2017) suggested superiority of other TIDs.

3.4. Risk of bias in studies

The RoB-2 quality assessment showed that all studies were rated as having some concerns due to issues in the randomization process (D1) and selection of the reported results, except for two studies (Filipas et al., 2022; Zapata-Lamana et al., 2018) that were rated as having low risk of bias in D1.

The ROBINS-I quality assessment showed that two studies (Selles-Perez et al., 2019; Treff et al., 2019) were rated as having low risk of bias due to having no concerns regarding confounding, selection of participants, classification of interventions, deviations from intended interventions, missing data, measurement of outcomes and selection of the reported results. One study (Schumann et al., 2017) was rated as having some concerns due to moderate bias in the

measurement of outcomes. The RoB-2 assessment of all RCTs and the ROBINS-I assessment of all N-RCTs is shown in Fig. 2 and Fig. 3, respectively.

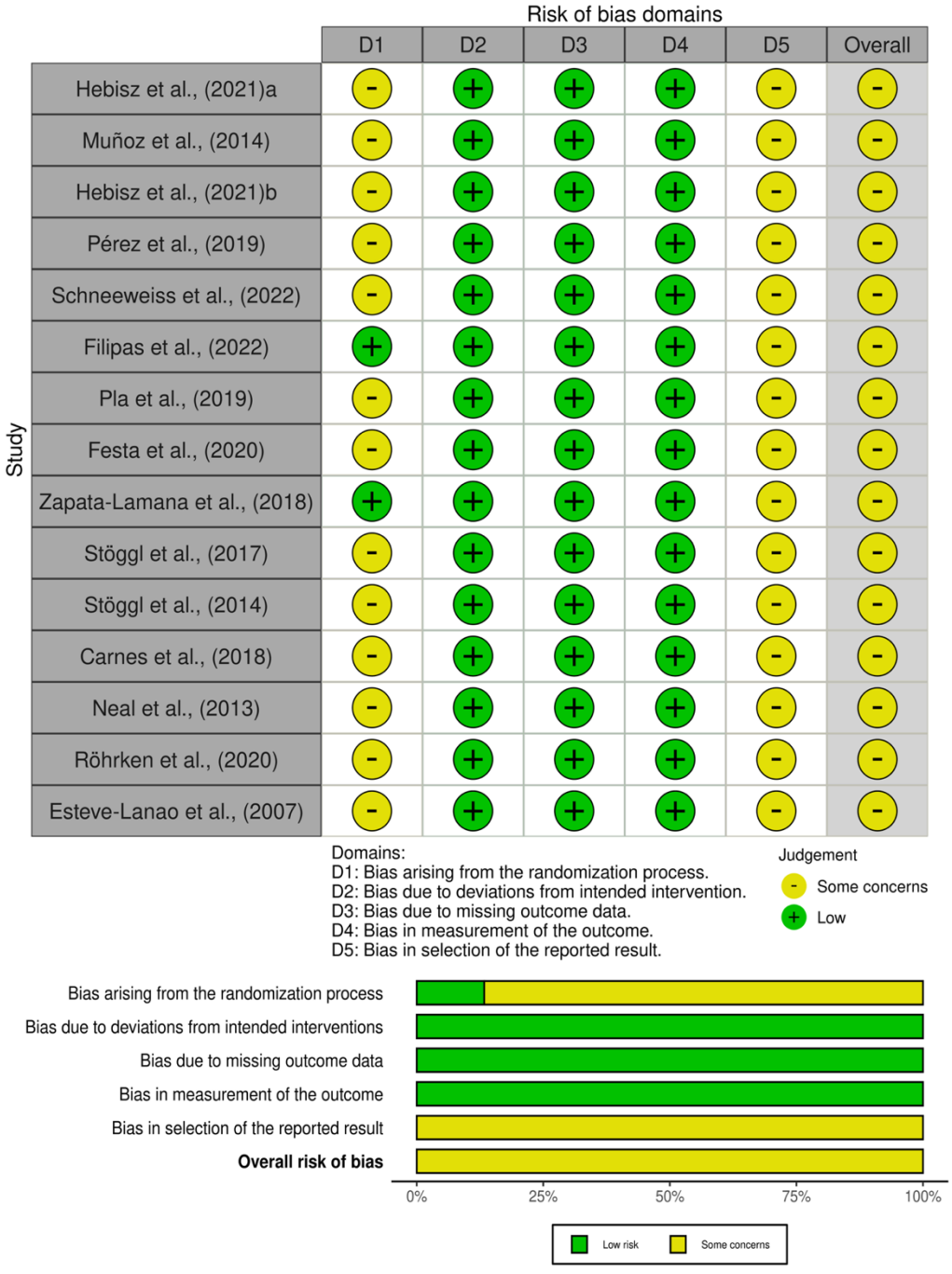


Fig. 2 Assessment of risk of bias of randomized trials with RoB-2

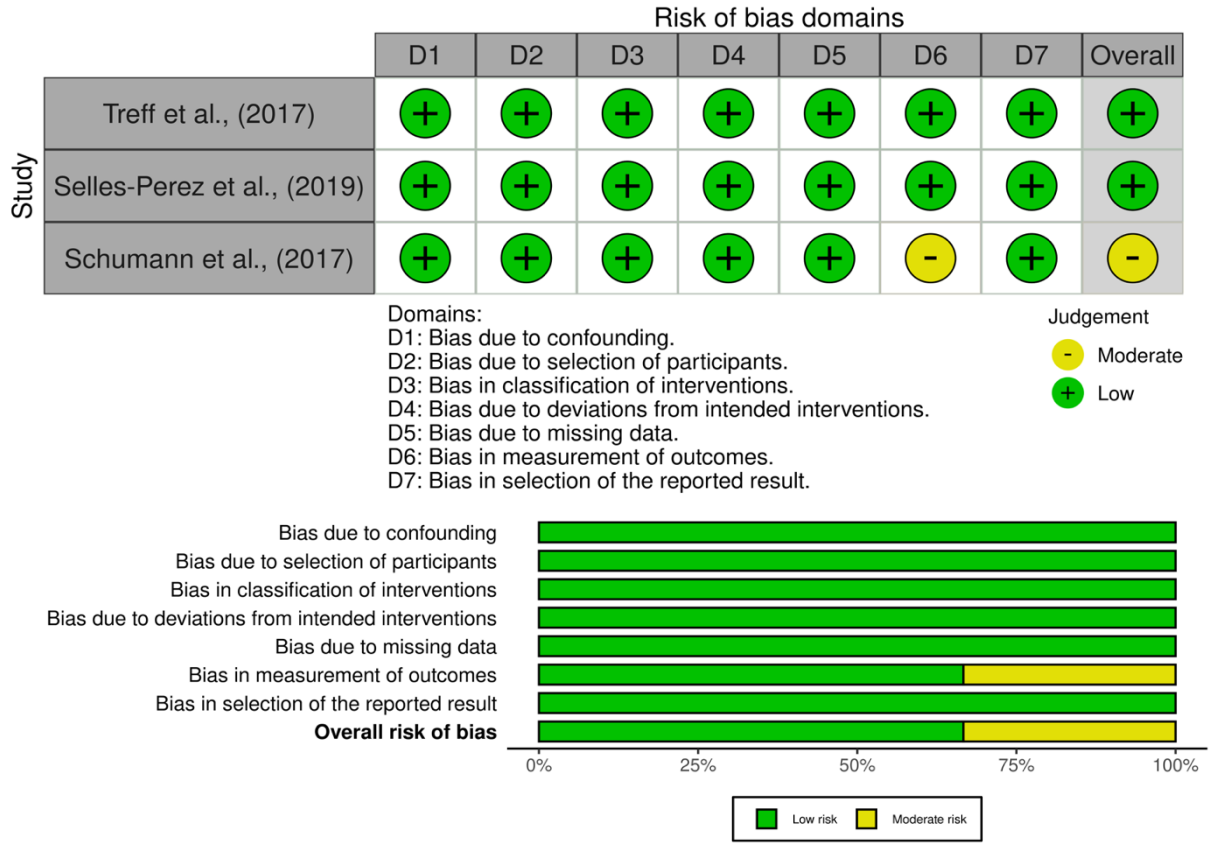


Fig.3 Assessment of risk of bias of non-randomized trials with ROBINS-I

3.5. Meta-analysis

3.5.1. Effect of POL compared to other TIDs on VO_2max/peak

Fig. 4 shows the pooled effect estimates of POL compared to other TIDs on VO_2max/peak. POL was shown to be superior to other TIDs in the improvement of VO_2max/peak, although with a small effect size (SMD=0.24; 95% CI 0.01, 0.48; z = 2.02; p = 0.040).

VO_2max/peak

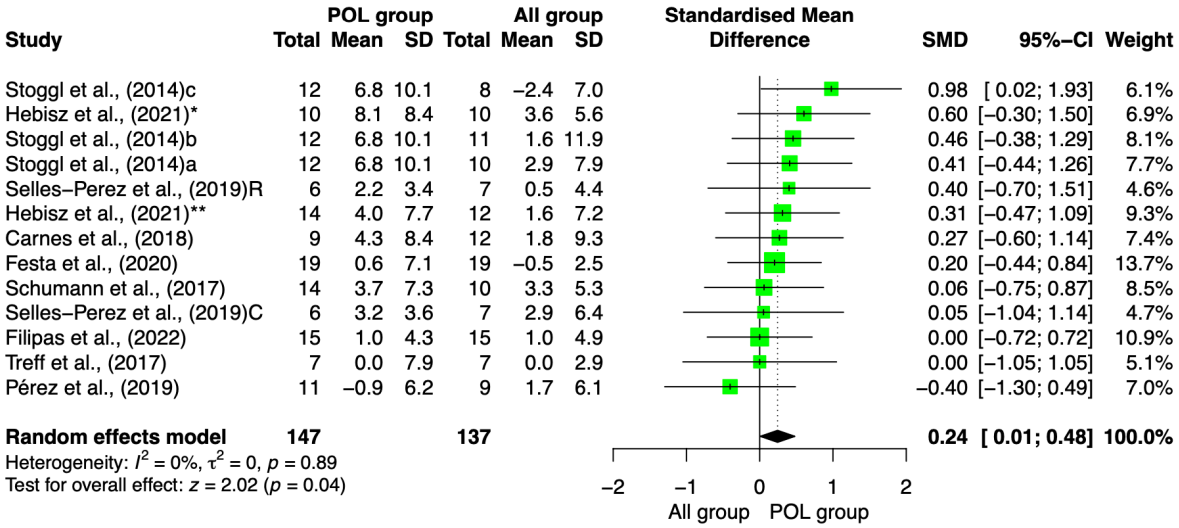


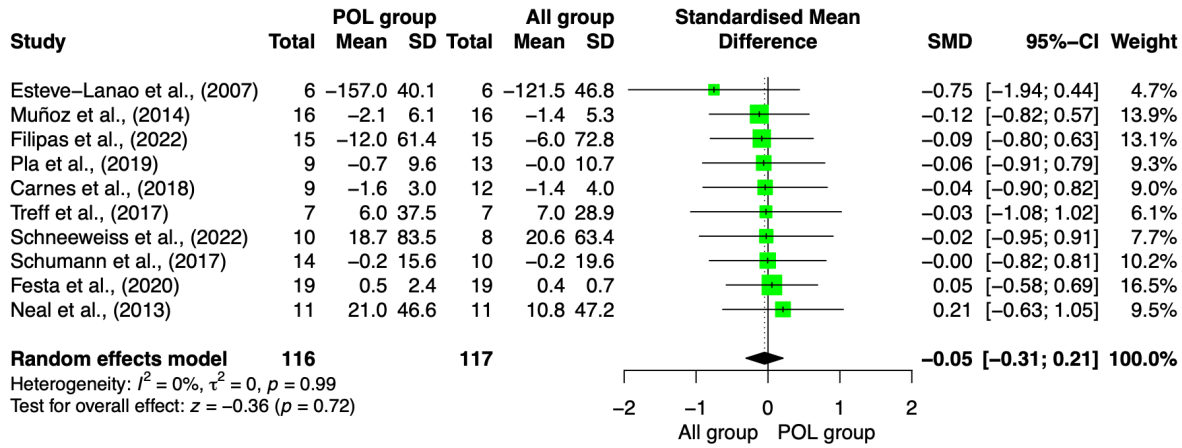
Fig. 4 Effect of POL compared to other TIDs on VO_2max/peak

Figure legend:
Stoggl et al., (2014)c = POL vs THR; Stoggl et al., (2014)b = POL vs HVT; Stoggl et al., (2014)a = POL vs HIIT; Selles−Perez et al., (2019)R = Running; Selles−Perez et al., (2019)C = Cycling; Selles−Perez et al., (2019)

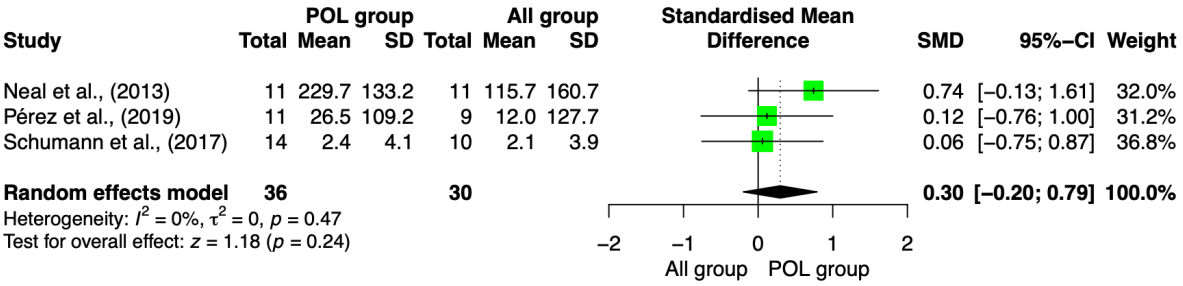
3.5.2. Effect of POL compared to other TIDs on TT, TTE, and V/P at VT_2/LT_2

POL in comparison to other TIDs was not shown to induce significant improvements on any of the study secondary outcomes, namely TT (SMD=-0.05; 95% CI -0.31, 0.21; z = −0.36; p = 0.72), TTE (SMD=0.30; 95% CI -0.20, 0.79; z = 1.18; p = 0.24) and V/P at VT_2/LT_2 (SMD=0.04; 95% CI -0.21, 0.29; z = 0.32; p = 0.75); Fig. 5.

Time trial



Time to exhaustion



Velocity or power at 2nd ventilatory or lactate threshold

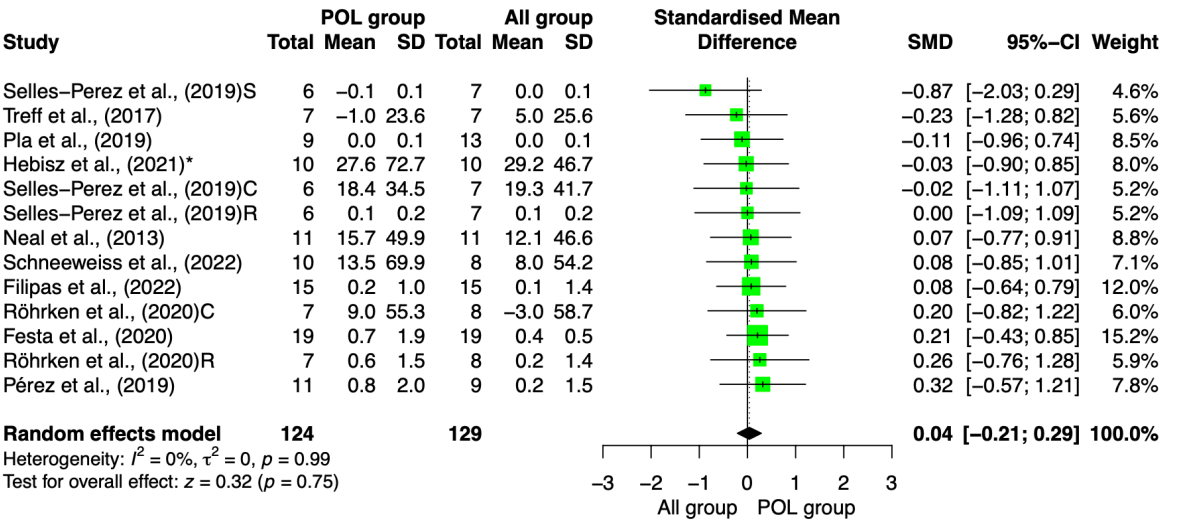


Fig. 5 Effect of POL compared to other TIDs on TT, TTE, and V/P at VT_2/LT_2

Figure legend:
Selles−Perez et al., (2019)S = Running; Selles−Perez et al., (2019)C = Cycling; Selles−Perez et al., (2019)R = Swimming; Röhrken et al., (2020)C = Cycling; Röhrken et al., (2020)R = Running.

3.5.3. Comparison of the effect of POL versus THR, PYR, HIIT and CG

No significant differences were identified when comparisons between POL and each one of the other TIDs were performed for any of the primary or secondary outcomes of the study. Detailed results are displayed on Table 2.

Table 2 Sub Analysis of the effect of POL *vs* THR, POL *vs* PYR, POL *vs* HIIT, POL *vs* CG

Intervention groups	**VO_2max/peak**				**TT**				**TTE**				**V/P at VT_2/LT_2**			
	N	SMD (95% CI)	I^2	Z (p)	N	SMD (95% CI)	I^2	Z (p)	N	SMD (95% CI)	I^2	Z (p)	N	SMD (95% CI)	I^2	Z (p)
POL *vs* THR	78	0.24 (-0.46; 0.94)	53%	0.67 (0.51)	126	-0.05 (-0.41; 0.30)	0%	-0.30 (0.76)	42	-0.43 (-0.18; 1.05)	0%	-1.38 (0.17)	132	0.15 (-0.19; 0.50)	0%	0.88 (0.38)
POL *vs* PYR	70	0.08 (-0.39; 0.55)	0%	0.34 (0.73)	44	-0.07 (-0.66; 0.52)	0%	-0.23 (0.82)	-	-	-	-	83	-0.14 (-0.57; 0.30)	0%	-0.61 (0.54)
POL *vs* HIIT	43	0.34 (-0.27; 0.95)	0%	1.10 (0.27)	-	-	-	-	-	-	-	-	-	-	-	-
POL *vs* CG	93	0.34 (-0.07; 0.76)	0%	1.63 (0.10)	42	-0.01 (-0.62; 0.60)	0%	-0.04 (0.97)	-	-	-	-	38	0.03 (-0.61; 0.66)	0%	0.08 (0.94)

Abbreviations: CG: Control Group; HIIT: High Intensity Interval Training; POL: Polarized Training; PYR: Pyramidal Training; THR: Threshold Training; TT: Time Trial; TTE: Time to exhaustion; V/P at VT_2/LT_2: Velocity or power at 2nd ventilatory or lactate threshold; VO_2max/peak: Maximal/peak oxygen uptake.
Note: SMD: standardized mean difference; I^2 (p): heterogeneity and p-value, Z (p): test for overall effect and p-value.
*Statistical significance: p= <0.05

3.5.4. Effect of POL compared to other TIDs according to intervention duration (<12 and ≥12 weeks)

A sub-analysis according to training intervention duration was also performed and is shown in Table 3, in which interventions were divided into two categories: <12 weeks duration and ≥12 weeks duration. POL was only shown to be superior to other TIDs on VO_2max when intervention duration was <12 weeks (SMD=0.33; 95% CI 0,04, 0.63; z = 2.21; p = 0.03; Fig. 6). For TT (p=0.98), and V/P at VT_2/LT_2 (p=0.65) there were no differences between POL and other TIDs according to intervention duration. When training duration was ≥12 weeks POL was not shown to be superior for any of the study outcomes (VO_2max/peak, p=0.83; TT, p=0.53; TTE, p=0.78; V/P at VT_2/LT_2, p=0.93).

Table 3 Sub Analysis of the effect of POL compared to other TIDs according to intervention duration and to athlete level

Intervention groups	VO_2max/peak				Time Trial				Time to Exhaustion				V/P at VT_2/LT_2			
	N	SMD (95% CI)	I^2	Z (p)	N	SMD (95% CI)	I^2	Z (p)	N	SMD (95% CI)	I^2	Z (p)	N	SMD (95% CI)	I^2	Z (p)
POL *vs* ALL (<12 weeks)	185	0.33 (0.04; 0.63)	0%	2.21 (0.03)	146	0.00 (-0.32; 0.33)	0%	0.03 (0.98)	-	-	-	-	164	0.07 (-0.24; 0.38)	0%	0.46 (0.65)
POL *vs* ALL (≥12 weeks)	121	0.04 (-0.32; 0.40)	0%	0.22 (0.83)	87	-0.14 (-0.56; 0.29)	0%	-0.63 (0.53)	44	0.09 (-0.51; 0.68)	0%	0.28 (0.78)	89	-0.02 (-0.44; 0.40)	0%	-0.09 (0.93)
POL *vs* ALL (Highly Trained)	56	0.15 (-0.38 0.68)	0%	0.55 (0.58)	54	-0.04 (-0.58; 0.50)	0%	-0.14 (0.89)	-	-	-	-	74	-0.06 (-0.52; 0.40)	0%	-0.27 (0.79)
POL *vs* ALL (Well/Mod Trained)	121	0.37 (0.01; 0.74)	0%	2.01 (0.04)	64	-0.10 (-0.59; 0.40)	0%	-0.39 (0.69)	-	-	-	-	84	0.13 (-0.30; 0.56)	0%	0.59 (0.56)
POL *vs* ALL (Recreational)	129	0.10 (-0.25; 0.45)	0%	0.55 (0.58)	115	-0.02 (-0.39; 0.34)	0%	-0.13 (0.90)	44	0.09 (-0.51; 0.68)	0%	0.28 (0.78)	97	0.04 (-0.36; 0.44)	0%	0.20 (0.84)

Abbreviations: All = Other Training Intensity Distribution; POL: Polarized Training; TT: Time Trial; TTE: Time to exhaustion; V/P at VT_2/LT_2: Velocity or power at 2nd ventilatory or lactate threshold; VO_2max/peak: Maximal/peak oxygen uptake.
Note: SMD: standardized mean difference; I^2 (p): heterogeneity and p-value, Z (p): test for overall effect and p-value.
*Statistical significance: p= <0.05.

VO_2max/peak

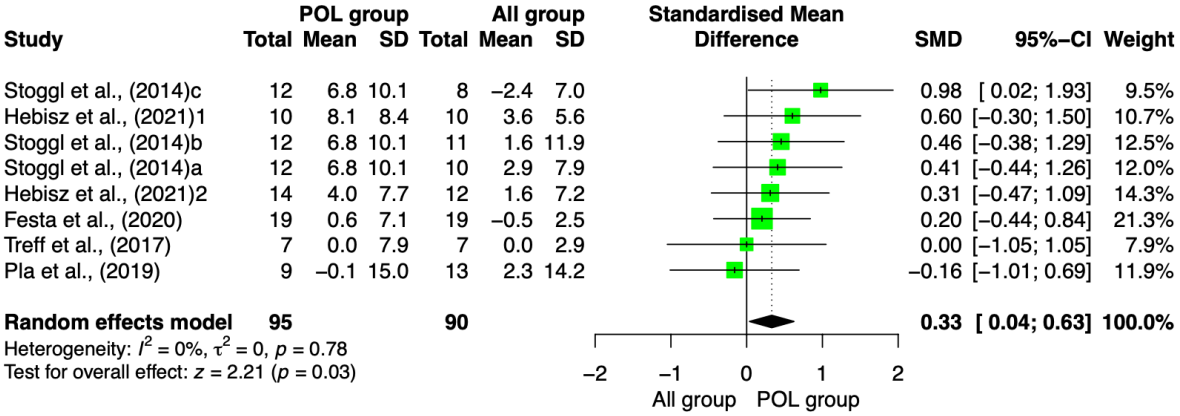


Fig. 6 sub-analysis of POL vs ALL <12 weeks

Figure legend:
Stoggl et al., (2014)c = POL vs THR; Stoggl et al., (2014)b = POL vs HVT; Stoggl et al., (2014)a = POL vs HIIT

3.5.5. Effects of POL compared to other TIDs according to athlete level

A sub-analysis comparing the effect of cardiorespiratory fitness starting level (recreational *vs* moderately/well trained *vs* highly trained athletes) on cardiorespiratory adaptations was performed (Table 3). In recreational athletes, POL was not superior to other TIDs on VO_2max/peak (p=0.58), TT (p=0.90), TTE (0.78) and V/P at VT_2/LT_2 (p=0.84). However, there were differences favoring POL on VO_2max/peak for moderate/well level athletes (SMD=0.37; 95% CI 0.01, 0.74; z = 2.01; p = 0.040), despite there were no differences on TT (p=0.69), and V/P at VT_2/LT_2 (p=0.56); Fig. 7. In the comparison between POL and other TIDs including only highly trained athletes there were no differences on VO_2max/peak (p=0.58), TT (p=0.89) and V/P at VT_2/LT_2 (p=0.79).

VO_2max/peak

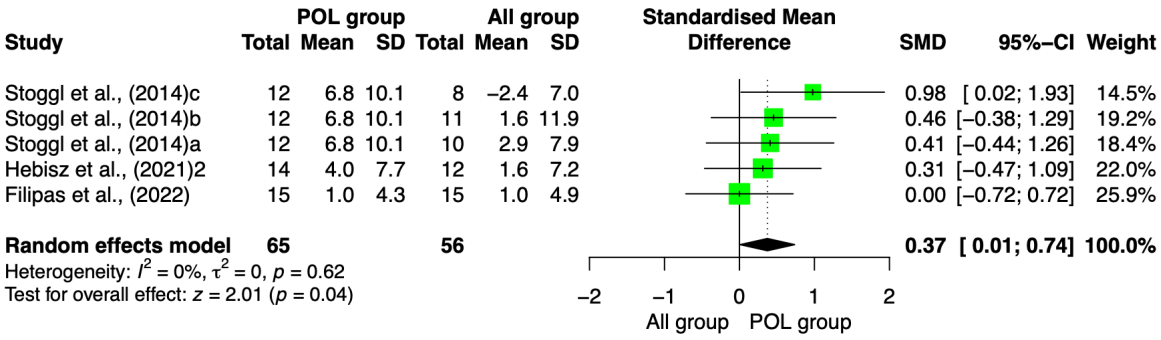


Fig. 7 Sub-analysis of POL vs ALL according to competitive level – moderate/well trained athletes

Figure legend:
Stoggl et al., (2014)c = POL vs THR; Stoggl et al., (2014)b = POL vs HVT; Stoggl et al., (2014)a = POL vs HIIT

3.5.6. Effect of Gender

Although in the pre-registration of this work a sub-analysis comparing the effect of POL vs other TIDs between males and females was proposed (gender effect on the adaptation to POL) it was not possible to perform this analysis due to insufficient gender reporting in the included studies.

3.6. Sensitivity analysis

All the analysis performed revealed low or inexistence of heterogeneity. The analysis with the highest heterogeneity was a secondary analysis of POL vs THR for VO_2max/peak (I^2 = 53%). A sensitivity analysis for this comparison was performed by removing one study at a time. The study that was shown to contribute the most to heterogeneity was the one by Stoggl et al., (2014)c (T. Stoggl & Sperlich, 2014). Nevertheless, removal of this study did not affect the results (p= 0.95) [Appendix S3 of the ESM].

3.7. Certainty of the evidence

According to the GRADE approach, all outcomes were classified as having a high certainty of the evidence [Appendix S4 of the ESM].

3.8. Publication bias

Publication bias was assessed for studies comparing athletes performing POL vs other TIDs on VO_2max/peak, TT and V/P at VT_2/LT_2. A non-significant publication bias was revealed by both the funnel plot symmetry and the Egger's test result adjusted to VO_2max/peak (Bias coefficient= -0.042 (intercept); p= 0.632), TT (Bias coefficient= 0.467 (intercept); p= 0.143) and V/P at VT_2/LT_2 (Bias coefficient= -0.708 (intercept); p= 0.092). Detailed results are depicted in Appendix S5 of the ESM.

4. Discussion

This study aimed to systematic review and meta-analyze the evidence comparing the effect of POL with other TIDs in cardiorespiratory function. POL was found to be superior to other TIDs for VO_2max/peak improvement, although with a small magnitude of effect (SMD=0.24 [95% CI 0.01, 0.48]; z = 2.02 (p = 0.040); n = 284; I2 = 0%). Regarding the secondary outcomes, there was no evidence of superiority of POL compared to other TIDs.

To date, only one systematic review with meta-analysis (Rosenblat et al., 2019) compared POL with THR but included only TT as a surrogate of endurance performance. In addition, this meta-analysis contained only 3 studies due to the scarcity of date available at that time. Their results suggested superiority of POL compared to THR for TT improvement which is in opposition to our findings. This disparity is due to difference in the number of studies included in the analysis. The increased interest on POL, has led to a surge in the number of experimental studies investigating this TID. Consequently, our study included a sample of n=449 participants compared to n=112 in Rosenblat et al. study (Rosenblat et al., 2019). Another important difference was the inclusion of other TIDs as well as a set of variables strongly correlated with cardiorespiratory fitness such as VO_2max/peak, TT, TTE and V/P at VT_2/LT_2, other than TT, which allows for a much thorough understanding of the effectiveness of POL.

Our results showed that VO_2max/peak was higher in POL compared to other TIDs. Results had a low heterogeneity but a small effect size. POL may have been more effective in promoting VO_2max adaptations as it exposes athletes to a combination of low and high intensity exercise, which appears to be particularly suited to the development of central and peripheral aerobic adaptations (Bartlett et al., 2012). The main central adaptation to endurance training is the increase in cardiac output, which results mainly from increases in blood volume, left ventricle end-diastolic volume and myocardial contractility (Jones & Carter, 2000; Rosenblat, Granata, & Thomas, 2022). Of these adaptations, the one that most likely contributes to improvements in VO_2max in studies with a short duration, such as those included in our systematic review, is an increase in blood volume. It has been shown that low-intensity aerobic training is effective in increasing plasma volume and cardiac output (Pollock, 1977). However, high-intensity training appears to be an even more effective strategy

for this purpose (Helgerud et al., 2007; Milanović, Sporiš, & Weston, 2015). Previous studies have shown that intensity is a crucial variable in exercise-induced hypervolemia and that higher exercise intensities seem particularly effective in inducing rapid elevations in plasma volume (Green et al., 1984; Warburton et al., 2004). For instance, adding a short period of high intensity exercise, between 90 to 95% VO_2max, to well-trained runners, significantly increased their blood volume by 4% (Richardson, Verstraete, Johnson, Luetkemeier, & Stray-Gundersen, 1996). Increases in blood volume of 10% 24h after a single exercise session at 85% VO_2max has also been reported (Gillen et al., 1991). Evidence suggesting that high intensity exercise rapidly increases blood volume is also in accordance with the findings of our sub-analysis showing that it was on shorter interventions (<12 weeks) that POL was particularly effective compared to other TIDs on improving VO_2max/peak (SMD=0.33; 95% CI 0.04, 0.63; z = 2.21; p = 0.03).

Improvements in VO_2max also depend, to a large extent, of peripheral skeletal muscle adaptations favoring capillary oxygen extraction and use by fiber mitochondria (van der Zwaard, Brocherie, & Jaspers, 2021). Although it is well demonstrated (A. Casado et al., 2022) that high-volume, low-intensity training favors mitochondrial biogenesis (Granata, Oliveira, Little, Renner, & Bishop, 2016a), increases lactate oxidation rate (González-Mohíno et al., 2016) and type I muscle fibers capillarization (Egan & Zierath, 2013; van der Zwaard et al., 2021), there is also evidence that high intensity is a key factor in peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) activation and mitochondrial biogenesis (M. J. Gibala, Little, Macdonald, & Hawley, 2012). A single high intensity exercise bout was shown to induce greater elevations in PGC-1α mRNA compared to low intensity exercise (Egan et al., 2010). This might be explained by the fact that high-intensity exercise is a major stimulus for ATP depletion and accumulation of ADP and AMP which will thereby activate PGC1-α and trigger mitochondrial biogenesis (Chandel, 2015; M. Gibala, 2009; Hawley, Hargreaves, Joyner, & Zierath, 2014). Training at higher intensities, has also been shown to lead to faster improvements in endurance performance (Driller, Fell, Gregory, Shing, & Williams, 2009; NJ, Harrison, & Warrington, 2017) and to faster peripheral adaptations compared to training at moderate or low intensity (Schubert, Clarke, Seay, & Spain, 2017). This may also be due to the greater

recruitment of type II muscle fibers which positively affects their oxidative capacity (Conwit et al., 1999; Poole & Gaesser, 1985; Torma et al., 2019). For instance, high intensity exercise seems to induce superior adaptations in type II fibers oxidative metabolism compared to low-intensity aerobic training (David J. Bishop, Granata, & Eynon, 2014). In addition, when high intensity exercise is performed at an intensity greater than VO_2peak, the oxidative capacity of type IIx muscle fibers is enhanced (Dudley, Abraham, & Terjung, 1982). Collectively, this evidence suggests that adding high intensity exercise to the training regimen favors a faster development of both central and peripheral adaptations optimally enhancing thereby VO_2max. This is in agreement with our sub-analysis showing that POL training is particularly effective in inducing faster increases in VO_2max/peak. Nevertheless, the advantage of POL seems to wane for interventions longer than 12 weeks, suggesting that subjects that undergo other TIDs might also develop adaptations to the same extent of those induced by POL, but more slowly. For this reason, POL might be a more interesting strategy to induce faster improvements in VO_2max, such as for instance in prehabilitation exercise contexts (Yue, Wang, Liu, Kong, & Qi, 2022).

However, despite high VO_2max is one of the determinants of cardiorespiratory fitness (di Prampero, 2003), other variables are also important for endurance performance (Jones et al., 2021; Pollock, 1977). Anaerobic threshold, which translates into the ability to maintain high workloads without exponentially increasing blood lactate concentration (Brooks, 1986) is a variable that is more strongly correlated with endurance sports performance (Støa et al., 2020). Interestingly, our results suggest that, for this variable, there is no evidence of superiority of POL compared to other TIDs ($p=0.75$).

Endurance training plays an important role in reducing blood lactate concentration for a given exercise intensity (Jones & Carter, 2000). This reduction seems to be a consequence of a lower rate of muscle glycogen utilization (Favier, Constable, Chen, & Holloszy, 1986), accelerated O_2 consumption kinetics (MacRae, Dennis, Bosch, & Noakes, 1992) and the ability to effectively remove blood lactate (Donovan & Pagliassotti, 1989). The most plausible physiological rationale for increasing velocity at the anaerobic threshold is increased lactate clearance (Bonen, Baker, & Hatta, 1997). After both low-intensity (Dubouchaud, Butterfield, Wolfel, Bergman, & Brooks, 2000) and high-intensity (Juel et al.,

2004) endurance training interventions, concentrations of monocarboxylic transporters (MCT), namely MCT1 and MCT4 seem to increase. A high abundance of MCT facilitates lactate and hydrogen ions transport and increases muscle lactate clearance (Juel, 1997; Juel & Halestrap, 1999). In fact, this is verifiable by analyzing the expression of MCT1 in well trained subjects which is much higher compared to less trained subjects (Thomas et al., 2005). Furthermore, the rate of lactate removal after maximal exertion correlates with MCT1 expression, favoring thereby high ATP utilization rates without major increases in blood lactate (Goulding & Marwood, 2023). Considering that both low intensity and high intensity exercise seem to induce similar adaptations in the mechanisms involved in lactate production and removal, it is not surprising that several studies using different exercise training intensities, such as threshold training (Evertsen, Medbø, & Bonen, 2001; Neal et al., 2013), training above the anaerobic threshold (Keith, Jacobs, & McLellan, 1992; Weltman et al., 1992) and POL (M. J. Gibala & Jones, 2013; Neal et al., 2013) were all effective in improving anaerobic threshold which is in agreement with our findings that POL is similar to other TIDs regarding V/P at VT_2/LT_2.

TT is a variable highly correlated with endurance performance (Russell, Redmann, Ravussin, Hunter, & Larson-Meyer, 2004). In our study, there was no evidence of superiority of POL compared to other TIDs in the improvement of TT ($p=0.72$). Nevertheless, the studies included in our meta-analysis displayed a high variability in terms of TT distances, ranging from 100m (Pla et al., 2019) to 40km (Neal et al., 2013), which correlate very differently with performance in aerobic activities, and therefore some of these results might not necessarily reflect adaptations of the aerobic metabolism. TT in endurance activities, is highly dependent of VO_2max and, especially for longer distances, on LT and running economy (di Prampero, 2003; Lucía et al., 2002; Ø Støren, Bratland-Sanda, Haave, & Helgerud, 2012; Øyvind Støren, Ulevåg, Larsen, Støa, & Helgerud, 2013). Considering our findings that POL is similar to other TIDs regarding improvements in P/V at VT_2/LT_2 and that POL is only able to marginally improve VO_2max, specially for shorter duration interventions, it is not surprising that the results from our meta-analysis suggest that TT can be similarly improved by several TIDs.

Our meta-analysis included only three studies (Neal et al., 2013; Perez et al., 2019; Schumann et al., 2017) analyzing TTE. The protocols by Perez et al., (Perez et al., 2019) and Schumann et al., (Schumann et al., 2017) consisted of incremental tests until exhaustion while Neal et al., (Neal et al., 2013) performed a protocol at 95% of peak power output in cycle ergometer to exhaustion. In this type of tests, in which athletes perform exercise at high intensity, there is a marked production and accumulation of lactate and hydrogen ions (Thomas, Bishop, Moore-Morris, & Mercier, 2007). Consequently, muscle pH will drop dramatically (Pilegaard et al., 1999) and, therefore, effective pH regulation, which is highly dependent of skeletal muscle buffering capacity (Edge, Bishop, & Goodman, 2006), is a crucial factor for prolonging TTE. Interestingly, since high intensity exercise recruits a substantial portion of fast-twitch muscle fibers, this has been shown to favor resistance to fatigue at higher intensities, therefore prolonging TTE (Poole & Gaesser, 1985; Weston et al., 1997). However, in recreational runners, continuous aerobic training at intensities between 60 and 80% VO_2max have also shown to effectively improve TTE by increasing cardiac output and oxidative enzyme activity (Bertuzzi et al., 2012). Therefore, different physiological adaptations, induced by different exercise intensities seem to be able to effectively improve TTE in endurance activities explaining the absence of differences between POL and other TIDs regarding improvements in TTE identified in our meta-analysis.

Knowing *a priori* that the starting cardiorespiratory level of the subjects could a differentiating factor in the magnitude of the cardiorespiratory response to the training stimulus, we carried out a sub-analysis of our variables of interest according to the initial cardiorespiratory fitness level of the subjects (recreational, moderately/ well trained, and highly trained athletes). Our results showed that baseline performance level did not influenced the effectiveness of POL compared to other TIDs in the improvement in each one of the variables included in our meta-analysis, except for moderately/well trained athletes regarding VO_2max/peak improvement in which POL was superior to other TIDs. This finding is in agreement with previous studies showing that adding a period of high intensity exercise to even well-trained athletes effectively induces several hematological adaptations (Richardson et al., 1996) that could favor VO_2max/peak increases (Astorino, Allen, Roberson, & Jurancich, 2012).

One of the main limitations of this study is that several of the included reports do not disclose the percentage of TID. Although the authors classify the training model as POL or PYR, without data on the %TID performed at each zone, it is problematic to robustly state which model was in fact followed. Another necessary criticism is the underreporting of weekly TRIMPs or variables such as volume, intensity, and frequency of the entire training program. Therefore, it is possible that groups might have differed not just in intensity but also in other crucial variables that were not accounted for. In addition, several studies lack adequate description of the training program variables, namely weekly frequency, type of sessions and robust measures of volume and intensity. It is also noteworthy to mention that although our study included several important variables for cardiorespiratory fitness and endurance performance, the success in endurance activities also depends on other aspects that were not assessed. For instance, Seiler et al., (S. Seiler, Haugen, & Kuffel, 2007) and Boullosa et a., (Boullosa & Nakamura, 2010) argue that the characteristics of POL favor a reduction in fatigue and therefore that when training volumes are substantially high, POL may be a superior strategy in reducing the risk of overtraining. Consequently, although our study has identified only marginal benefits of POL in improving variables related with endurance performance, future studies should further investigate other determinants of endurance performance success, namely those related with recovery (Cadegiani & Kater, 2017; Laurent et al., 2011; Nuuttila, Nummela, Häkkinen, Seipäjärvi, & Kyröläinen, 2021; Stanley, Peake, & Buchheit, 2013).

5. Conclusions

In conclusion, the results of our systematic review and meta-analysis suggest that POL is superior to other TIDs for the improvement of VO_2max/peak, but with a small effect size, particularly for shorter duration interventions and for the case of moderately/well trained subjects. There was no evidence of superiority of POL regarding TT, TTE and V/P at VT_2/LT_2. These findings do not seem to be influenced by the subjects starting cardiorespiratory fitness level. POL could be a more effective strategy to increase VO_2max/peak in a short period of time.

Chapter II

Experimental Work

1. Material and Methods

1.1. Ethical approval

All experimental procedures involving animals were performed in accordance with the guidelines for the Care and Use of Laboratory Animals in Research recommended by the European Federation of Associations for the Science of Laboratory Animals (FELASA). The Ethics Committee of the Faculty of Sport of the University of Porto approved the experimental protocol (CEFADE 19-2023). The study was carried out in the Laboratories of Experimental Morphology and Biochemistry (LBME) of the Faculty of Sport of the University of Porto (FADE-UP).

1.2. Animals and Experimental Groups

Fifteen Wistar Han rats, strain code 273, aged four weeks (males, 150.3 ± 55.20g, n = 15) were obtained from Charles River Laboratories (Les Oncins, France). Immediately after arrival animals were housed in groups of three in five cages until the beginning of the intervention, with a light-dark cycle of 12:12 h, room temperature 24 °C and 50 ± 10% humidity and fed with a standard laboratory diet. Food and water were provided *ad libitum*. After one week of quarantine and acclimatization in the animal facility, rats were randomized assigned into three groups based on their initial weight, in order to minimize differences in baseline body weight between groups. The groups were named according to the type of intervention they received during the experiment: Polarized Training (POL), Threshold Training (LIM), Untrained Controls (CON). One month after arrival, the animals were housed in individual cages with access to freewheeling and the training protocol was initiated.

1.3. Experimental Design

The animals underwent a two-week treadmill habituation period. At the end of the adaptation period, the animals were submitted to an incremental maximal oxygen consumption (VO_2max) and endurance capacity (EC) exercise test. The VO_2max test served to determine baseline VO_2max and to define training intensity. After the assessment week, the animals started the training protocol

and the treadmill intensity was maintained for four weeks. Intermediate VO_2max and EC tests were repeated at four weeks of the training protocol to evaluate the effect of training and to readjust the intensity of the exercise protocol. This intensity was again maintained for four additional weeks of training. At the end of 8 weeks training, after 48 hours of rest, all animals performed the final VO_2max and EC test. The animals than underwent a final week of training at the same intensity of the previous weeks to ensure stable training conditions (stabilization week) and to allow recovery from any potential muscle damage that could have resulted from the VO_2max and EC tests (Table 1). All procedures were performed on a motorized treadmill between eight (am) and two (pm).

The defined inclusion criteria were: (i) all animals that fulfilled at least 95% of the planned training protocol; (ii) all animals that remained healthy during the protocol. The defined exclusion criteria were: (i) animals that were injured during the intervention; (ii) animals showing clear signs of stress; (iii) animals that become ill during the intervention; (iv) animals that failed to complete the stipulated assessments. For each experimental group, the animals not included in the analysis and the respective reason will be reported, as well as the total number of animals in each group.

Table 1 Schedule of the experimental design

Week nº	**Experimental protocol phase**
0 - 4	Animals arrive to the facility/ quarantine
5 - 6	Treadmill adaptation period
7	VO_2max and EC - Baseline tests
8 - 11	Endurance Training protocol
12	VO_2max and EC - Mid Tests
13 - 16	Endurance Training protocol
17	VO_2max and EC - Final Tests
18	Stabilization week
19	Euthanasia and necropsy

1.4. Adaptation period

The treadmill adaptation protocol lasted two weeks (10 days). On the first day (D1), the rats were placed on the immobile treadmill for 10 minutes to adapt to the new environment. On the second day (D2), the treadmill was turned on at 10m/min for 10 minutes. The speed remained constant and there was an increment of 10 minutes each day until the protocol duration reached 60 minutes. In the following days, the speed increased by two m/min until the last adaptation day (Table 2). The CON group protocol did not suffer any increase in speed or duration, consisting of 10min at 10m/m every day. Electrified grids were used to stimulate rats to run. The intensity of electric stimulus was maintained at the lowest necessary to prevent animals from stopping at the rear end of the treadmill lane.

Table 2 Adaptation period program

Acclimatization	Week 1					Week 2				
Days	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Duration (min)	10	10	20	30	40	50	60	60	60	60
Speed (m/min)	0	10	10	10	10	10	10	12	14	16

1.5. Exercise Training program

The 15 Wistar Han rats started the training protocol at eight weeks of age and 336.1 ± 30.4g weight. The endurance training protocols were performed on a motorized treadmill (Panlab/Harvard Treadmill Apparatus) All groups performed 5d/week training sessions (Monday to Friday) during eight weeks of training, with rest on Saturday and Sunday. The temperature of the training room was maintained at 19ºC to avoid thermal stress of the animals. All training and evaluations were carried out at the same time (13h ± 1h) to avoid the effect of circadian variation on fatigue and muscle glycogen concentration. At the end of each training all animals receive a positive reinforcement with 0.5 g of chocolate. Animals that received >5 shocks/min were temporally removed from the treadmill for two min to rest.

1.5.1. POL Exercise Training Program

In order to fulfill the assumption of POL (Z1>Z3>Z2), we assumed a training intensity distribution that would allow the animals to perform 80% of the total training volume at low intensity (60% VO_2max) and 20% at high intensity (90% VO_2max). For practical reasons, the training protocol did not assume a block periodization pattern, typical in athletes, having been constituted with high and low intensity sessions performed in the same training session. Each session consisted of 60 minutes (48 minutes of low intensity and 12 minutes of high intensity). The training session started with a 5min warm-up at 60% VO_2max, followed by 3 intervals of 4min at 90% VO_2max, interspersed with 6min at 60% VO_2max, remaining 29min at 60% VO_2max. The principle of load progression was applied to the interval training of the POL group, as described in Table 3.

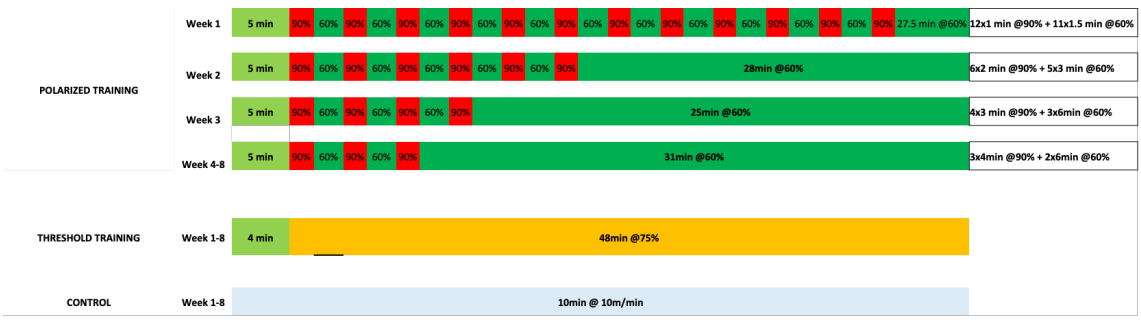
1.5.2. THR Exercise Training Program

THR training was carried out through the method of continuous running at a moderate intensity (Z2). Each session consisted of 52 min (four min warm-up at 50% VO_2max and 48 min at 75% VO_2max.

1.5.3. CON Exercise Training Program

The Control group performed 10min of running at 10m/min daily to ensure similar exposure to the strass of daily manipulation and contact with the treadmill. In this way, the animals were familiarized with the running pattern on the treadmill, allowing them to perform the VO2max and EC assessment, without the exercise performed representing a sufficient stimulus to cause significant physiological adaptations.

Table 3 Exercise Training Protocol



1.6. Assessments

During the intervention, evaluations of VO_2max and EC were performed in three different moments: (i) initial moment, after the adaptation phase and before the beginning of the training protocol; (ii) intermediate moment, at the end of the fourth week of training, precisely at the middle of the training protocol; (iii) final moment, after the eighth week of the training protocol. The initial and intermediate evaluations also served to define and adjust the intensity of the training protocols, respectively. Assessments were performed at least 48 hours after the last training session/functional assessment. According to Poole et al., (2020) (Poole et al., 2020) the intensities were determined as low (60% VO_2max), moderate (75% VO_2max) and high (90% VO_2max).

1.6.1. Maximal oxygen consumption (VO_2max) assessment protocol

VO_2max was determined through a maximal exercise test on a motorized treadmill (Metabolic Modular Treadmill; Columbus Instruments, Columbus, OH, USA) coupled to a computer with the Oxymax software (Columbus Instruments, Columbus, OH, USA) for respiratory gas analysis. Before each testing session, the Oxymax software and the open circuit indirect calorimetry treadmill were calibrated and verified for hardware malfunctions according to manufacturer instructions. This procedure was repeated every three tests. A primary standard grade calibration gas (Acail gás, Portugal) with 20.50 ± 0.02% O_2 and 0.50 ± 1.0% CO_2 was used for calibration and drierite columns (Calcium Sulfate with Indicator, Sigma-Aldrich; St. Louis, MO, USA) were used to assure that moisture in the sample gas was properly absorbed during testing. During testing the software was set to a constant flow inside the chamber of 0.6 L/min and to collect gas sample measurements every 5 seconds. Oxygen consumption (VO_2) and RER (VCO_2/VO_2) were continuously displayed during the testing.

Immediately before testing, animals were weighed on a precision balance to allow determination of their relative oxygen consumption (VO_2max ml/Kg/min). Then, they were placed inside the sealed treadmill chamber and went through a phase of acclimatization for a few minutes (five - 10min) until RER was considered stable. The exercise testing protocol consisted of steps lasting two minutes with increments of three m/min at each step with the initial speed starting at 10m/min. The inclination remained constant at 10º. VO_2max was determined

by the peak oxygen consumption reached during the test and the measurement was considered valid when RER was >1.0 and [blood lactate] >6mM. Blood lactate concentration was determined in a capillary blood sample of ~1μL collected via tail vein prick and analyzed on a handheld lactate meter (Lactate Pro 2) within one minute of test completion. The testing protocol was also terminated if animals remained more than 5 consecutive seconds in the electrified grid in the rear end of the treadmill due to exhaustion or refusal to collaborate with the test. At the end of the exercise testing protocol animals were removed from the treadmill chamber and placed back on their cage.

After four weeks of training, animals were subjected to an intermediate VO_2max test. It was necessary to modify the VO_2max assessment protocol of the POL and LIM groups due to the effect of training and to prevent the test duration from being excessively long and final treadmill speed excessively high. To achieve this, the inclination was changed to 15º and the protocol started with a warm-up at 16m/m. The 2nd and 3rd levels underwent increments of six m/min so that the test was not excessively prolonged in time, as described in Fig 3. This same adapted protocol was used in the last VO_2max assessment.

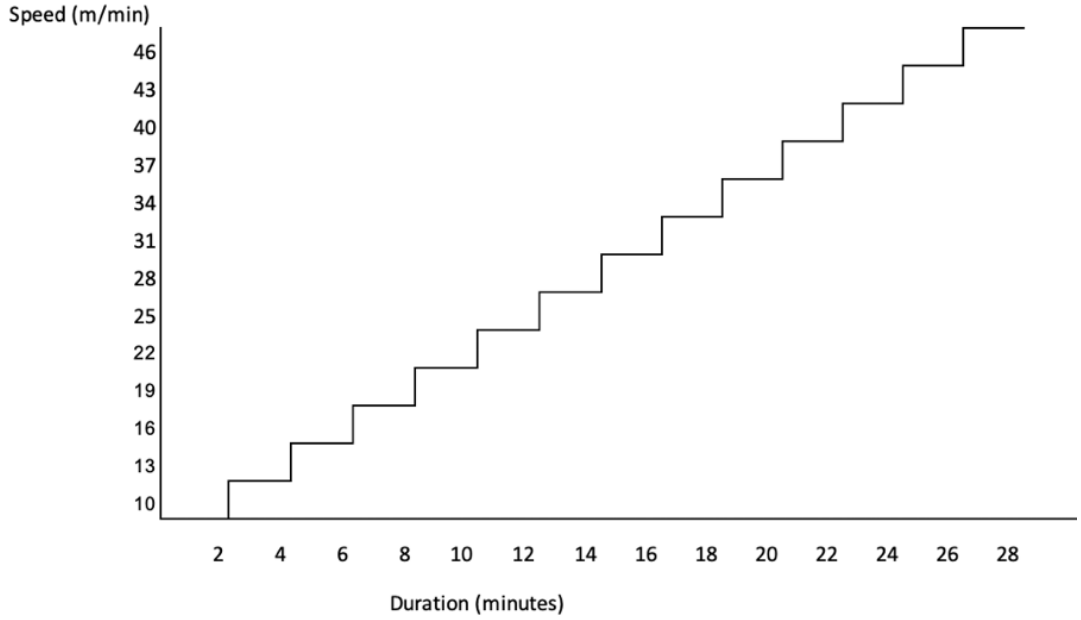


Figure 1 Maximal oxygen consumption (VO_2max) assessment protocol

1.6.2. Aerobic capacity assessment protocol

Aerobic capacity was determined by an exercise test on a motorized treadmill until exhaustion. The aerobic capacity test started with a 5min warm-up at 10m/m. Afterwards, the speed was increased 5m/min every five minutes until exhaustion (Figure 2). The treadmill incline was kept constant at 10%. During warm-up, the electrified grid intensity was set at 1mA and changed to 2mA during the incremental steps of the protocol. To determine the animals exhaustion the following predetermined criteria were established: (i) remain on top of the electrified grid >5 consecutive seconds; (ii) reveal alterations in the gait pattern or present a decrease in the reflex to return to the 4-legged position (righting reflex) when positioned in dorsal decubitus. The main variables collected during the aerobic capacity test were the total distance covered by the animal and the total time until exhaustion.

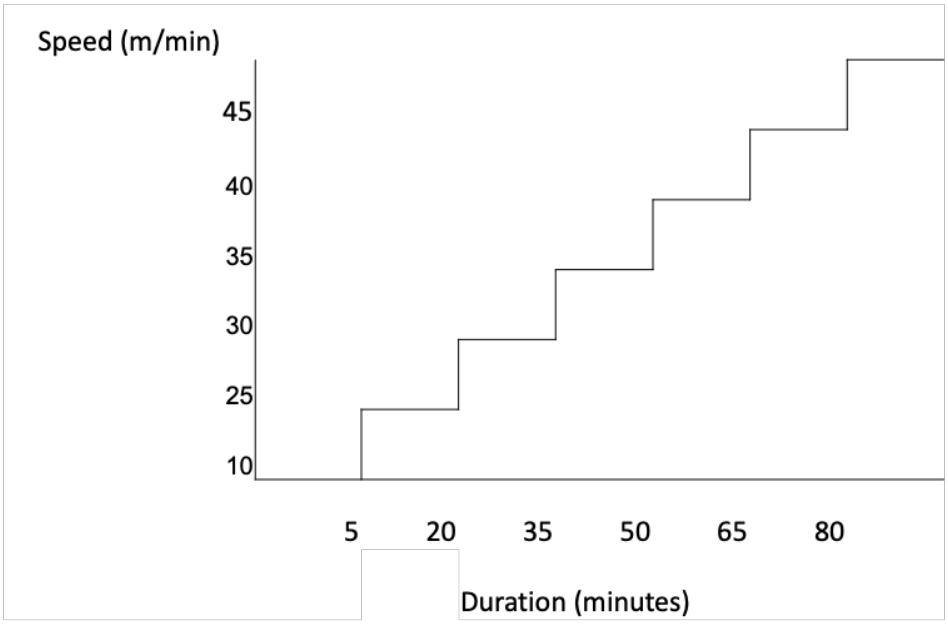


Figure 2 Endurance capacity assessment protocol

1.7. Training load quantification

In order to equalize the training load of both POL and THR groups, each training protocol was designed to result in similar values of oxygen consumption per session between the POL and THR groups, regardless of the specific running speeds that each group performed. For this, it was necessary to determine the oxygen consumed at the specific predefined running intensities (POL = 60% and 90%, LIM = 50% and 75% VO_2max) by each animal recorded during the VO_2max

test and adjust the running time of both protocols to achieve VO_2 equalization between groups.

1.8. Sacrifice and necropsy

At the end of the eight weeks of training and at least 48h after the last training session or functional evaluation, all animals were sacrificed by an overdose of the anesthetics Ketamine (Nimatek 100 $mg.mL^{-1}$, Dechra) and Xylazine (Rompun 2%, Bayer). After confirmation of the animal death by absence of heart rate, the abdominal and thoracic cavities were opened and inspected. Biological samples were then collected, cleared of blood and surrounding tissue weighted on a precision balance and prepared for analysis according to each specific protocol, namely for determination of mitochondrial respiration by high-resolution respirometry, histological analysis or biochemical analysis. The following samples were collected: heart, soleus, gastrocnemius, tibialis anterior, triceps brachii and diaphragm muscles. Tissue samples were immediately processed according to appropriate technical procedures or stored at -80°C for later biochemical analysis. The remaining tissues not analyzed were disposed of in accordance with the legislation by a company certified in the disposal of group III hospital waste.

1.9. Histological analysis of diaphragm muscle

Samples of the *diaphragm* muscle with approximately $5mm^2$ were routinely prepared for histological analysis by fixation in buffered 4% formaldehyde in phosphate buffered saline (v/v) by diffusion during 48h, subsequently dehydrated in graded ethanol (Panreac) solutions, cleared in xylene and included in paraffin blocks (Merck). Serial cross-sections with 5 µm of thickness were cut from the paraffin blocks using a rotary microtome (Leica RM 2125) and mounted on silane-coated slides. After drying in air during 48-72h, the slides were dewaxed in xylene (Panreac), rehydrated through graded ethanol (Panreac) solutions and stained with haematoxylin and eosin (Sigma). After adequate washing, dehydration and clearing, slides were mounted with DPX (Sigma) and glass coverslip for analysis of the muscle fibers cross sectional area in a photomicroscope (Zeis Phomi 3).

Representative photomicrographs were taken from the tissue sections stained with hematoxylin and eosin and the images were analyzed with ImageJ software

(NIH, Bethesda, MD) by individually contouring each fiber manually. The diaphragm muscle fibers cross-sectional area was determined for each animal as the average area of ≈250 fibers.

1.10. *Left ventricle*, *diaphragm*, *soleus* and *tibialis anterior* homogenate preparation

A portion of each muscle was homogenized in the proportion of 10 mg of tissue *per* 1 mL of phosphate buffer (50 mM KH_2PO_4 pH 7.5, 0.5% Triton X-100 and 200 mM PMSF), using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer. The obtained samples were preserved at -80ºC for subsequent analysis.

1.11. Total protein quantification

An aliquot of tissue homogenate (5 µL) was used for total protein quantification using the commercial kit *DC Protein Assay* (Bio-Rad®, Hercules, CA, USA). This kit is based on Lowry *et al (Lowry, Rosebrough, Farr, & Randall, 1951)* assay, on which proteins react with copper in an alkaline solution, thereby leading to the reduction of Folin reagent by the copper-treated protein, and to the formation of a blue color with maximum absorbance at 750 nm. Hence, a calibration curve with standard solutions of bovine serum albumin (BSA) at concentrations between 0.625 and 10 mg/mL was made. Briefly, to 5 µL of samples or standards was added 25 µL of reagent A' (prepared by the addition of 1 mL of reagent A and 20 µL of reagent S) and, after 5 minutes at room temperature, 400 µL of reagent B was added. After 15 minutes at room temperature, the absorbance was measured at 750 nm in a microplate reader (Multiskan GO, Thermo Fischer Scientific®, Northumberland, UK).

1.12. O2k-Technology High-Resolution Respirometry Assay - OROBOROS

Approximately 5 mg of cardiac muscle (*left heart ventricle*) and skeletal muscles (*soleus, tibialis anterior and diaphragm*) were collected used soon after de animals were sacrificed (1h~ after) for determination of mitochondrial respiration. Mitochondrial respiration was assessed using a high-resolution respirometry system O2k (Oroboros Instruments, Innsbruck, Austria) at 37°C in

a O2k-Chamber with 2.0 mL for different types of tissues. The cells were added to the respirometry chambers containing 2 mL of mitochondrial respiration medium MiR05-Kit (ID: 60101-01 Lot.20J01923, Oroboros Instruments, Innsbruck, Austria) preequilibrated at 37°C. The chambers were closed after approximately 40 min of calibration. A Substrate Uncoupler Inhibitor Titration protocol (SUIT-001 O2 mt D001) was used to assess the functionality of different components of the mitochondrial electron transport chain (ETC) (Table 4) for O2k-sV-Module. This SUIT protocol reflects the maintenance of flux with the addition of complex I substrates pyruvate and malate (PM), followed by a saturating amount of ADP. Cytochrome c was added to test potential problems linked to mitochondrial inner membrane integrity. A progressive titration of two steps of Carbonyl Cyanide-m-Chlorophenylhydrazone (CCCP) was subsequently performed until no further stimulation could be observed (i.e. maximal stimulation was reached), glutamate oxidized in the mitochondrial matrix by glutamate dehydrogenase to α-ketoglutarate, representing the control state of the glutamate-anaplerotic pathway. Complex II substrate (succinate) was then added to stimulate mitochondrial respiration powered by both complexes I and II. Rotenone is a complex I inhibitor and thus inhibits NADH oxidation. The glycerophosphate pathway is a level 3 ETC pathway control state, supported by glycerophosphate fuel substrate and electron transfer through the glycerophosphate dehydrogenase complex to Q function. Then, mitochondrial respiration was completely inhibited using the complex III inhibitor antimycin A. Lastly, the maximal activity of complex IV was assessed using ascorbate and TMPD. While there is a known consumption of chemical O_2 linked to the auto-oxidation of ascorbate and TMPD, the consumption of chemical O_2 was subtracted from the ascorbate and TMPD oxygen specific flux, as demonstrated in the results for the normalization of complex IV.

Table 4 O2k manual titrations of SUIT-001 O2 mt D001 protocol for skeletal and cardiac muscle tissues in O2k-sVModule - Mitochondrial Physiology Network 09.12(20):1-4 (2020).

Substrates	Event	Concentration in syringe (solvent)	Storage [°C]	Final concentration in 2 mL	Titration [µL]	Syringe [µL]
Pyruvate	P	2 M (H_2O)	fresh	5 mM	5	25
Malate	M	0.4 M (H_2O)	-20	2 mM	10	25
ADP+ Mg2+	D	0.5 M (H_2O)	-20	2.5 mM	10	25
Cyt. c	c	4 mM (H_2O)	-20	10 µM	5	25
CCCP	U	1 mM (EtOH)	-20	0.5 µM	1, 2steps	10
Glutamate	G	2 M (H_2O)	-20	10 mM	10	25
Succinate	S	1 M (H_2O)	-20	10 mM	20	50
Octanoylcarnitine	O	0,1 M (H_2O)	-20	0.5 mM	10	25
Rotenone	Rot	1 mM (EtOH)	-20	0.5 µM	1	10
Glycerophosphate	Gp	1 M (H_2O)	-20	10 mM	20	50
Antimycin A	Ama	5 mM (EtOH)	-20	2.5 µM	1	10
Ascorbate	As	0.8 M (H_2O)	-20	2 mM	5	25
TMPD	Tm	0.2 M (H_2O)	-20	0.5 mM	5	25
Sodium azide	Azd	4 M (H_2O)	-20	≥100 mM	≥50	100

Note: Ama, Antimycin A (inhibitor); As, Ascorbate; Azd, Sodium azide (inhibitor); c, Cyt. c - Cytochrome c; CCCP, Uncoupler CCCP - Carbonyl Cyanide-m-Chlorophenylhydrazone (obtained at optimum uncoupler concentration for maximum flux); D, Adenosine diphosphate (ADP) + Mg2+; DMSO, Dimethyl sulfoxide; EtOH, Ethanol; G, Glutamate; Gp, Glycerophosphate; H2O, Water; M, Malate; O, Octanoylcarnitine; P, Pyruvate (fresh preparation); Rot, Rotenone (inhibitor); S, Succinate; Tm, TMPD - N,N,N',N'-Tetramethyl-phenylenediamine dihydrochloride. Updates: http://wiki.oroboros.at/index.php/MiPNet09.12_O2k-Titrations

1.13. Western blotting analysis

The expression of the mitochondrial markers PGC-1α, TFAM, Mitofusin 1 and Mitofusin 2 was determined in tissue samples of the left heart ventricle, diaphragm, soleus and tibialis anterior muscles by western blot.

A given volume of sample (total homogenate or mitochondrial extract) corresponding to 20 µg of protein was diluted 1:1 in reduction buffer (0.5 M Tris-HCL, pH 6.8%; 10% SDS (m/v) ; 20% glycerol (v/v); 10 mM B-mercaptoethanol; 0.05% bromophenol blue (m/v) and incubated at 100º for 5 min. Reduced and denatured samples of each of the experimental groups were applied in gels of SDS-PAGE with 10 and 12.5%, prepared according to Laemmli (Laemmli, 1970).

After separation, the proteins were transferred into a nitrocellulose membrane (Whatman®, Protan®) by TURBO transblot (Bio-Rad, USA) at 2500 mA , 25V for 7 minutes (Mixed MW). After transfer, the membranes were stained with Ponceau S for applied and transferred protein control, washed with distilled water, and then blocked with Every Blotting Blocking Buffer (#12010020, Bio-Rad) for 10 minutes at room temperature, in order to avoid non-specific antibody binding. Subsequently, the membranes were incubated with a solution of primary antibody (rabbit polyclonal anti-mtTFA (sc-28200), diluted 1:200 in EveryBlot Blocking Buffer, mouse monoclonal anti-Mfn2/Mitofusin 2 (F-5) (sc-515647) diluted 1:750 in EveryBlot Blocking Buffer, mouse monoclonal anti-Mfn1/Mitofusin 1 (D-10) (sc-166644), diluted 1:750 in EveryBlot Blocking Buffer, acquired from Santa Cruz and rabbit polyclonal anti-PGC1 alpha (ab54481), diluted 1:1000 in EveryBlot Blocking Buffer, acquired from Abcam (Cambridge, UK)). All membranes were incubated for 3 hours at room temperature under continuous shaking. Then, membranes were washed three times (10/15/10 minutes each) with TBS-T to remove the unbound antibodies. Subsequently, membranes were incubated with the appropriate secondary horseradish peroxidase-conjugated antibody (anti-mouse or anti-rabbit, GE Healthcare, UK), diluted 1:5000 in EveryBlot Blocking Buffer, for 1h with shaking at room temperature. After washing with TBS-T, the membranes were exposed, for 2 minutes, to the enzymatic substrate (Clarity Western ECL Substrate, Bio-Rad, USA) and digitized in the ChemiDoc system (Bio-Rad, USA). The intensity of the bands was determined using ImageLab v4.1 software (Bio-Rad Laboratories). Optical densities were expressed in arbitrary units.

1.14. Citrate Synthase activity

The activity of citrate synthase (CS) was measured in left heart ventricle, diaphragm, soleus and tibialis anterior tissue sample homogenates according to Coore et al (Coore, Denton, Martin, & Randle, 1971). This method evaluates the presence of free thiol groups in CoASH by its reaction with 5,5'-dithiobis-(2-nitrobenzoate) (DTNB). The resulting 2-nitro-5-thiolbenzoate (TNB) anion has a strong absorption at 412 nm, which might be followed spectrophotometrically. Briefly, 10 µL from each tissue sample were incubated with 190 µL of reaction

buffer (200 mM Tris buffer pH 8.0, 10 mM acetyl-CoA, 10 mM DTNB and 0.1 % (v/v) Triton X-100) and the absorbance was measured at 412 nm for 2 minutes at 30 ºC in a microplate reader (Multiskan GO, Thermo Fischer Scientific®, Northumberland, UK). Thereafter, 10 µL of 10 mM oxaloacetate (OAA) was added to each well of the microplate and absorbance was immediately measured at 412 nm for 2 minutes at 30 ºC. The absorbance values were plotted against time and the difference between the second and the first measurement, in relation to the slope of the equation ((ΔA412)/min) was divided by the extinction coefficient of TNB at 412 nm (13.6 $mM^{-1}.cm^{-1}$). Then, the absorbance values were divided by total protein to obtain the enzymatic activity values (mmol/min/mg) on each sample.

1.15. Statistical analysis

Descriptive statistics (mean and standard deviation) were determined for all relevant variables collected in the study. Each variable distribution was assessed for normality and homogeneity of variance with the Shapiro-Wilk and Levene test, respectively. Whenever the necessary assumptions to perform parametric statistical testing were met, comparisons between multiple groups were performed by one-way analysis of variance (one-way ANOVA). If differences were detected, multiple post-hoc comparisons were then performed with the Bonferroni or Games-Howell test, if homogeneity of variance was present or not, respectively. Whenever variables lacked normal distribution, comparisons were performed by one-way ANOVA with bootstrapping, using 1000 bootstrap samples. No outliers were removed from the analysis unless stated otherwise. Statistical analysis was performed with IBM SPSS statistics version 27. The significance level of the statistical testes was defined at 5%, two-tailed.

2. Results

2.1. Bodyweight, food intake and voluntary physical activity

At the beginning of the experiment there were no differences in weight between groups (CON = 352 ± 40 g, POL = 324 ± 26 g, LIM = 331 ± 21 g; p = 0.362). During the experiment, all groups increased body weight (CON = 47.2 ± 16.6 g, POL = 33.8 ± 14.1 g, LIM = 41.0 ± 11.2 g). Although there were no observed differences in body weight variation between groups (p = 0.370), body weight at the end of the experiment was significantly lower in POL (359 ± 21 g) compared to CON group (399 ± 28; p = 0.028). There were no differences in final body weight between POL and LIM (365 ± 10; p = 1.0) groups (Figure 3A).

There were also no observed differences in average weekly food intake (CON = 142.5 ± 17.1 g, POL = 136.4 ± 10.4 g, LIM = 152.2 ± 14.1 g; p = 0.245; Figure 3B) or average running distance in the cage activity wheel (CON = 11.1 ± 17.9 km, POL = 11.1 ± 16.4 km, LIM = 24.0 ± 25.3 km, p = 0.181) between groups (Figure 3C). Despite the lack of observed differences in voluntary running activity between groups, average weekly distance in the activity wheel tended to be higher in the LIM group. During necropsy, several organs were collected and weighted (Table 5). Gastrocnemius weight was lower in POL group compared to CON (p = 0.038) and tibialis anterior was lower in both POL (p < 0.01) and LIM (p < 0.01) compared to CON.

Table 5 Morphometric analysis of the organs collected during necropsy

Variable	CON		POL		LIM		p
	mean	SD	mean	SD	mean	SD	
Heart (g)	0.986	0.080	0.948	0.061	1.000	0.032	0.401
Diaphragm (g)	1.003	0.057	0.952	0.065	1.014	0.066	0.304
Soleus (g)	0.324	0.039	0.278	0.033	0.276	0.029	0.078
Gastrocnemius (g)	5.07	0.34	4.61[a]	0.14	4.74	0.23	**0.033**
Tibialis anterior (g)	1.640	0.057	1.444[a]	0.434	1.498[a]	0.073	**<.001**
Triceps brachii (g)	3.41	0.37	3.44	0.14	3.51	0.12	0.825

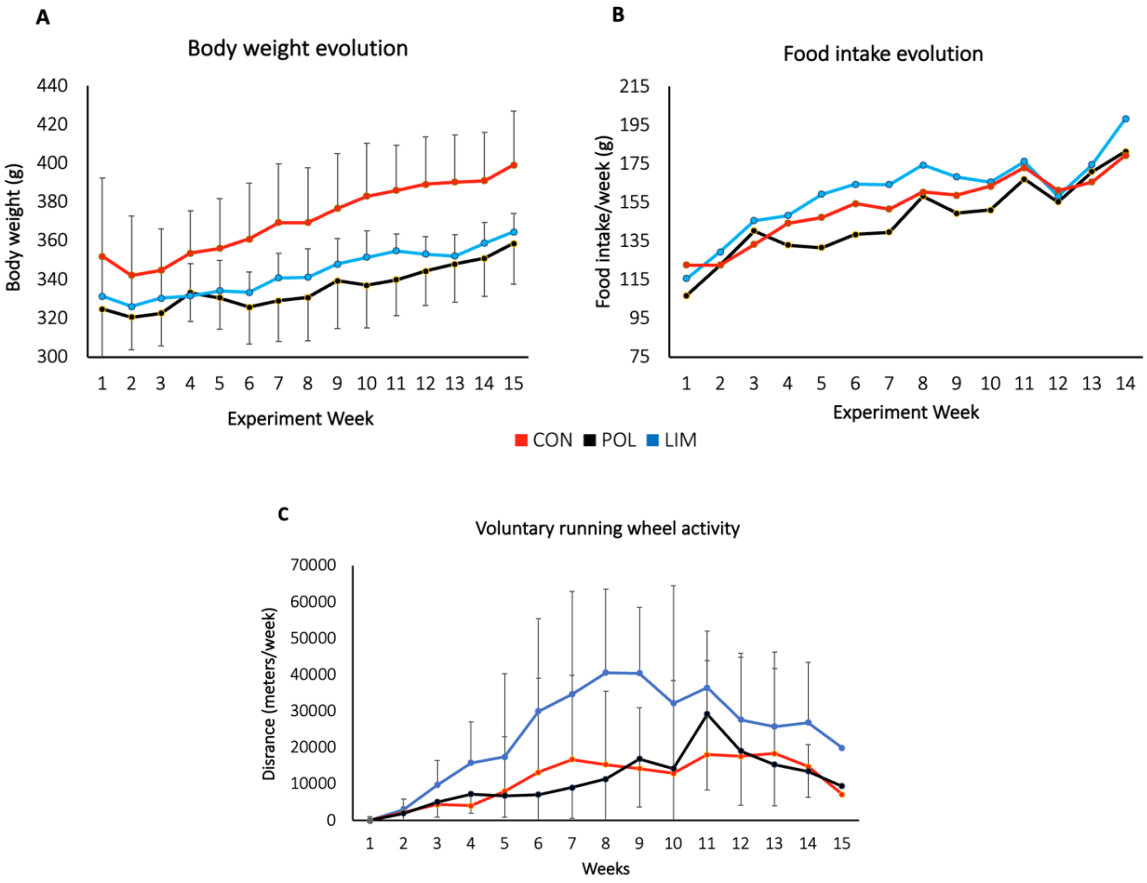


Figure 3 Evolution of A) body weight in grams (*g*), B) food intake per week in *g* and C) voluntary running wheel activity per week in meters (m). Data are presented as mean±standard deviation.

2.2. VO_2max and endurance capacity

There were no differences in baseline relative VO_2max between groups (CON = 71.0 ± 9.1, POL = 79.0 ± 6.6, LIM = 83.0 ± 8.5 mL.Kg.min^{-1}; p = 0.101). At the fourth week of the training protocol, VO_2max was reassessed and was shown to be significantly higher in both POL (84.8 ± 7.4 mL.Kg.min^{-1}) and LIM (89.2 ± 8.5 mL.Kg.min^{-1}) groups compared to CON (70.4 ± 6.3 mL.Kg.min^{-1}; p = 0.005), but there were no differences between POL and LIM groups (p = 1.00). At the end of the training protocol (eighth weeks), relative VO_2max was maintained in the CON and tended to decrease in POL and LIM animals due to the increase in body weight. There were no observed differences in relative (Figure 4A) or absolute (Figure 4B) VO_2max between groups at the end of the eight weeks of the exercise protocol.

Endurance capacity, determined by the total running distance during a treadmill test until exhaustion, was initially found to be similar between groups at

baseline, intermediate and final timepoint assessment (Figure 4C). Nevertheless, a sensitivity analysis was performed, and an extreme outlier was removed from the CON group at the intermediate and final assessments. Data analysis after removal of this outlier showed that POL and LIM groups had a significantly higher endurance capacity compared to CON at the intermediate timepoint assessment (CON = 782 ± 178, POL = 1963 ± 173, LIM = 2218 ± 516 m; $p < 0.001$) and at the end of the training protocol (CON = 824 ± 223, POL = 2057 ± 122, LIM = 2134 ± 413 m; $p < 0.001$). There were also no differences between POL and LIM groups at the intermediate ($p = 0.785$) and final ($p = 1.00$) timepoint assessments.

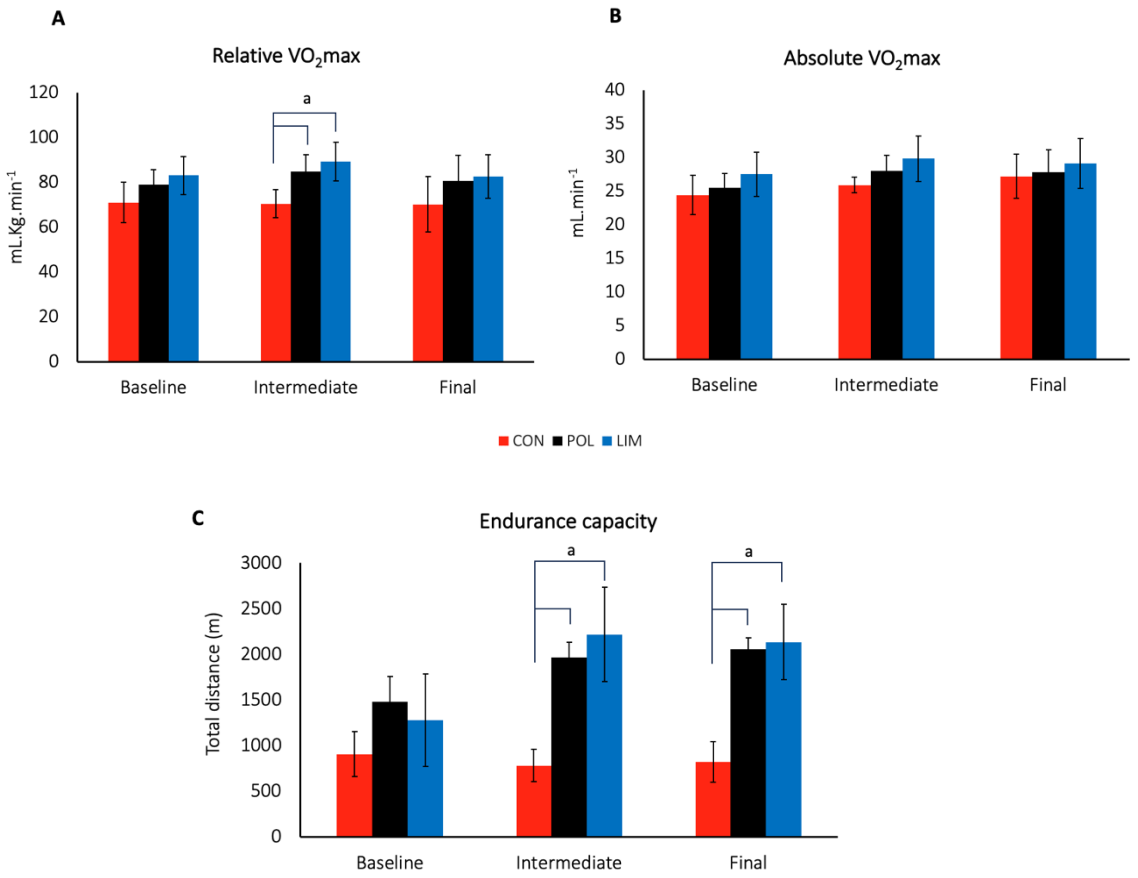


Figure 4 A) evolution of relative maximal oxygen consumption in VO2max baseline, intermediate (four weeks) and final assessment (eight weeks); B evolution of absolute maximal oxygen consumption in VO2max baseline, intermediate and final assessment C) Evolution of total distance covered in meters (m) Endurance capacity baseline, intermediate and final assessment. Data are presented as mean ± standard deviation. A difference between groups was considered as statistically significant if [a] $p \leq 0.05$.

2.3. High resolution respirometry

Mitochondrial respiration was measured using high-resolution respirometry Oxygraph-2k (O2k, Oroboros Instruments, Innsbruck, Austria).

The non-phosphorylating mitochondrial resting state (LEAK state) was determined following the addition of a mixture of pyruvate and malate in the absence of adenylates (LEAK respiration L(n)) in tissue sample homogenates of the left heart ventricle, diaphragm, soleus and tibialis anterior muscles. The LEAK state represents the flux of H^+ across the inner mitochondrial membrane due to intrinsic uncoupling in the absence of utilization of the protonmotive force for driving ATP synthesis. There were no observed differences in LEAK respiration state between groups for any of the tissue samples analyzed, namely left heart ventricle ($p = 0.622$), diaphragm ($p = 0.656$), soleus ($p = 0.977$) and tibialis anterior ($p = 0.154$), (Figure 5).

The oxidative phosphorylation (OXPHOS) capacity corresponds to the respiratory capacity of mitochondria in saturating conditions of ADP, oxygen and necessary substrates and represents the maximal capacity to generate a protonmotive force to fuel the synthesis of ATP in the mitochondria. There were no observed differences in OXPHOS respiration between groups for any of the tissue samples analyzed, namely left ventricle ($p = 0.234$), diaphragm ($p = 0.618$), soleus ($p = 0.549$) and tibialis anterior ($p = 0.434$), (Figure 6).

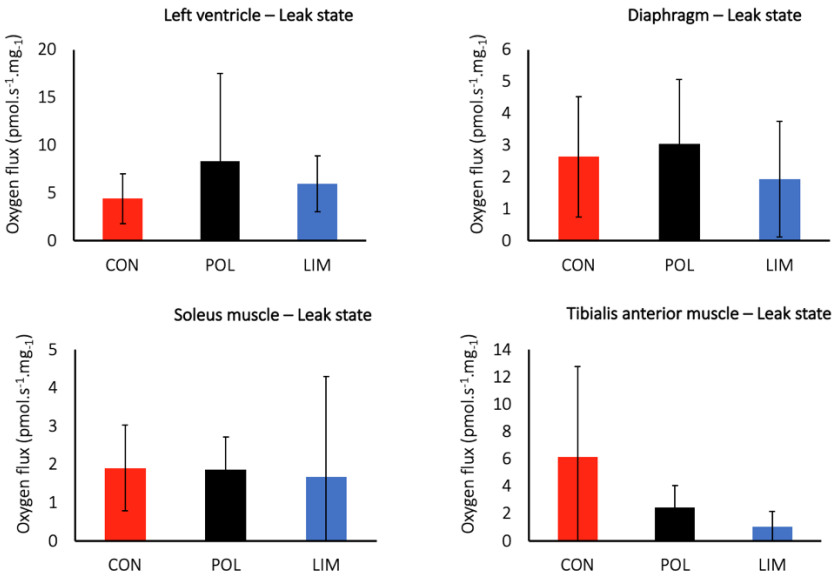


Figure 5 Titrations for the assessment LEAK state. Data are presented as mean±standard deviation. A difference between groups was considered as statistically significant, if [a] $p \leq 0.05$.

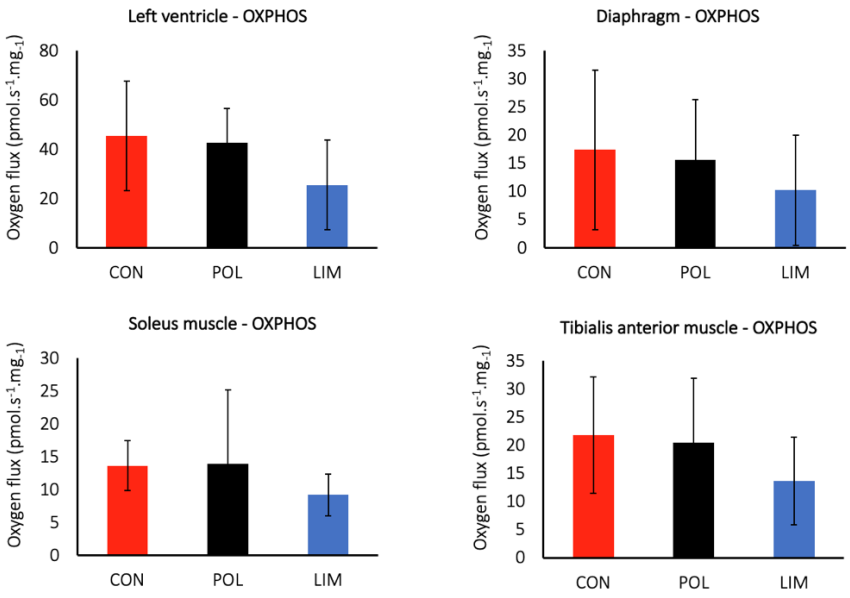


Figure 6 Titrations for the assessment OXPHOS respiration. Data are presented as mean±standard deviation. A difference between groups was considered as statistically significant if [a] $p \leq 0.05$.

2.4. Diaphragm histological analysis

Approximately 250 muscle fibers of the diaphragm of each animal were analyzed. Average cross-sectional area was CON = 2191 ± 368 μm^2, POL = 1790 ± 466 μm^2 and LIM = 1951 ± 412 μm^2. There were no observed differences between groups regarding diaphragm cross-sectional area ($p = 0.343$). There was also a similar distribution frequency of fibers cross-sectional area between groups, with the highest number of muscle fibers within the range of 700 and 1700 μm^2 (Figure 7).

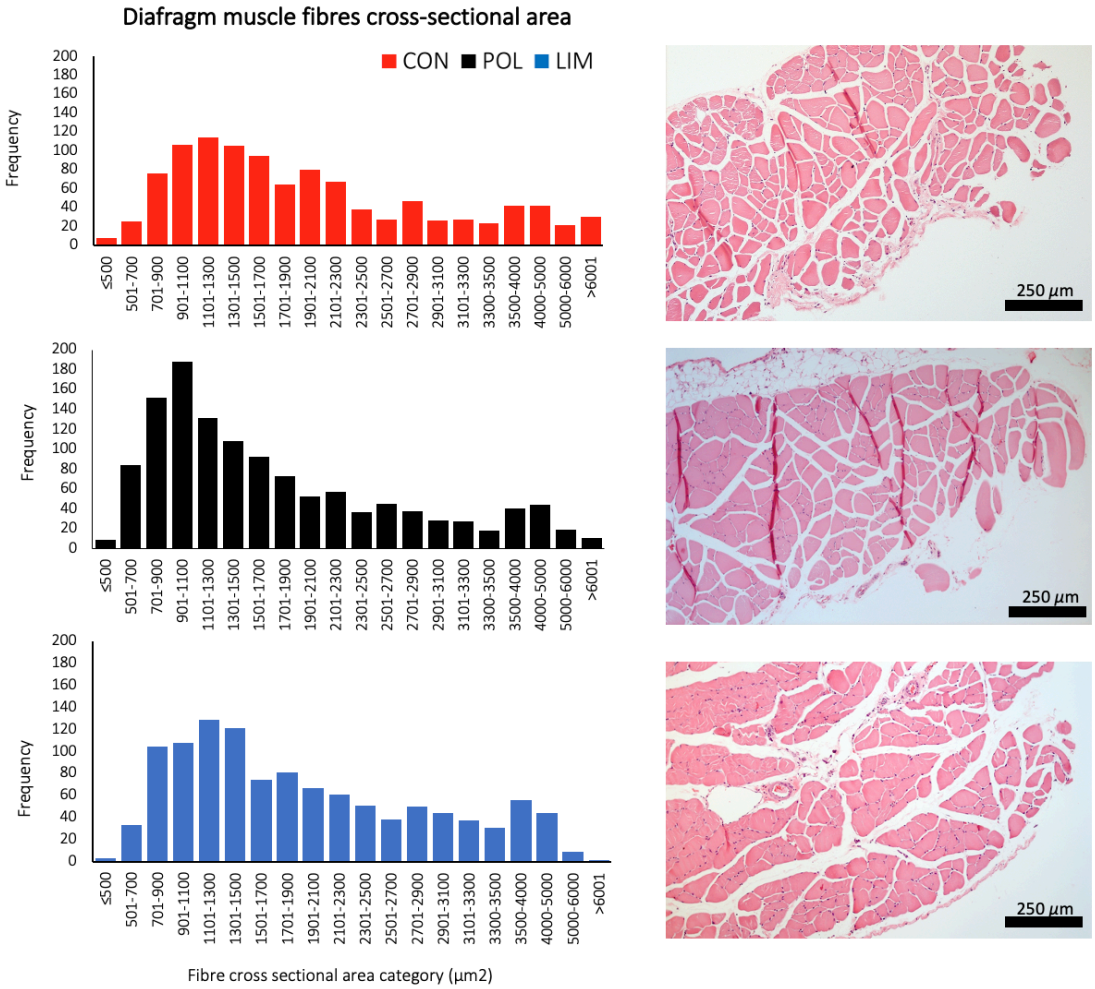


Figure 7 Diaphragm muscle fiber cross-sectional area ($\mu m^{2)}$

2.5. Citrate synthase activity

Citrate synthase activity was determined in tissue samples of the left heart ventricle, diaphragm, soleus, and tibialis anterior muscles collected at the end of the training protocol. Differences were not observed between groups regarding left heart ventricle (CON = 456 ± 69, POL = 499 ± 79, LIM = 422 ± 92 $nmol.mg.min^{-1}$; p = 0.348), diaphragm (CON = 313 ± 36, POL = 296 ± 39, LIM = 316 ± 57 $nmol.mg.min^{-1}$, p = 0.753), soleus (CON = 204 ± 23, POL = 248 ± 52, LIM = 245 ± 19 $nmol.mg.min^{-1}$; p = 0.121) and tibialis anterior muscles (CON = 245 ± 80, POL = 243 ± 57, LIM = 282 ± 48 $nmol.mg.min^{-1}$, p = 0.558) regarding citrate synthase activity (Figure 8).

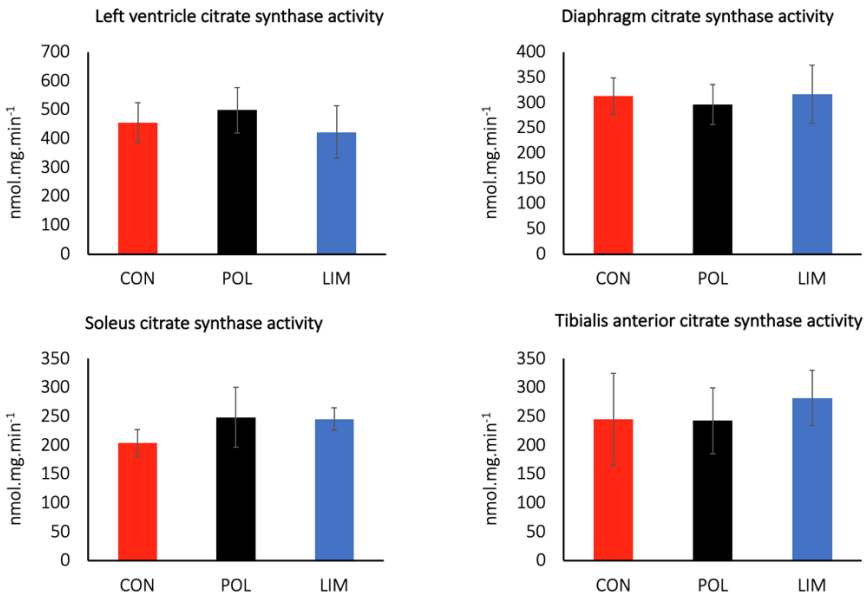


Figure 8 Citrate synthase activity. The values (mean ± SD) are expressed in nmol.min.mg^{-1} of n=5 for CS activity.

2.6. Mitochondrial biogenesis markers

There were no differences between groups regarding the expression of any of the markers of mitochondrial biogenesis assessed (Figures 9, 10, 11 and 12), namely in MTF1 expression in the left heart ventricle (CON = 944 ± 123, POL = 1110 ± 602, LIM = 844 ± 169; p = 0.557), diaphragm (CON = 178 ± 45, POL = 218 ± 99, LIM = 268 ± 199; p = 0.621), soleus (CON = 159 ± 66, POL = 124 ± 25, LIM = 165 ± 34; p = 0.313) and tibialis anterior muscles (CON = 550 ± 159, POL = 388 ± 181, LIM = 545 ± 412; p = 0.613), MTF2 expression in the left heart ventricle (CON = 114 ± 28, POL = 147 ± 61, LIM = 122 ± 23; p = 0.518) diaphragm (CON = 752 ± 334, POL = 907 ± 419, LIM = 1197 ± 580; p = 0.373), soleus (CON = 760 ± 315, POL = 806 ± 378, LIM = 713 ± 426; p = 0.929) and tibialis anterior (CON = 560 ± 204, POL = 612 ± 244, LIM = 534 ± 52; p = 0.789), PGC1-alpha expression in the left heart ventricle (CON = 228 ± 51, POL = 227 ± 57, LIM = 263 ± 30; p = 0.423), diaphragm (CON = 466 ± 225, POL = 467 ± 194, LIM = 667 ± 290; p = 0.363), soleus (CON = 188 ± 26, POL = 187 ± 57, LIM = 204 ± 65; p = 0.858) and tibialis anterior muscle (CON = 557 ± 334, POL = 648 ± 316, LIM = 762 ± 239; p = 0.595), and TFAM expression in the left heart ventricle (CON = 890 ± 651, POL = 630 ± 315, LIM = 474 ± 216; p = 0.353), diaphragm (CON = 230 ± 75, POL = 326 ± 134, LIM = 303 ± 140; p = 0.515), soleus (CON = 303 ±

132, POL = 352 ± 127, LIM = 286 ± 161; p = 0.757) and tibialis anterior muscles (CON = 265 ± 20, POL = 272 ± 31, LIM = 267 ± 39; p = 0.950).

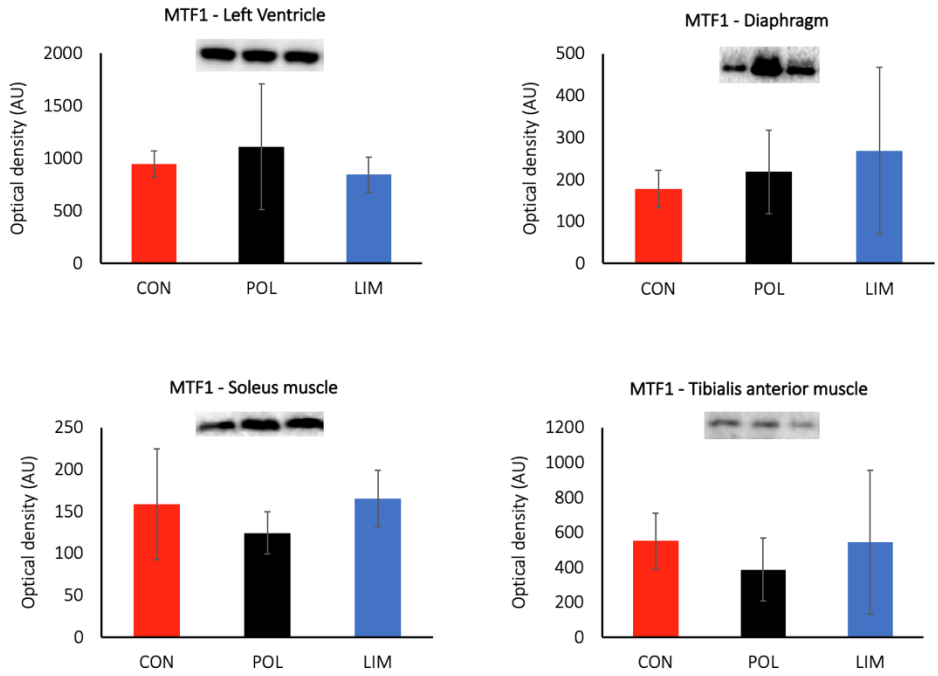


Figure 9 Effects of training in MTF1 expression measured by western blotting. Above each graph is a representative image of the western blot bands obtained. Samples were loaded in the gel one per group side-by-side. The values (mean ± SD) are expressed in arbitrary units of optical density (OD) of n=5.

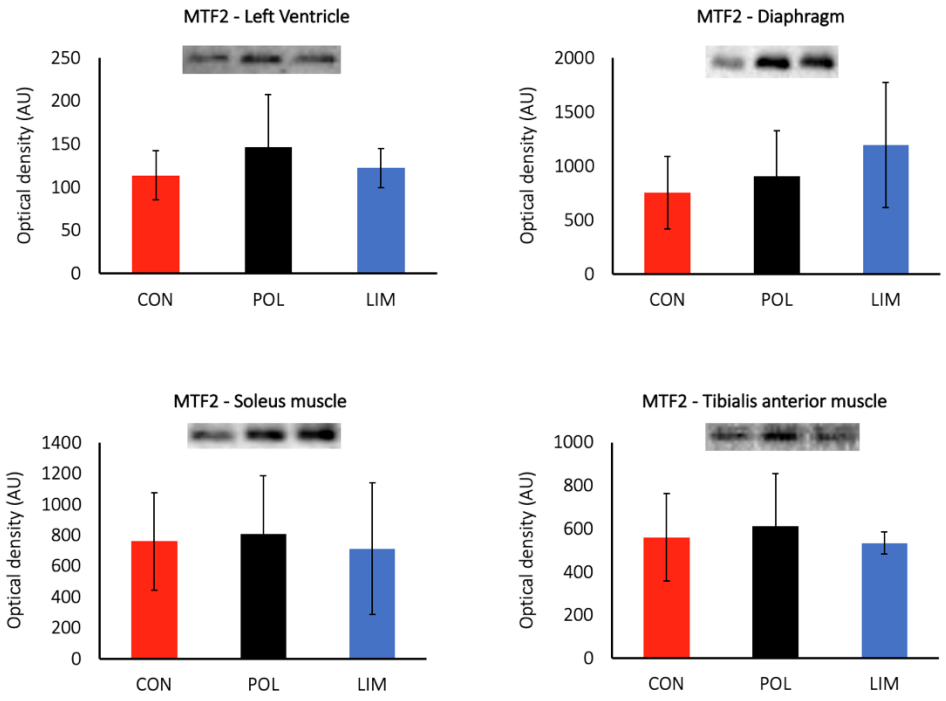


Figure 10 Effects of training in MTF2 expression measured by western blotting. Above each graph is a representative image of the western blot bands obtained. Samples were loaded in the gel one per group side-by-side. The values (mean ± SD) are expressed in arbitrary units of optical density (OD) of n=5.

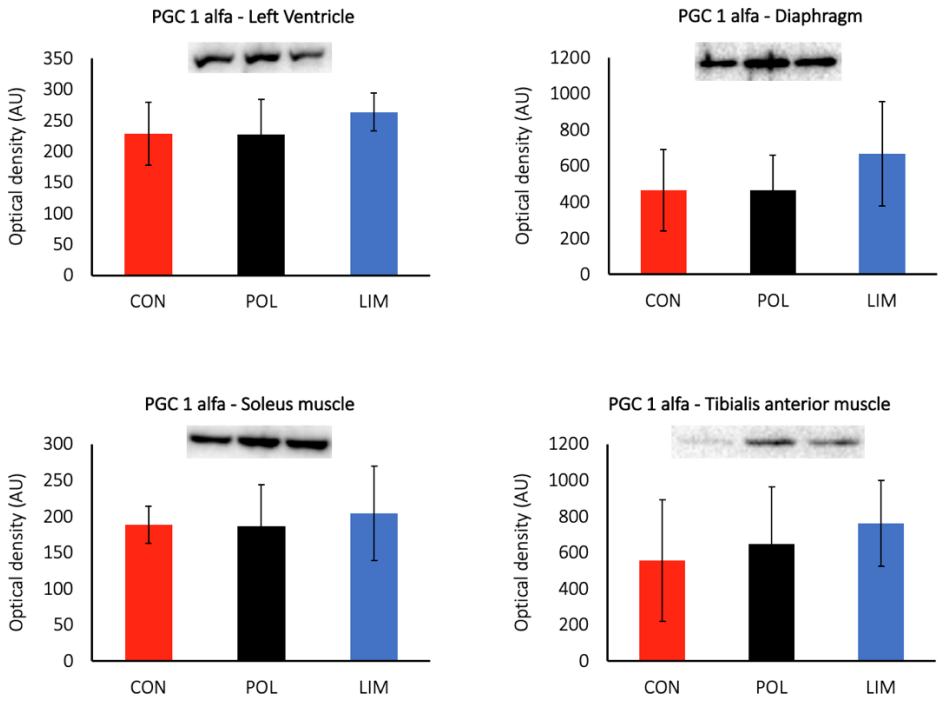


Figure 11 Effects of training in PGC1 alpha measured by western blotting; above each graph is presented a representative image of the western blots obtained – samples were loaded in the gel one per group side-by-side. The values (mean ± SD) are expressed in arbitrary units of optical density (OD) of n=5.

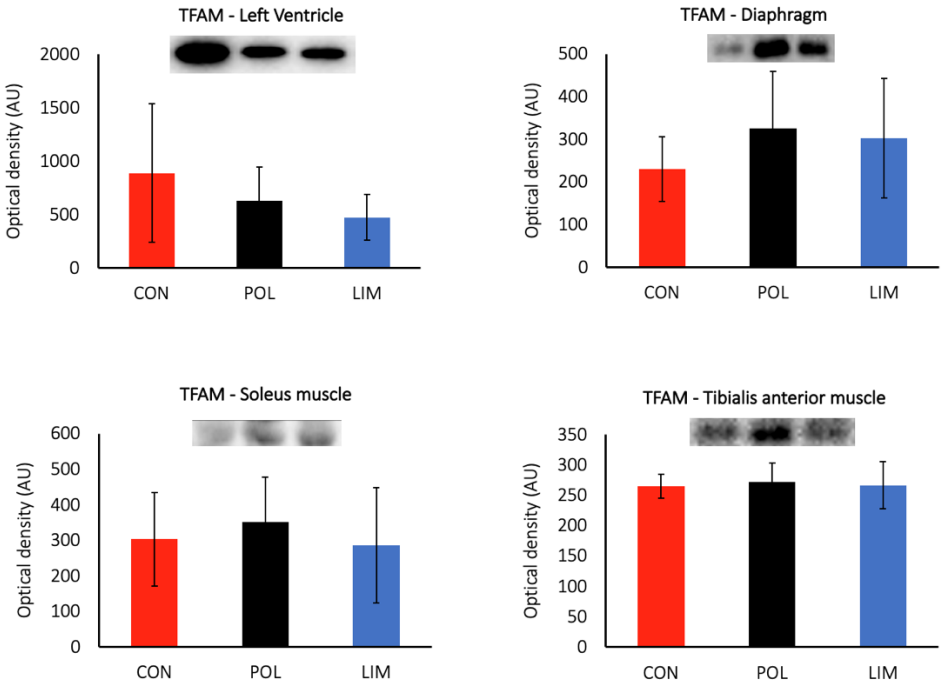


Figure 12 Effects of training in TFAM expression measured by western blotting. Above each graph is a representative image of the western blot bands obtained. Samples were loaded in the gel one per group side-by-side. The values (mean ± SD) are expressed in arbitrary units of optical density (OD) of n=5.

Discussion

Since the available evidence in the literature regarding the superiority of POL compared to other TIDs is uncertain we performed a systematic review with meta-analysis comparing POL with other TIDs regarding a set of indicators of endurance performance. Those results are described in the first chapter of this dissertation. In addition, considering that a potential cause of the conflicting results in the literature might stem from poor control of the training protocol variables and considering that this could be surpassed by rigorously controlled animal model experiments, we also carried out a study aiming to compare an eight-week POL *vs* THR (LIM) training protocol on endurance performance indicators (VO_2max and endurance capacity) and physiological mechanisms associated with endurance performance in Wistar Han rats. These results are described in the second chapter of this dissertation.

The results of our systematic review with meta-analysis suggest that POL is superior to other TIDs for the improvement of VO_2max/peak. However, to date, animal model studies that compared the effect of POL vs other TIDs on VO_2max have never been performed. In our experimental work, after four weeks of training, both VO_2max values of POL and LIM were shown to be significantly higher compared to the CON group ($p = 0.005$). However, there were no differences between POL and LIM ($p = 1.00$). Our results seem to support the rationale that previously untrained subjects are equally sensitive to various TIDs, and have the potential to improve similarly regardless of the training stimulus (Festa et al., 2020). Nevertheless, at the end of the training protocol, differences were not observed in relative or absolute VO_2max between groups. This absence of differences could also be attributable to the existence of outliers in the CON group that were naturally very active and that ran three-to-four-fold higher distances daily compared to the remaining animals, particularly from the fourth week of the experiment onwards. Given that the rats running pattern in the wheel is composed of intermittent high intensity runs (Beleza et al., 2019), this may have contributed to the increase in their VO_2max.

The Rat EC can be assessed by having it run at a submaximal speed or work rate until it becomes exhausted (Poole et al., 2020). POL and LIM groups displayed a significantly higher EC compared to CON at the intermediate ($p <$

0.001) and final timepoint assessments ($p < 0.001$). However, there were also no differences between POL and LIM groups at the intermediate ($p = 0.785$) and final ($p = 1.00$) timepoint assessments. These findings are in accordance with our meta-analysis, that suggest that POL and THR training induce similar adaptations in TTE.

Endurance training constitutes a powerful stimulus to enhance mitochondrial respiration function (Leick et al., 2008). One of the mitochondrial benefits linked to aerobic exercise is increased OXPHOS efficiency (Silva et al., 2022). However, there is a lack in research comparing the effect of different training intensities on alterations of mitochondrial respiratory function (D. J. Bishop et al., 2019). Furthermore, most studies investigating mitochondrial adaptations to endurance exercise training to date have studied subjects who performed moderate intensity continuous training, high intensity interval training or sprint interval training and not a combination of different intensities (D. J. Bishop et al., 2019). This contrasts with typical endurance athletes, who often spread their training throughout a variety of training zones (K. S. Seiler & Kjerland, 2006). To the best of our knowledge, this is the first study to get close to real TIDs, which are replicated by athletes, to understand how best to optimize mitochondrial adaptations. Our findings did not showed any significant differences between groups in Leak respiration state for the diaphragm muscle ($p = 0.656$), left ventricle ($p = 0.622$), soleus ($p = 0.977$) and tibialis anterior ($p = 0.154$) neither in OXPHOS respiration for the diaphragm ($p = 0.618$), left ventricle ($p = 0.234$), soleus ($p = 0.549$) and tibialis anterior muscles ($p = 0.434$).

Citrate synthase (CS) is a crucial citric acid cycle regulatory enzyme involved in the metabolic pathways that produce ATP through the aerobic metabolism and is frequently used as a surrogate for measuring oxidative and respiratory capacity (Siu, Donley, Bryner, & Alway, 2003). In line with the previously described findings, the activity of CS was not found to be significantly different between groups for the left heart ventricle ($p = 0.348$), diaphragm ($p = 0.753$), soleus ($p = 0.121$) and tibialis anterior muscles ($p = 0.558$). These findings are also in accordance to the study of Carvalho et al., (2022) (de Carvalho, Valentim, Navegantes, & Papoti, 2022) which also did not observed differences in either CS or OXPHOS complexes between a low, moderate, and high intensity aerobic training groups with equalized loads. These findings suggest that the

soleus muscle's protein level of these complexes were unaffected by the five weeks of training, demonstrating that there was no discernible change in mitochondrial activity in this particular muscle. Although our eight-week training protocol had a higher duration, it may not have been enough to trigger significant differences in adaptations in mitochondrial biogenesis markers. Nevertheless, another possible explanation, as demonstrated by Burgomaster et al. study (Burgomaster, Hughes, Heigenhauser, Bradwell, & Gibala, 2005) could be that CS and PGC1-alpha increased similarly after six weeks high intensity interval training or traditional endurance training.

According to the available evidence, within a few weeks of endurance training, there are significant increases in the synthesis of skeletal muscle mitochondrial proteins which lead to improvements in skeletal muscle metabolic activity (Holloszy, 1967). To determine which TID is optimal to stimulate mitochondrial biogenesis markers, we analyzed PGC-1α, TFAM, Mfn1 and Mfn2 expression in several organs directly involved in aerobic performance. There are multiple signaling pathways that act to regulate PGC-1α in skeletal muscle (Zhang et al., 2014). As it was discussed above, one of these mechanisms, which is primarily potentiated by completing high training volumes at low intensity, is derived from intracellular calcium signaling (Rose et al., 2007). The other pathway is connected to the cell low energy availability and involves the activation of AMPK dependent intracellular signaling pathways. High-intensity activities are what largely activate this route (Bartlett et al., 2012; M. Gibala, 2009). Our findings suggest there were no differences regarding PGC1-α expression in the left heart ventricle ($p = 0.423$), diaphragm ($p = 0.363$), soleus ($p = 0.858$) and tibialis anterior muscles ($p = 0.595$), suggesting that there is no superiority between training at moderate intensity or a combination of high and low intensity regarding the stimulation of PGC1-α expression.

TFAM is regarded as a measure of skeletal muscle mitochondrial protein synthesis since it regulates mtDNA transcription and replication and is also a gene responsive to exercise, namely endurance exercise (Erlich et al., 2016). However, to date, there are conflicting findings on training-induced changes in TFAM protein content (Granata, Jamnick, & Bishop, 2018). In fact, TFAM protein content has been found to be both unchanged (Granata, Oliveira, Little, Renner, & Bishop, 2016b; Gurd, Perry, Heigenhauser, Spriet, & Bonen, 2010) or

increased (Bengtsson, Gustafsson, Widegren, Jansson, & Sundberg, 2001; Granata et al., 2016a; Little, Safdar, Wilkin, Tarnopolsky, & Gibala, 2010) after different exercise training protocols including moderate, high intensity interval training or sprint interval training. In the present study, there were no differences between groups regarding TFAM expression in the left heart ventricle ($p = 0.353$), diaphragm ($p = 0.515$), soleus ($p = 0.757$) and tibialis anterior muscles ($p = 0.950$).

Mitochondria are dynamic organelles that may suffer fusion or fission according to the conditions of the cell and its mitochondria. Mitofusins are proteins involved in the regulation of the mitochondrial fusion dynamics in mammalian cells (Ding et al., 2010). Endurance exercise has been shown to enhance mitochondrial fusion, whether it is an acute exercise session or a short-term training period (Balan et al., 2019). It is also known that the protein expression of markers of mitochondrial fusion Mtf2 was higher in active subjects than in sedentary counterparts (Balan et al., 2019). Despite these evidences, we found no differences in the expression of Mfn1 in the left heart ventricle ($p = 0.557$), diaphragm ($p = 0.621$), soleus ($p = 0.313$) and tibialis anterior muscles ($p=0.613$) as well as in Mfn2 expression in the left heart ventricle ($p = 0.518$) diaphragm ($p = 0.373$), soleus ($p = 0.929$), tibialis anterior ($p = 0.789$). These findings could be explained by the fact that CON animals were not really sedentary and, on the contrary, did a substantial amount of high-intensity voluntary physical activity on the cage running wheel.

Thus, the results of our experimental work suggest that there does not seem to be evidence justifying the superiority of POL as a strategy to improve endurance performance nor does it seem to be evidence of a higher degree of cardiac or muscle bioenergetic adaptations as a result of POL training. Nevertheless, in our experimental study we did not investigate hematological adaptations to training. Considering the importance of the blood O_2 transport capacity for endurance performance, future studies should address this aspect in order to be able to determine if, in fact, there are no major differences in physiological adaptations to POL and other TIDs that might support its superiority as an optimal training strategy for improving endurance performance.

Conclusions

This master's dissertation is comprised by both a systematic review with meta-analysis and an experimental study. Our key findings regarding both studies were that:

- Studies on humans suggest that POL is superior to other TIDs for the improvement of VO_2max/peak. There was no evidence of POL superiority for any of the remaining cardiorespiratory function surrogates investigated.
- POL superiority was mostly evident for interventions lasting less than 12 weeks. When exercise interventions were longer than 12 weeks, VO_2max/peak was shown to increase similarly in those undergoing POL or other training intensity distribution models.
- Baseline cardiorespiratory fitness level was shown to influence the effect of POL on VO_2max/peak improvement for the case of moderately/well trained subjects.
- After four weeks of training, both VO_2max values of POL and LIM were shown to be significantly higher compared to CON. However, there were no differences between POL and LIM. At the end of the eight-week training protocol, there were no observed differences in relative or absolute VO_2max between POL and LIM groups.
- There were no differences between POL and LIM groups at the intermediate and final timepoint assessments in EC. However, both groups had a significantly higher EC compared to CON at the intermediate and final timepoint assessments.
- There were no observed differences in mitochondrial respiration between groups for any of the tissue samples analyzed.
- There were no observed differences between groups regarding diaphragm muscle fiber cross-sectional area.
- Differences were not observed between groups regarding citrate synthase activity for any of the tissue samples analyzed.
- There were no differences between groups regarding the expression of any of the markers of mitochondrial biogenesis assessed.

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Attachments

Electronic Supplementary Material Appendix S1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 Checklist.

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	3
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	6
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	6
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	8
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	8
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	8
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	9
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	9
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	9
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	9
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	10

Section and Topic	Item #	Checklist item	Location where item is reported
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	10
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	10
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	10
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	10
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	11
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	11
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	11
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	10
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	12
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	13
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	13
Study characteristics	17	Cite each included study and present its characteristics.	13
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	15
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	15
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	15
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	15
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	15
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	17

Section and Topic	Item #	Checklist item	Location where item is reported
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	17
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	13
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	19
	23b	Discuss any limitations of the evidence included in the review.	22
	23c	Discuss any limitations of the review processes used.	22
	23d	Discuss implications of the results for practice, policy, and future research.	22
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	3, 8
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	3, 8
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	8
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	4
Competing interests	26	Declare any competing interests of review authors.	4
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	4

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

For more information, visit: http://www.prisma-statement.org/

Electronic Supplementary Material Appendix, S2. Preprints of search strategy

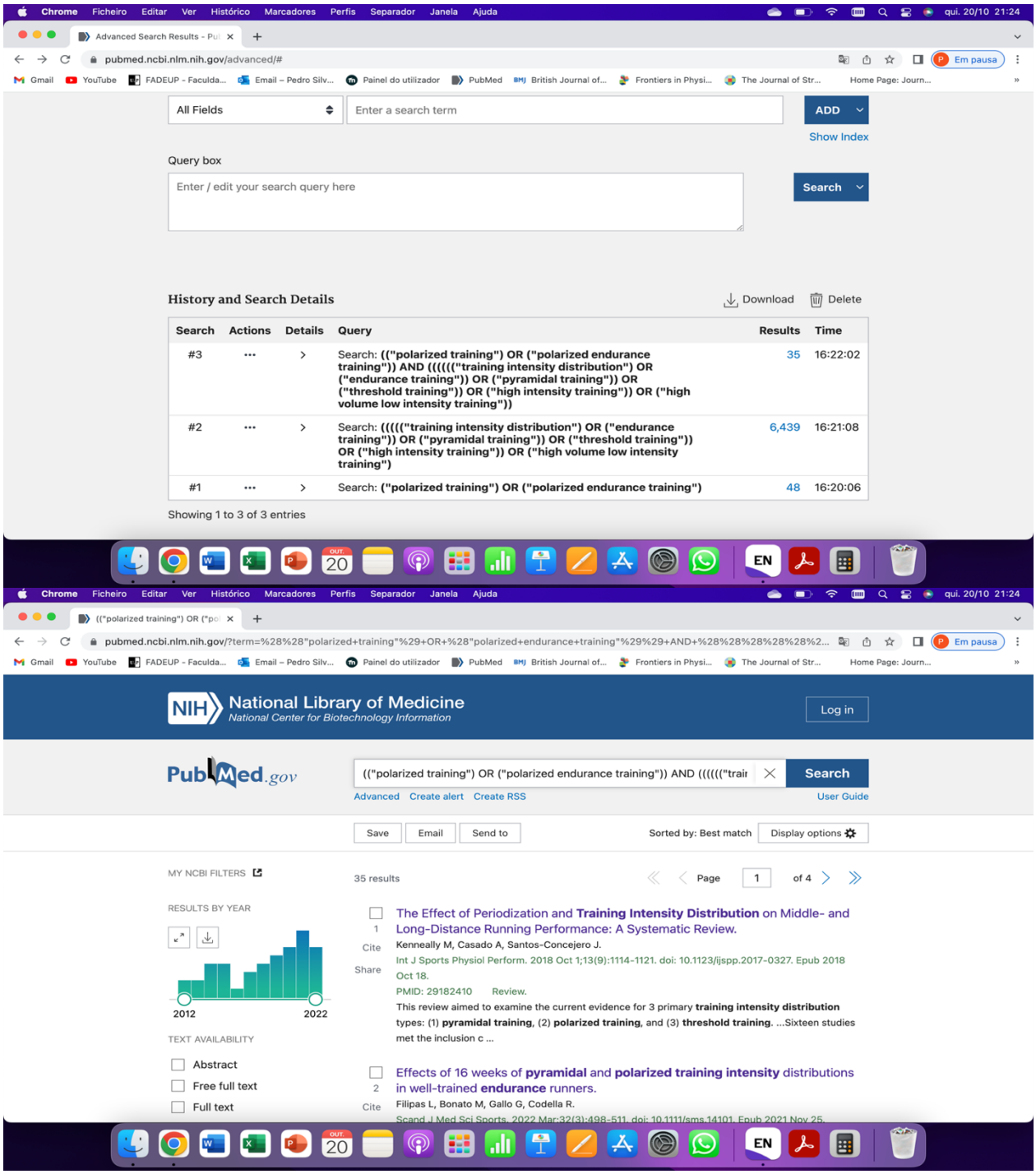

Fig. S1 Preprint search strategy PubMed

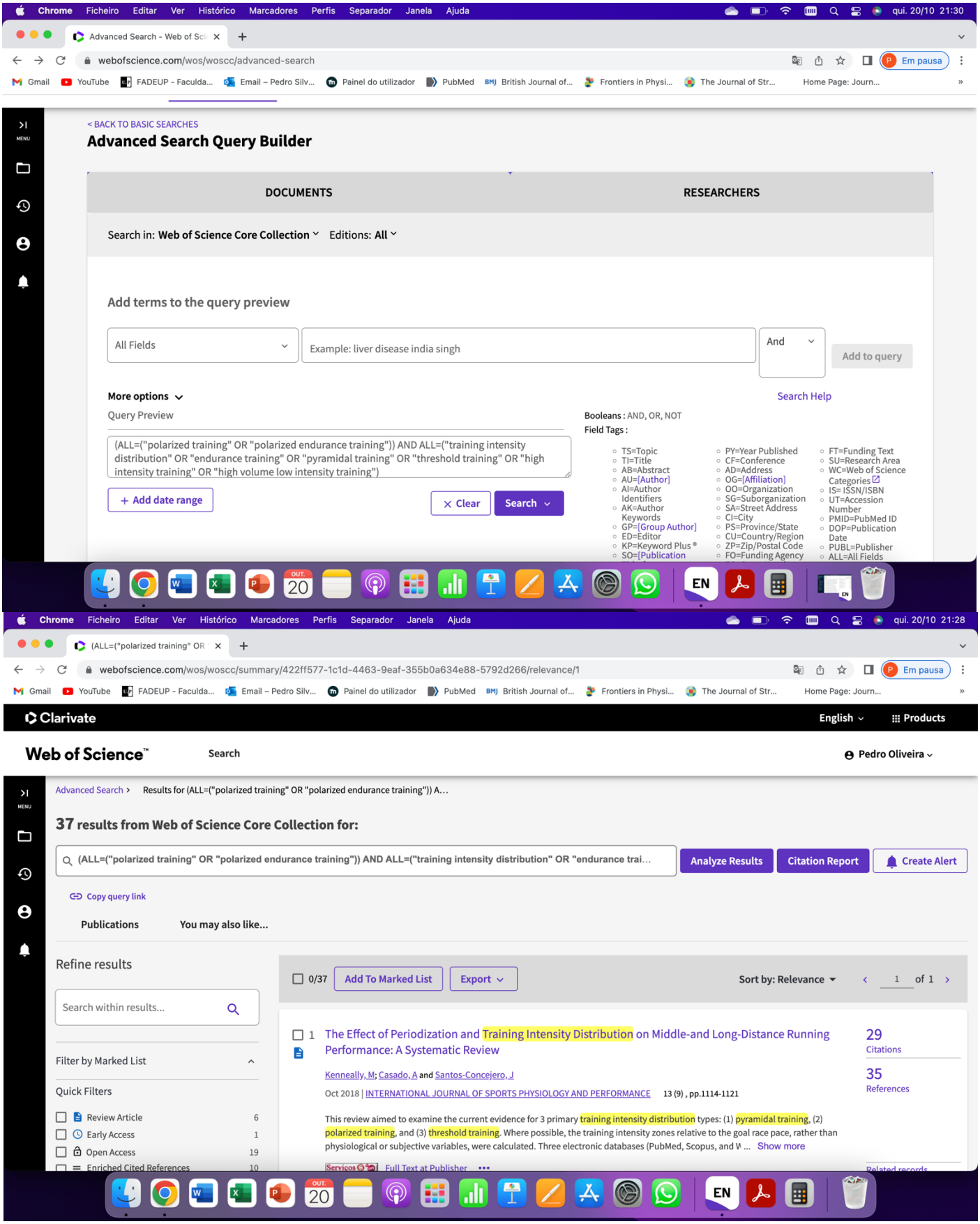

Fig. S2 Preprint search strategy Web of Science

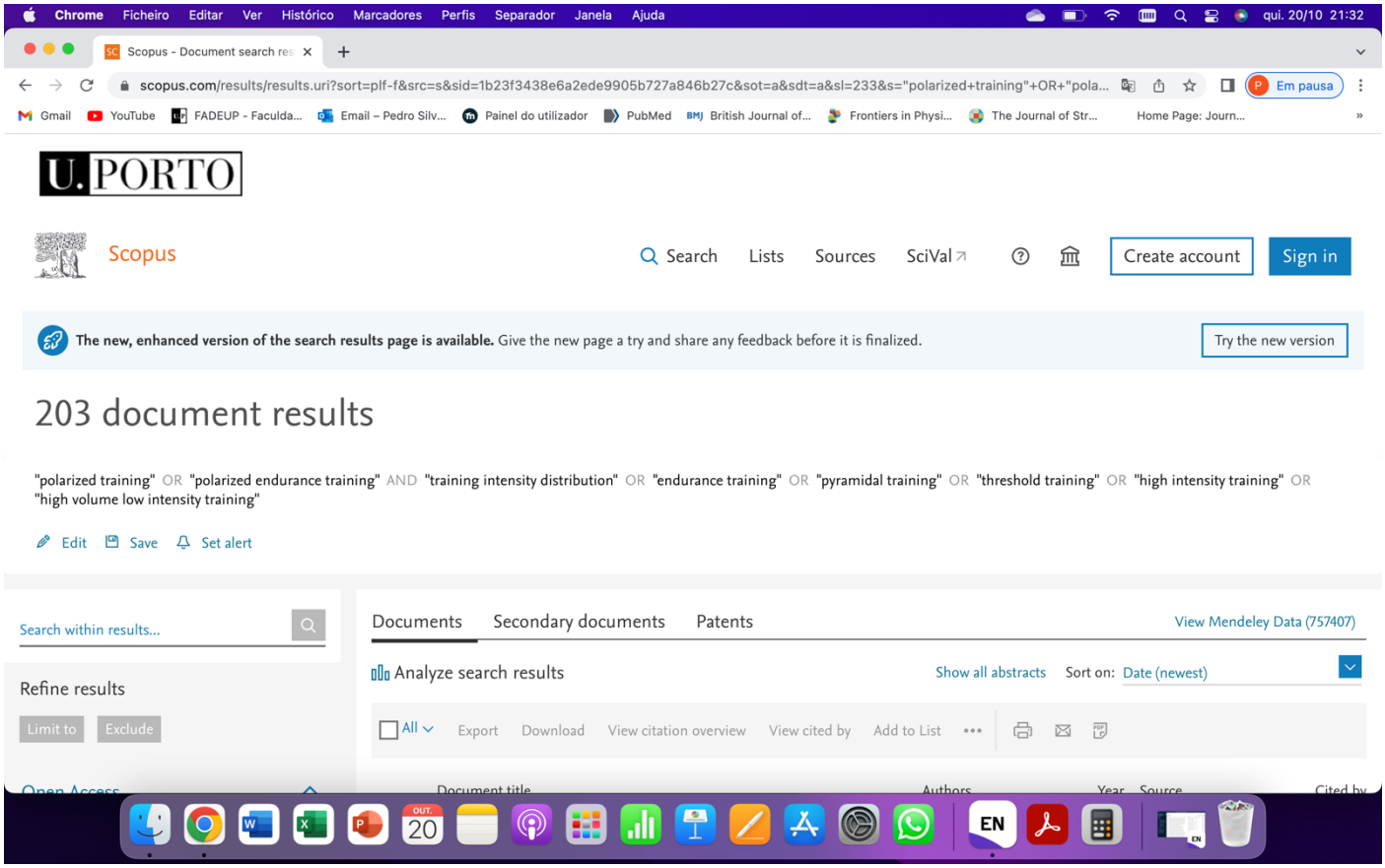

Fig. S3 Preprint search strategy Scopus

Electronic Supplementary Material Appendix S3. Sensitivity analysis

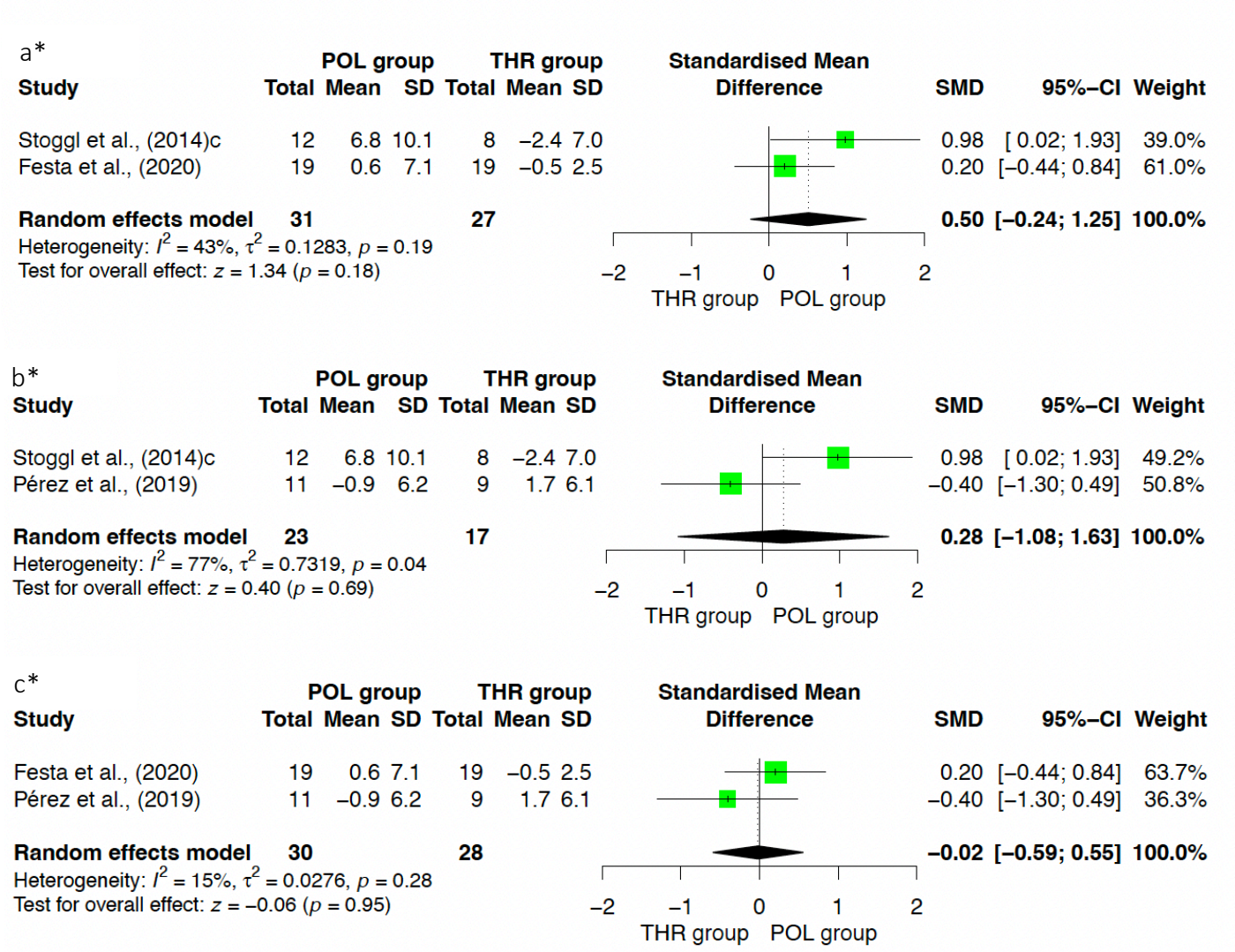

Fig. S1 Sensitivity analysis of POL vs THR regarding VO_2max/peak

Note: A sensitivity analysis for this comparison was performed by removing one study at a time. The study that was shown to contribute the most to heterogeneity was the one by Stoggl et al., (2014)c (T. Stoggl & Sperlich, 2014). Nevertheless, removal of this study did not affect the results (p= 0.95), c*.

Electronic Supplementary Material Appendix S4. GRADE – Summary of findings

Table S1 GRADE - Summary of findings

№ of studies	Study design	Certainty assessment: Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	№ of patients: POL	№ of patients: All	Absolute effect (95% CI)	Certainty
VO_2max/Peak										
13	RCT and Non-RCT [a]	not serious [g]	not serious	not serious	not serious	none	147	137	SMD **0.24** (0.01 to 0.48)	⊕⊕⊕⊕ High
TT										
10	RCT and Non-RCT [b]	not serious [h]	not serious	not serious	not serious	none	116	117	SMD -**0.05** (-0.31 to 0.21)	⊕⊕⊕⊕ High
TTE										
3	RCT and Non-RCT [c]	not serious [i]	not serious	not serious	not serious	none	36	30	SMD **0.3** (-0.2 to 0.79)	⊕⊕⊕⊕ High
V/P at VT_2/LT_2										
13	RCT and Non-RCT [d]	not serious [j]	not serious	not serious	not serious	none	124	129	SMD **0.04** (-0.21 to 0.29)	⊕⊕⊕⊕ High

Abbreviations: All: Other Training Intensity Distributions; CI: confidence interval; POL: Polarized Training; SMD: standardized mean difference; TT: Time trial; TTE: Time to exhaustion; V/P at VT_2/LT_2: Velocity or Power at 2nd ventilatory or lactate threshold; VO_2max/peak: Maximal/peak oxygen uptake.
Notes: a) Nine studies out of 13 are randomized controlled trials (RCTs); b) 8 studies out of 10 are RCTs; c) 2 studies out of 3 are RCTs; d) 9 studies out of 13 are RCTs; g) Of the 4 non-RCT studies, one was rated as moderate and 3 as low risk of bias assessed by ROBINS-I, and 9 studies were rated as some concerns risk of bias assessed by RoB-2; h) Of the 2 non-RCT studies, 1 was rated as moderate and 1 as low risk of bias assessed by ROBINS-I, and 8 studies were rated as some concerns risk of bias assessed by RoB-2; i) One non-RCT study was rated as moderate risk of bias assessed by ROBINS-I, and 2 RCTs studies were rated as some concerns risk of bias assessed by RoB-2; j) Of the 4 non-RCT studies, one was rated as moderate and 3 as low risk of bias assessed by ROBINS-I, and 9 studies were rated as some concerns risk of bias assessed by RoB-2.

Electronic Supplementary Material Appendix S5. Funnel plot and Egger's test

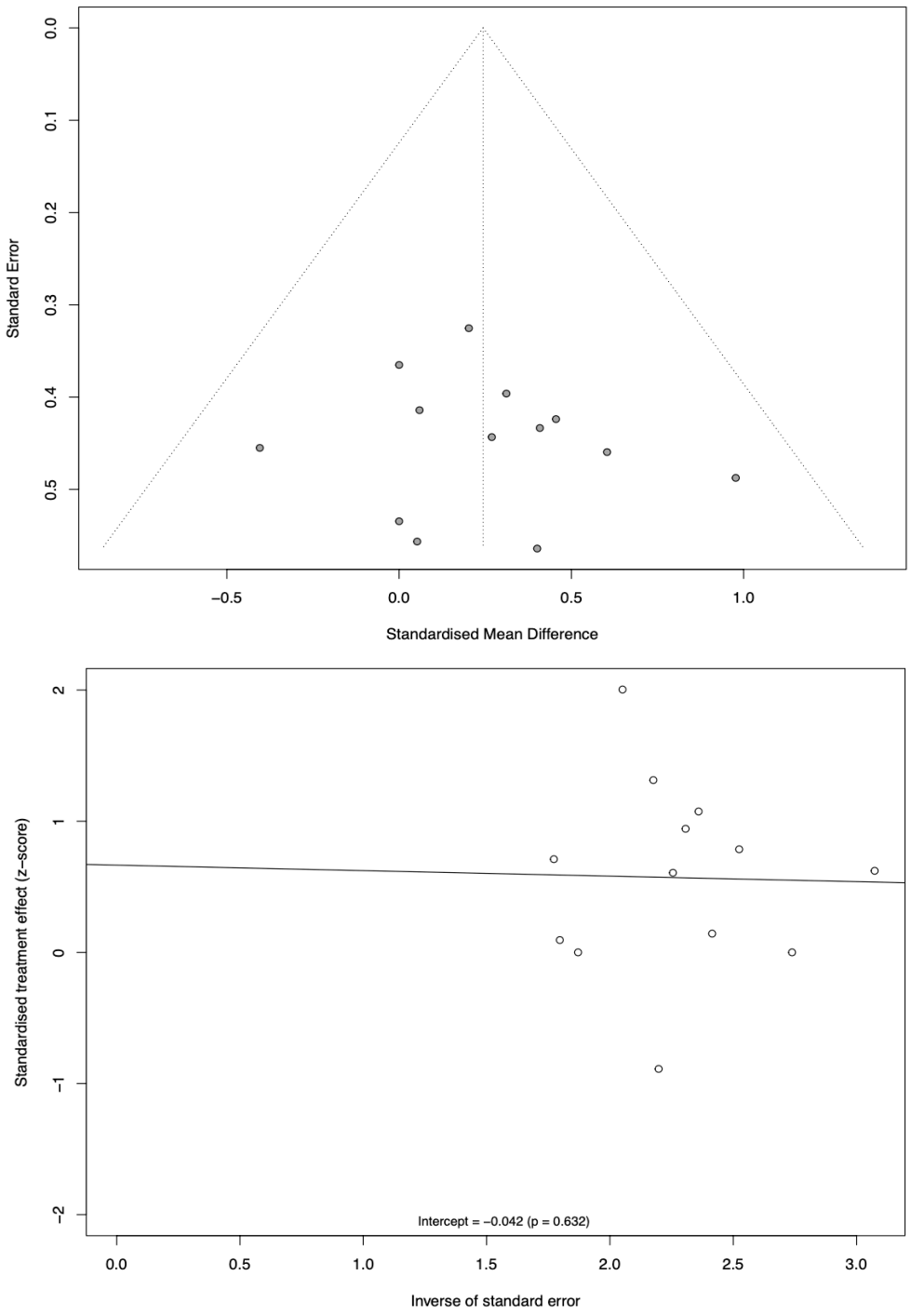

Fig. S1 Funnel plot and the Egger's test results adjusted to VO_2max/peak

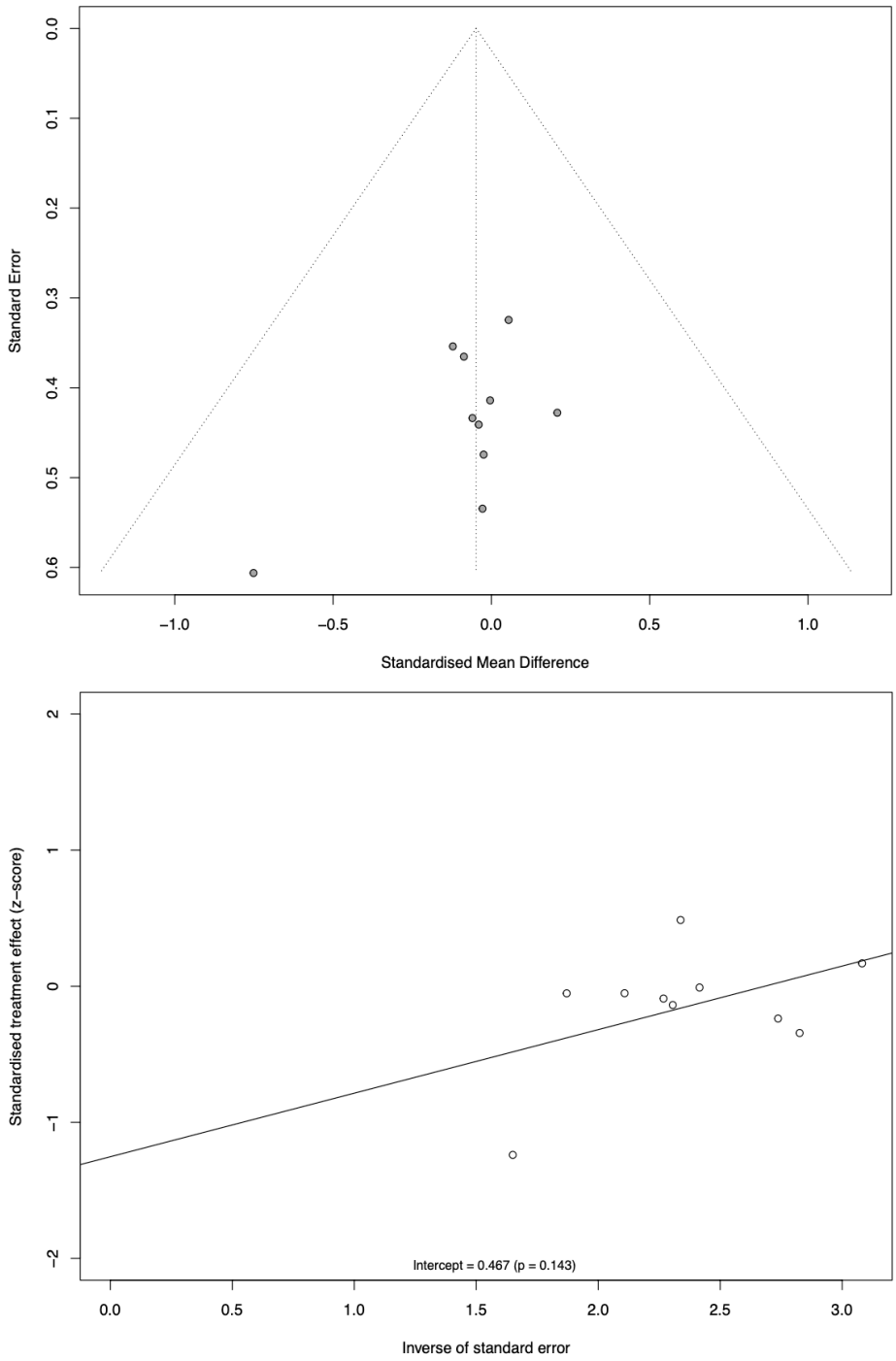

Fig. S2 Funnel plot and the Egger's test results adjusted to TT

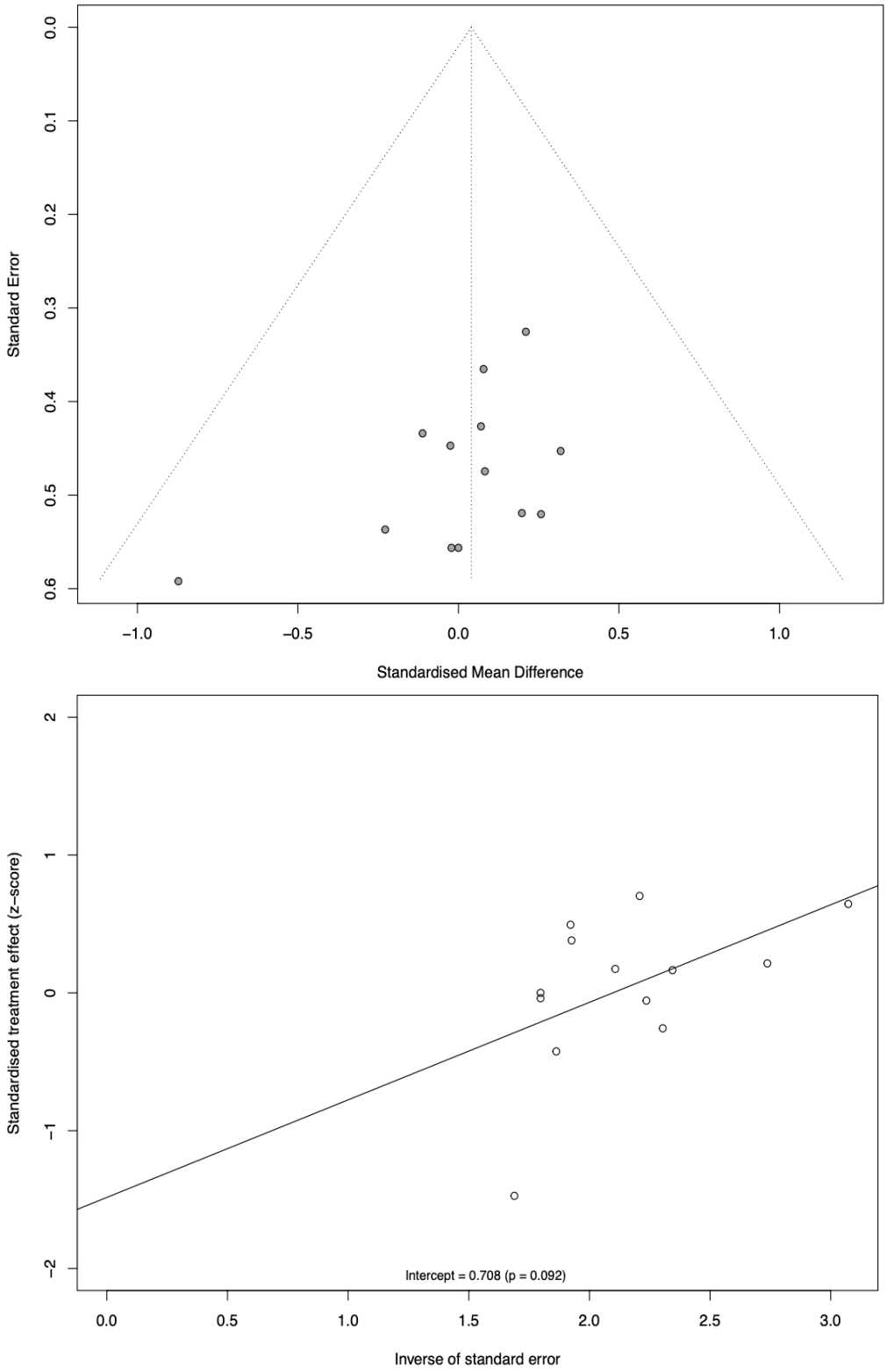

Fig. S3 Funnel plot and the Egger's test results adjusted to V/P at VT_2/LT_2