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Similar flight performance but sex-specific mitochondrial responses in free-flying pigeons

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Sex differences in metabolic responses of animals performing natural behaviours remain poorly understood, particularly under free-living conditions. Here, we examined how locomotor effort relates to mitochondrial metabolism in red blood cells in homing pigeons (*Columba livia*) by combining biologging of flight behaviour with measurements of mitochondrial respiration taken before and immediately after flight. Birds were equipped with global positioning system (GPS) loggers and tri-axial accelerometers, and movement effort was quantified using vectorial dynamic body acceleration (VeDBA). Flight was associated with a marked increase in mitochondrial respiration linked to aerobic energy production in both sexes, with increases ranging from 58.2% to 87.3% across traits. Males and females showed similar flight speeds, flight durations and social flight behaviour. Nevertheless, females showed higher VeDBA and higher post-flight mitochondrial aerobic metabolism than males, indicating sex differences in both mechanical and physiological responses. The relationship between VeDBA and post-flight mitochondrial metabolism was also sex-specific: as predicted, higher VeDBA was associated with higher ROUTINE and OXPHOS in males, but with lower values in females, a pattern for which we discuss possible physiological and behavioural explanations. Independent of flight status, males exhibited higher mitochondrial coupling efficiency than females, which was explained by their higher body mass. Together, these results provide evidence that flight effort was mirrored in blood mitochondrial metabolism and show that similar flight performance can mask potential sex-specific energetic signatures.

1. Introduction

Locomotion is essential for free-living animals, enabling behaviours such as foraging, predator avoidance or navigation [1]. Because movement is energetically demanding and may be constrained by different factors, understanding how animals fuel locomotion is a central question in ecology [2]. As a simplification, behavioural performance is often used as a proxy for energetic expenditure, but recent work shows that similar behavioural performance does not necessarily imply similar energetic costs [3]. One likely, but often overlooked, source of variation in energetics is sex. Theory on metabolic variation already recognizes sex as a major axis of physiological diversity, alongside age, life-history stage and environmental context [4]. Males and females can differ in substrate use, endurance and mitochondrial function [5,6]. Yet,

studies of locomotor energetics in free-living animals often assume that similar performance reflects similar energetic strategies in both males and females.

Direct measurements of energy expenditure during movement remain difficult in free-living vertebrates [7,8]. Mitochondrial physiology could offer one way to address this limitation. Mitochondria generate adenosin triphosphate (ATP) to meet the high energetic demands of physical activity [9,10]. Muscles engaged in intense work can increase ATP consumption by 50- to 100-fold compared to resting conditions [11–14]. Despite newly produced ATPs last only a few seconds, cellular ATP concentrations remain remarkably stable during exercise [14], underscoring the efficiency of mitochondrial function in preventing ATP depletion. However, individuals differ in mitochondrial efficiency, which may influence their ability to cope with energy-intensive locomotion [15]. These subcellar differences in organismal performance can also be sex-specific, at least in humans and animal models [16–19]. Understanding whether males and females differently allocate energy during periods of high demand is therefore crucial, as it directly relates to fitness and survival outcomes [20–23]. Yet, a recent synthesis of physiological studies in wild birds and mammals showed that sex differences are still rarely considered in work on metabolism, thermoregulation, stress and immune function [24].

Recent advances in high-resolution respirometry have enabled real-time measurement of mitochondrial metabolism in low-metabolic-rate tissues, including blood [4,25–27]. In humans, blood mitochondrial traits respond to aerobic training [28–33], and in birds, they can predict flight performance and correlate with organismal metabolic rate [34–36]. In birds, erythrocyte mitochondrial metabolism correlates with organismal basal metabolic rate [37–39] and is positively associated with pectoral muscle oxygen consumption, suggesting a link between systemic and muscle-specific metabolic capacities [37]. These findings indicate the potential of blood-based mitochondrial measures as proxies for whole-body activity, although this relationship has not yet been tested directly.

Flapping flight is an especially powerful context in which to test this idea because it is among the most energetically expensive forms of locomotion in vertebrates [40–42]. Homing pigeons (*Columba livia*) offer an excellent system in which to address this problem, owing to their almost exclusive reliance on flapping flight, reflected by the extraordinary development of their pectoral muscles that, similarly to hummingbirds, constitute over 30% of body mass [43]. Males are typically heavier than females, which could influence the energetic demands of flight. Studies from pigeon racing and homing experiments generally never found differences between males and females in flight performance or homing success [44–50]. Accordingly, most physiological studies of flight in pigeons have focused on energetic, cardiovascular, endocrine, oxidative or immune responses without explicitly testing for sex differences [51–56]. This raises the possibility to test whether males and females achieve similar behavioural outcomes because of comparable metabolism or whether they have different strategies.

To test whether mitochondrial metabolism reflects the energetic demands of homing flight, we equipped pigeons with global positioning system (GPS) and tri-axial accelerometer loggers that provide a well-established index of locomotor effort through vectorial dynamic body acceleration (VeDBA) [7,8,45,57,58,59,60]. VeDBA reflects wingbeat kinematics, postural adjustments and active control required to maintain stability during flight [7,45,47,49–52,61,62] and has been used to predict daily energy expenditure during flight [53,54]. We also considered mitochondrial respiration in red blood cells (RBCs) at rest and immediately after flight ('in flight' for brevity), and related flight mitochondrial traits to VeDBA as an index of mechanical flight effort. If blood mitochondrial metabolism reflects the energetic cost of flight, then it should increase after flight and covary with flight effort. If similar behavioural performance is regulated by similar energetic strategies, then these relationships may be consistent among individuals, including between the sexes. We focused on complementary respiratory traits (see Methods for details) that together describe how mitochondria meet energetic demand: overall cellular respiration, respiration coupled to ATP production and the respiration coupled with heat (and not with ATP). We addressed two main questions. First, does blood mitochondrial metabolism increase after flight, consistent with a systemic upregulation of aerobic energy production? Second, do individuals showing greater mechanical effort during flight (i.e. VeDBA) also show higher post-flight mitochondrial metabolism? Because sex-associated differences in mitochondrial function have been reported, we asked whether males and females may achieve comparable flight outcomes through different physiological strategies by considering the sex effect in the two previous questions. We also quantified circulating corticosterone to evaluate whether homing flight imposed any stress response [63] that can impair mitochondrial coupling efficiency [64].

2. Methods

(a) Study area and model species

The study was conducted in 2021 and 2025 using homing pigeons housed in a loft located in Guidonia (Rome, Italy). Birds were allowed daily free flight and trained with weekly releases.

(b) Pigeon releases and blood sampling

Twenty days before each annual experiment (2-3 July 2021; 17–18 June 2025), we randomly selected 17 males and 15 females, aged 2–4 years, from a pool of 150 birds. To habituate birds to carrying devices during homing flights, we attached a plasticine dummy matching the size and weight of the GPS logger (40 × 20 × 20 mm, 12 g; Technosmart Europe, Italy), equipped with a tri-axial accelerometer. The load corresponded to 2.8% of male and 3.4% of female body mass and was attached to the central back feathers with a Velcro strip [65]. Birds wearing the dummy were trained twice per week to fly home, with the release distance from their

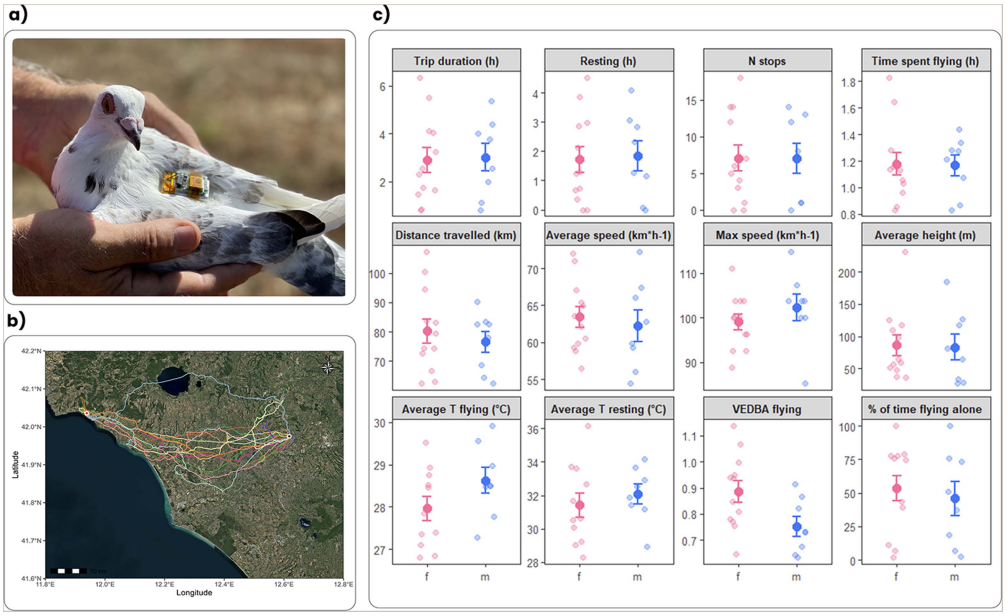

Figure 1. (a) Pigeon equipped with a biologger. Photo credit: Stefania Casagrande. (b) Homing tracks from the release site (red) to the home loft (black), illustrating individual variation in route choice. (c) Flight parameters of males (blue) and females (pink). Small pale dots show individual values, and large coloured dots show mean ± s.e.

loft increasing by approximately 15 km each session, up to a distance of about 75 km. During both training and experimental flights, birds were released together.

The afternoon before each year's experiment (16.00–18.00 hours), birds were blood sampled (200 µl from the tarsal vein with heparinized syringes), weighed and returned to the aviary. The next morning, they were placed in two transport baskets (80 × 50 × 40 cm) and driven by car from their loft to the release site at approximately 60 km (figure 1a), where the dummy was replaced with the GPS device before release (around 10.20 and 13.30 hours). Upon return, birds were sampled again, the devices retrieved and body mass re-measured with a Pesola scale with a precision of 2 g. Blood samples were collected in heparinized tubes and kept in a cooling box until processing. Plasma was separated and stored for corticosterone assays, whereas RBCs were used immediately for mitochondrial analyses. The birds were then returned to the aviary.

(c) Processing of global positioning system and accelerometer data

GPS positions were recorded every 1 s and acceleration at 25 Hz. Based on ground-speed distributions, locations with speeds of less than 18 km h^{-1} were classified as pauses and those of 18 km h^{-1} or more as flight. Positions within 2 km of the release site and 300 m of the loft were excluded (figure 1b). From GPS data, we quantified duration and length of the trip, number and duration of stops (>5 s), total pause duration and total flying time. Average speed, altitude, air temperature, wingbeat frequency and VeDBA were calculated for flight periods, with the latter two derived from acceleration data. Wingbeat frequency was estimated from the heave axis using a fast Fourier transform over a 4 s moving window. VeDBA was calculated from dynamic acceleration obtained by subtracting static acceleration, estimated with a 3 s running mean, from raw acceleration to obtain the dynamic acceleration [66] that was then smoothed using a 1 s running mean [67]. We also quantified whether birds flew alone or in company, defined as at least one bird within 50 m and 3 s [41].

(d) Mitochondrial analysis

Aerobic metabolism of intact RBC was quantified by high-resolution respirometry (O2k Oxygraph, Oroboros Instruments, Austria) following [68] with modifications from [69]. Briefly, 50 µl of washed RBC pellet was resuspended in 1.0 ml of pre-equilibrated MiR05 buffer and measured at 40°C, close to resting pigeon body temperature (40.5 ± 1.2°C; [70]). Oxygen consumption was recorded following a standard sequential substrate/inhibitor addition protocol (SUIT-003_ceD009, https://wiki.oroboros.at). We quantified ROUTINE respiration (basal oxygen consumption in intact cells under endogenous conditions, reflecting cell metabolic rate, CMR), LEAK respiration (oxygen consumption not coupled to ATP synthesis, mainly reflecting proton leak across the inner mitochondrial membrane) after 1 mg ml^{-1} oligomycin addition, OXPHOS (oxygen consumption coupled to ATP production through oxidative phosphorylation) as ROUTINE minus LEAK, ETS (maximal capacity of the electron transport system) after carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 1 mmol l^{-1} titration), and residual oxygen consumption (ROX) after antimycin A (5 mmol l^{-1}); ROX was used to correct all preceding states. We also calculated mitochondrial coupling efficiency ($FCR_{OXPHOS/ROUTINE}$), representing the proportion of endogenous respiration allocated to ATP production via oxidative phosphorylation. We reported other details in the supplementary material.

(e) Statistical analysis

Statistical analyses were performed in R (v. 4.3.1; R Core Team 2023), with full details provided in the supplementary material. Linear mixed-effects models and linear models were fitted using the lme4 and base R packages, respectively. For all models, uncertainty in fixed-effect estimates was quantified from the posterior distribution of model coefficients generated using the sim() function in the arm package. We report the posterior median, 95% credible interval (CrI) and posterior probability (P) that the effect was positive or negative. Effects were considered strongly supported when 95% CrIs did not include zero, while $P \geq 0.95$ were considered indicative of meaningful trends. Pre- versus post-flight changes in physiological traits were analysed using linear mixed-effects models, with time (pre/post), sex and their interaction included as fixed effects, and individual identity (TagID) fitted as a random intercept to account for repeated measurements of the same birds. Because males were larger than females, scaled body mass was included as an additional covariate. To test whether flight effort predicted post-flight mitochondrial metabolism, we fitted separate linear models for post-flight ROUTINE, OXPHOS and ETS, with VeDBA, sex and their interaction as predictors. Because VeDBA was measured only during flight, these analyses were restricted to post-flight values.

Because birds did not always enter the aviary immediately upon arrival, the delay between the actual arrival recorded with the GPS and blood sampling (hereafter 'elapsed time', mean: 32.8 ± 9.09 min, range: 1–162 min, $n = 20$) varied among individuals. Elapsed time had no effect on mitochondrial traits and was also excluded from the final post-flight models (full results are provided in the supplementary material, table S4). Year effects were also evaluated; although some differences in flight context occurred between 2021 and 2025, year did not change conclusions, and these analyses are therefore provided in the supplementary material, table S5, together with year differences in flight behaviour (supplementary material, table S6).

3. Results

Of the 32 birds released, 20 (12 females and eight males) returned home within the same day (figure 1b,c), while the other 11 individuals returned on the day after and were not sampled because their physiological state would no longer represent the immediate post-flight metabolic response to homing. Two post-flight ETS measurements from females were lost owing to technical problems, so ETS analyses at post-flight are based on 18 rather than 20 individuals.

(a) Question 1: effect of flight on mitochondrial bioenergetics

On average (mean ± s.e.), the homing trip lasted 2.94 ± 0.38 h, of which individuals spent 1.17 ± 0.38 h flying. The return flight was interrupted by stops (7 ± 5.80) that lasted on average 15.15 ± 19.37 min (figure 1c). Pigeons travelled an average distance of 78.71 ± 2.85 km (see also figure 1b) at an average speed of 62.96 ± 1.17 km h^{-1}, reached a maximum speed of 100.22 ± 1.59 km h^{-1}, and performed 5.41 ± 0.43 wingbeats s^{-1}. Mean flight altitude was 84.84 ± 54.64 m. During flight, birds experienced an air temperature of 28.24 ± 0.94°C, whereas resting periods were associated with higher temperatures (31.72 ± 0.48°C). Males and females did not differ in any of the measured flight behavioural traits, including flight duration, number of stops, distance travelled, speed or altitude (figure 1c). The only exception was dynamic body acceleration, with males exhibiting lower mean VeDBA values during flight than females (figures 1c and 3; table 2; supplementary material, table S1).

ROUTINE increased by 58.2% from resting to flying status (table 1a; figure 2a), similarly in both sexes, although females showed higher levels of ROUTINE than males (table 1a), driven by post-flight measures (figures 2a and 3a). Repeatability was moderate ($R = 0.51$).

OXPHOS increased by 62.8%. Females showed a stronger increase than males (sex × time: $P = 0.96$), although the CrI slightly overlapped zero (table 1b; figures 2b and 3a). Repeatability was moderate ($R = 0.52$).

LEAK did not change clearly with flight state but decreased with body mass (table 1c; figure 2c). Without body mass in the model, females showed higher LEAK than males (supplementary material, tables S4c and S5c; figure 2c), suggesting this sex difference was partly masked by size variation. LEAK showed low repeatability ($R = 0.14$).

ETS increased by 87.3% from rest to flight condition (table 1d; figure 2d) with no clear sex × time interaction (table 1d). Females showed overall higher ETS values than males (table 1d; figure 2d). Repeatability was low to moderate ($R = 0.29$).

Mitochondrial coupling efficiency ($FCR_{OXPHOS/ROUTINE}$) did not vary clearly with flight status or sex, but increased with body mass (table 1e; supplementary material, figure S2). In models without body mass, males showed overall higher mitochondrial coupling efficiency than females (supplementary material, tables S4e and S5e), indicating that this sex difference became apparent only when variation associated with body mass was not accounted for. Repeatability was low ($R = 0.18$). Body mass was not affected by the flight state or the interaction between sex and flight state (table 1f).

(b) Question 2: relationship between aerobic mitochondrial metabolism and flight mechanical effort

ROUTINE showed a clear sex-specific association with flight effort, reflected by a sex × VeDBA interaction (figure 3b; table 2a). In females, ROUTINE declined with increasing VeDBA, whereas in males, it increased, suggesting opposite mitochondrial responses to increasing flight effort (table 2a; figure 3b). A similar sex-specific pattern was observed for OXPHOS: it decreased with VeDBA in females but increased in males (table 2b; figure 3b).

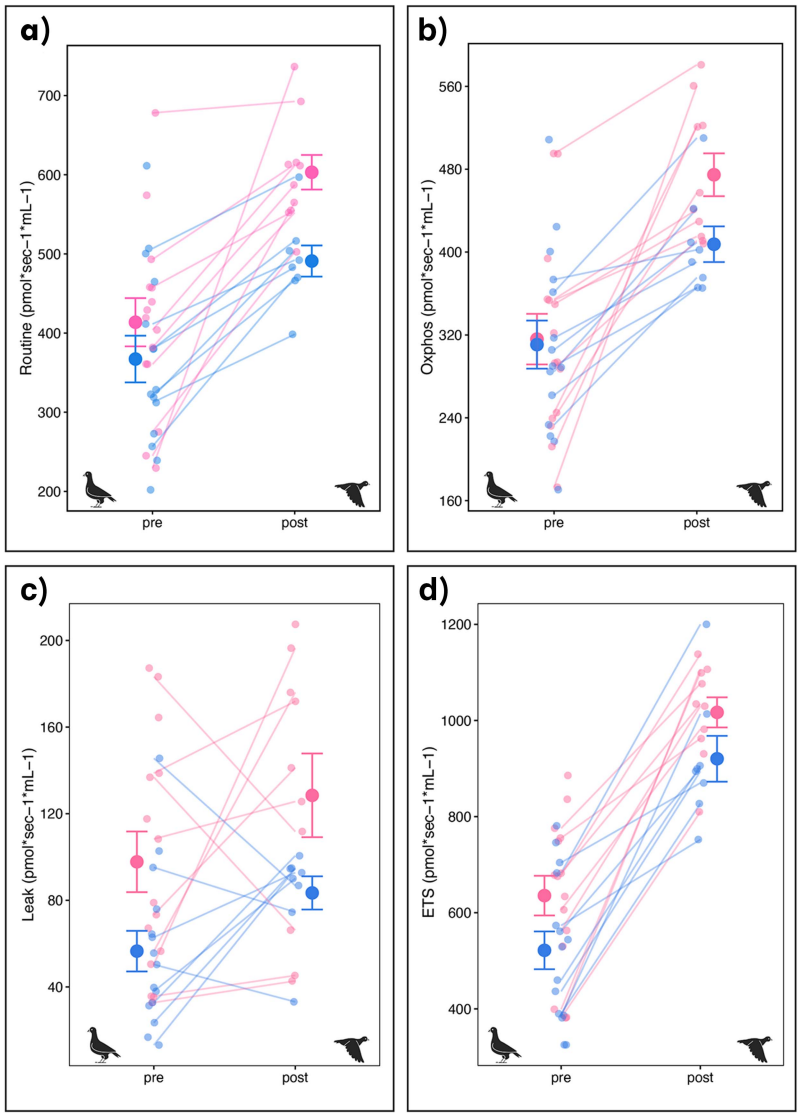

Figure 2. Change in mitochondrial bioenergetic traits from rest ('pre') to post-flight ('post'). (a) ROUTINE respiration equivalent to CMR; (b) OXPHOS; (c) LEAK; and (d) ETS. Pink = females, blue = males; small dots show individuals, and large dots show mean ± s.e. $n = 30$ at rest; post-flight ($n = 20$), except ETS ($n = 18$).

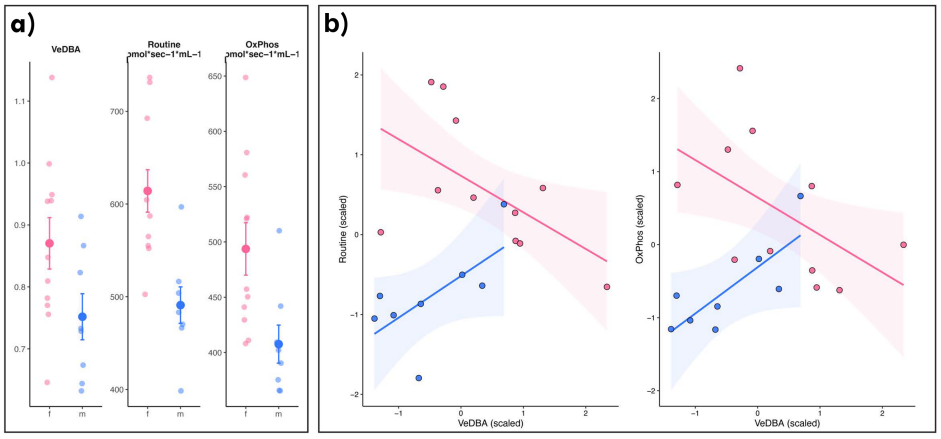

Figure 3. (a) Sex differences in VeDBA, ROUTINE and OXPHOS. Pink = females, blue = males; small dots show individual values, and large dots show mean ± s.e. (b) Relationships between mean VeDBA during flight and post-flight ROUTINE and OXPHOS. Points show scaled individual values coloured by sex; lines show sex-specific predictions from models including the sex × VeDBA interaction, with shaded 95% confidence intervals.

Table 1. Effect of flight activity on mitochondrial measures. (Number of observations: 52 on 31 individuals (except for ETS, for which observations are 48 on 31 individuals). Bold text indicates statistically meaningful results. Asterisks indicate significance levels from the corresponding frequentist analyses: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Notes: '*P*' represents the posterior probability that the parameter of interest falls within a specific range, given the observed data. σ^2_TagID Among-individual variance ['lower 95% CrI, upper 95% CrI'], σ^2_*residual* 'residual variance' ['lower 95% CrI, upper 95% CrI']. *R* = repeatability.)

variable (R^2 marginal, R^2 conditional)	estimate [95% CrI] – (*P* = posterior probability)	*p*-values
(a) ROUTINE (0.54, 0.77)		
intercept	**803.23 [481.06, 1118.44]**	***
time (rest)	**−203.00 [−257.67, −143.62] – (*P* = 1.00)**	***
sex (male)	**−108.13 [−214.27, −4.68] – (*P* = 0.98)**	**
sex× time	61.61 [−26.72, 148.15] – (*P* = 0.92)	ns
mass	−0.23 [−0.67, 20.53] – (*P* = 0.87)	ns
σ^2TagID: 5049.29 [3145.07, 7875.69]; σ^2 residual: 4968.51 [3408.93, 7739.35] – *R* = 0.51		
(b) OXPHOS (0.46, 0.75)		
intercept	**501.16 [216.46, 788.92]**	***
time (rest)	**−175.45 [−224.96, −126.69] – (*P* = 1.00)**	***
sex (male)	**−94.93 [−185.96, −3.42] – (*P* = 0.98)**	**
sex × time	**64.34 [−8.95, 142.04] – (*P* = 0.96)**	**(*)**
mass	−0.75 [−38.13, 36.40] – (*P* = 0.51)	ns
σ^2TagID: 4036.21 [2524.36, 6250.11]; σ^2 residual: 3707.24 [2534.44, 5692.10] – *R* = 0.52		
(c) LEAK (0.35, 0.43)		
intercept	**279.96 [153.62, 412.04]**	***
time (rest)	−26.68 [−57.84, 5.03] – (*P* = 0.93)	ns
sex (male)	−11.53 [−59.90, 35.58] – (*P* = 0.69)	ns
sex × time	−5.03 [−52.30, 42.08] – (*P* = 0.58)	ns
mass	**−21.12 [−38.98, −3.52] – (*P* = 0.99)**	**
σ^2TagID: 250.39 [133.37, 440.35]; σ^2 residual: 1606.60 [1094.10, 2479.51] – *R* = 0.14		
(d) ETS (0.70, 0.78)		
intercept	**1325.36 [828.90, 1800.04]**	***
time (rest)	**−392.17 [−495.63, −286.63] – (*P* = 1.00)**	***
sex (male)	−71.37 [−222.58, 85.22] – (*P* = 0.82)	ns
sex × time	−20.94 [−170.37, 131.53] – (*P* = 0.61)	ns
mass	−37.75 [−102.12, 25.59] – (*P* = 0.88)	ns
σ^2TagID: 5665.42 [3199.84, 9812.02]; σ^2 residual: 14104.94 [9463.37, 22343.93] – *R* = 0.29		
(e) $FCR_{OXPHOS/ROUTINE}$ (0.24, 0.38)		
intercept	**0.49 [0.24, 0.73]**	***

(*Continued.*)

Table 1. (*Continued.*)

variable (R^2 marginal, R^2 conditional)	estimate [95% CrI] – (P = posterior probability)	p-values
time (rest)	−0.03 [−0.09, 0.03] – ($P = 0.87$)	ns
sex (male)	−0.03 [−0.11, 0.06] – ($P = 0.73$)	ns
sex × time	0.05 [−0.04, 0.14] – ($P = 0.89$)	ns
mass	**0.04 [0.01, 0.08] – ($P = 0.99$)**	*
σ^2TagID: 0.00 [0.00, 0.00]; σ^2residual: 0.01 [0.00, 0.01]		
(f) body mass (0.94, 0.95)		
intercept	**379.37 [361.28,397.78]**	***
time (rest)	−4.27 [−12.40,3.81] – ($P = 0.86$)	ns
sex (male)	**32.90 [10.93,54.62] – ($P = 1.0$)**	**
sex × time	−0.72 [−13.39,11.54] – ($P = 0.45$)	ns
σ^2TagID: 1524.93 [1212.40, 2013.68]; σ^2 residual: 91.09 [62.23, 139.14] – $R = 0.95$		

ETS showed little association with VeDBA, although males tended to have lower ETS values than females (table 2c). Mitochondrial efficiency was not clearly related to VeDBA, and there was no evidence for a sex × VeDBA interaction (table 2).

4. Discussion

Our study reveals that relatively short homing flights (about 70 km and a bit more than 1 h of sustained flight) trigger substantial and coordinated changes in avian energetics. ROUTINE, OXPHOS and ETS all increased markedly from rest to flight values, indicating rapid upregulation of aerobic metabolism in response to the energetic demands of flapping flight. This increase was not explained by sampling delay or year effects, supporting the robustness of the pattern. To our knowledge, this is the first study to show such an effect in free-flying birds. Although blood cells contribute minimally to total energy expenditure [37], blood mitochondrial metabolism may provide an integrated indicator of whole-organism physiological adjustment to flight, including indirect signals associated with flight-muscle activity. Blood circulates through all organs and is exposed to substrates, hormones, redox signals, extracellular vesicles and other metabolic cues released during intense exercise [72]. This interpretation is consistent with evidence that exercise requires coordinated communication among tissues, with skeletal muscle acting as a signalling organ providing an additional route for tissue-to-tissue communication [72]. We suggest that during flapping flight, signals originating from highly active tissues, particularly the pectoral muscles, may therefore be reflected in circulating blood cells.

Despite that flight performance was similar between sexes—including distance flown, speed, altitude, time flying alone or with others and time spent flying or resting—the sexes differed consistently in their energetic phenotype. Females showed higher ROUTINE, LEAK and ETS than males and increased more OXPHOS from rest to flight. Consistent with their higher energy expenditure, females also showed higher VeDBA values than males. In addition, females were less efficient in coupling respiration with energy production, largely driven by higher LEAK. The most likely interpretation is therefore that females achieved similar behavioural performance through higher mitochondrial throughput than males. These sex differences were not fully explained by body mass: the main flight-related increases in ROUTINE, OXPHOS and ETS remained after accounting for size, whereas LEAK and coupling efficiency were more sensitive to body mass adjustment. This interpretation is consistent with growing evidence from mammals and birds that mitochondrial organization and respiration can differ between sexes [16–18,73]. In our system, sex-specific bioenergetic strategies would probably have remained undetected from behaviour alone. For instance, studies in pigeons have extensively characterized substrate use, lipid mobilization and protein catabolism during flight and recovery [52,56,70,74–76], and recent work has further refined our understanding of the aerodynamic and energetic costs of flight in free-flying pigeons [41,47–49,79,82], but all ignored sex as a biological variable. These findings may also have implications for birds that rely on sustained flight over longer distances. If similar sex-specific bioenergetic strategies occur in migratory species, comparable movement performance could conceal differences in endurance, recovery, refuelling needs, stopover duration or exposure to oxidative and energetic stress. These possibilities remain to be tested, but they suggest that blood mitochondrial metabolism may help reveal hidden physiological variation in flight costs that is not apparent from movement metrics alone.

Unlike in many previous studies in a wind tunnel [77–79], flight was voluntary rather than forced in our study and thus reflected self-paced effort and spontaneous intermittent resting, which allowed control of physiological stress. Circulating corticosterone, which mobilizes stored fuels during energetic challenges [80] at the potential expenses of coupling efficiency [64], did

Table 2. Models evaluating the association between VeDBA and the mitochondrial parameters. Bold text indicates statistically meaningful results. Asterisks indicate significance levels from the corresponding frequentist analyses: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

response var (R^2)	estimate	95% CI	posterior *P*
(a) ROUTINE (0.61)			
intercept	**628.00**	**589.35, 667.35**	***
sex (male)	**−108.63**	**−176.68, −39.76**	***P* = 1.00 ** **
VeDBA	**−37.21**	**−75.14, −0.93**	***P* = 0.97 ***
sex (male) × VeDBA	**87.82**	**12.21, −159.22**	***P* = 0.99 ***
(b) OXPHOS (0.52)			
intercept	**504.00**	**466.43, 540.92**	***
sex (male)	−66.26	−133.36, 1.74	$P = 0.97$
VeDBA	−45.25	−81.20, −8.30	***P* = 1.00 ***
sex (male) × VeDBA	**98.13**	**24.53, 169.75**	***P* = 1.00 ** **
(c) ETS (0.31)			
intercept	**1050.21**	**963.19, 1136.47**	***
sex (male)	**−144.48**	**−278.76, −3.15**	***P* = 0.98***
VeDBA	−56.09	−130.91, 18.79	$P = 0.93$
sex (male) × VeDBA	19.62	−132.93, 164.64	$P = 0.60$
(d) $FCR_{OXPHOS \times ROUTINE\text{-}1}$ (0.19)			
intercept	**0.80**	**0.75, 0.85**	***
sex (male)	0.04	−0.05, 0.13	$P = 0.82$
VeDBA	−0.02	−0.07, 0.03	$P = 0.82$
sex (male) × VeDBA	0.04	−0.06, 0.14	$P = 0.81$

not increase significantly after flight and showed no association with any of the mitochondrial traits measured (see the supplementary material for full results), suggesting that our birds met the demands of homing flight without entering an emergency state.

Our repeated-measures design further showed that ROUTINE and OXPHOS retained moderate repeatability across rest and post-flight states, indicating that blood mitochondrial traits capture not only short-term plasticity but also stable among-individual differences [81–83], supporting their use as biologically meaningful traits in free-flying birds.

The relationship between VeDBA and post-flight mitochondrial metabolism was also sex-specific. In males, higher VeDBA was associated with higher ROUTINE and OXPHOS, consistent with the expectation that greater flight effort requires greater aerobic upregulation, as mirrored in the blood. In females, however, the relationship was reversed. We do not yet have a definitive explanation for this pattern, but lower coupling efficiency in females may weaken the link between mechanical effort and ATP-producing respiration. In addition, VeDBA integrates not only propulsive accelerations but also body stabilization and manoeuvring. Consistent with this, females showed higher VeDBA without higher wingbeat frequency, which was similar in males and females (supplementary material, figure S1), suggesting that their higher acceleration may reflect differences in stroke amplitude or control-related movements, rather than faster wingbeats alone. We would like to add that sex differences in thermal physiology may also contribute to this pattern. Some studies suggest that female birds can show higher body temperature than males [84], raising the possibility that females experience a different thermal state during flight. Higher temperature can increase membrane leakiness and may therefore contribute to elevated LEAK respiration and lower coupling efficiency. Conversely, increased LEAK itself can influence heat production and mitochondrial membrane potential, making the causal direction difficult to resolve. Thus,

sex-specific thermal physiology may partly underlie the negative association between VeDBA and ATP-linked respiration in females, but this possibility remains to be tested. These factors may obscure a simple positive relationship between VeDBA and ATP-linked respiration at the individual level in females. Nevertheless, at the broader level, females showed both higher VeDBA and higher mitochondrial metabolism than males, indicating overall concordance between biomechanical and physiological measures.

5. Conclusions

Current knowledge of animal energy expenditure largely relies on approaches such as respirometry (respiratory masks/wind tunnels), doubly labelled water, or implanted heart-rate loggers. These methods can be invasive, stressful and difficult to apply under natural conditions, limiting ecological validity and comparability across techniques [85,86]. Wearable accelerometers have therefore become popular to estimate energy expenditure from body acceleration [7,8,87], but they cannot capture non-locomotor metabolic costs (e.g. thermoregulation, digestion, growth). In addition, the relationship between acceleration and energetic investment is not necessarily straightforward because similar mechanical output can reflect different physiological states or conditions [88–90]. Together, these limitations motivate the need for complementary, high-resolution measures of metabolic rates linked to specific behaviours or life-history stages. Our results show that blood mitochondrial metabolism responds strongly to prolonged flight, supporting its use as a minimally invasive measure of aerobic metabolism in free-ranging animals. They also reveal that similar flight performance can conceal sex differences in metabolic investment, highlighting sex as an important source of hidden physiological variation.

Ethics. The study was conducted following scientific approval by the Italian Institute for Environmental Protection and Research (ISPRA) and authorization from Regione Lazio (permit no. G10618-27045), following the strict ethical and animal welfare guidelines of animal experimentation laws of the European Union (Directive 2010/63/EU).

Data accessibility. The data supporting this study are available from Edmond, the Open Research Data Repository of the Max Planck Society [91].

Supplementary material is available online [92].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. S.C.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, writing—original draft, writing—review and editing; A.G.-L.: data curation, formal analysis, methodology, writing—review and editing; G.D.: investigation, methodology, resources, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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