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# Effect of physiological hyperthermia on mitochondrial fuel selection in skeletal muscle of birds and mammals

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ABSTRACT

Both birds and mammals have important thermogenic capacities allowing them to maintain high body temperatures, i.e., 37 °C and 40 °C on average in mammals and birds, respectively. However, during periods of high locomotor activity, the energy released during muscular contraction can lead to muscle temperature reaching up to 43–44 °C. Mitochondria are responsible for producing the majority of ATP through cellular respiration and metabolizing different substrates, including carbohydrates and lipids, to generate ATP. A limited number of studies comparing avian and mammalian species showed preferential utilization of specific substrates for mitochondrial energy at different metabolic intensities, but authors always measured at body temperature. The present study evaluated mitochondrial respiration rates and OXPHOS coupling efficiencies at 37 °C, 40 °C and 43 °C associated with pyruvate/malate (carbohydrate metabolism) or palmitoyl-carnitine/malate (lipid metabolism) as substrates in pigeons (*Columba livia*) and rats (*Rattus norvegicus*), a well-known pair in scientific literature and for their similar body mass. The data show different hyperthermia-induced responses between the two species with (i) skeletal muscle mitochondria from rats being more sensitive to rising temperatures than in pigeons, and (ii) the two species having different substrate preferences during hyperthermia, with rats oxidizing preferentially carbohydrates and pigeons lipids. By analyzing the interplay between temperature and substrate utilization, we describe a means by which endotherms deal with extreme muscular temperatures to provide enough ATP to support energy demands.

## 1. Introduction

Mammals and birds are endothermic animals, meaning that their thermogenesis capacities allow them to maintain relatively high and constant internal body temperatures. Although the average temperature in placental mammals and birds is close to 37 °C and 41 °C respectively, substantial variation has been described in response to extrinsic factors, such as environmental temperature (Geiser, 2004; Krüger et al., 1982; Mckechnie and Lovegrove, 2002) and fasting (Monternier et al., 2017; Severinsen and Munch, 1999) and intrinsic factors, such as gender, reproductive and locomotion activities (Fernández-Peña et al., 2023; Prinzinger et al., 1991). During active phases or high locomotor activity, the production of heat by muscular contraction triggers a rise in the body temperature reaching up to ~43–44 °C in birds (McNab, 1966) and mammals (Brooks et al., 1971a; Saltin et al., 1968). It is well known that an increase in temperature has the ability to increase biochemical reactions and cellular processes, at least until the denaturation of protein and destabilization of the cell membrane. Although an increase in temperature can benefit muscle performance (contractile properties, velocity, mechanical power) (James, 2013; Ranatunga, 1998), it also affects the mitochondrial energy metabolism by increasing oxygen consumption, proton leakage, and membrane destabilization, resulting in a decrease in the mitochondrial coupling efficiency (Brooks et al., 1971b; Willis and Jackman, 1994; Jarmuszkiewicz et al., 2015; Roussel and Voituron, 2020; Thoral et al., 2023; Zoladz et al., 2016). The mitochondrial oxidative phosphorylation (OXPHOS) system relies on a tight coupling between electron transport and ATP synthesis that provides the energy necessary for cellular processes. Thus, a decrease in the mitochondrial coupling efficiency (ATP/O ratio) indicates a loss of energy transfer between the electron transport and ATP synthesis, which could ultimately decrease ATP delivery and cell performance. As skeletal muscles are the main source of heat production during exercise, their mitochondria may be specifically affected by exercise-induced hyperthermia.

The first step of the OXPHOS metabolism is the ability of mitochondria to transport and oxidize respiratory substrates. Animals use

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carbohydrates and lipids to fuel mitochondrial oxidative phosphorylation (Rasmussen and Rasmussen, 2000). The management and utilization of fuel differ between birds and mammals when sustaining high metabolic intensity. In mammals, there is a general dependence on carbohydrate oxidation, particularly on muscle glycogen, which dominates at higher exercise intensities (Hargreaves and Spriet, 2020; Weber, 2011). On the contrary, birds are able to maintain a high lipid oxidation during intense skeletal muscle activity such as shivering or flight (Guglielmo, 2010; Vaillancourt and Weber, 2007). Fuel preferences have been observed at the mitochondrial level, with birds exhibiting higher oxidation of fatty acids relative to pyruvate (the end product of glycolysis and a central metabolite involved in the carbohydrate catabolism under aerobic conditions) compared with mammals (Kuzmiak et al., 2012; Kuzmiak-Glancy and Willis, 2014; Rasmussen et al., 2004).

In birds and mammals, the muscular thermal adjustment from resting metabolic activity to high metabolic activity (flying or running) does not occur with the same magnitude of temperature change. In fact, muscle temperature in mammals increases from a physiological body temperature of 37 °C to a physiological hyperthermia of 42 °C. The variation is similar in birds: from 40 °C (or even 38 °C for certain non-flying species) to an average of 44 °C (Prinzinger et al., 1991). Few comparative studies between these two clades consider the increase in muscle temperature during intense activity, and even fewer are coupled with a preference for the use of energy fuels (carbohydrate, lipid). Thus, the aim of this study is to assess how much physiological hyperthermia influences the mitochondrial fuel selection of skeletal muscle between these two endothermic clades. For this study, we used the most common mammal-bird comparison in the scientific literature, which is the rat-pigeon pair. Their similar body mass excludes an allometric effect on mitochondrial bioenergetics as described in endotherms (Boel et al., 2019; Brand et al., 2003; Emmett and Hochachka, 1981; Porter and Brand, 1993). To reproduce muscle temperatures within the typical range of metabolic intensity shift, mitochondria from the skeletal muscle supporting the main locomotion in birds (*pectoralis* muscle) and mammals (hindlimb muscles) were isolated and exposed to 3 assay temperatures (37 °C, 40 °C, and 43 °C). Both 37 and 40 °C represent physiological muscular temperatures during low metabolic activity (rest) and 43 °C represents the realistic hyperthermia in these two endotherms.

## 2. Material and methods

### 2.1. Animals

All experiments were conducted in accordance with the animal care guidelines of the Ministère de la Recherche et de l'Enseignement Supérieur. We complied with Article R. 427-6 of the Environmental Code regarding wildlife species.

Wistar rats (*Rattus norvegicus;* $n = 8$; 276 ± 29 g) and domestic pigeons (*Columba livia*; $n = 8$; 293 ± 17 g) of equivalent body masses were selected for all the experiments. Body masses between pigeon and rat were not statistically different ($W = 34$, $p = 0.88$; $d = 0.25$, 95% CI: 0.76 to 1.27). Therefore, any variation in mitochondrial bioenergetics of the rat-pigeon pair should not be attributed to an effect of the body mass. Rats were collected from the laboratory animal platform (ASCED), and housed at 25 °C (12 h:12 h light:dark cycle). A certified trapper provided the pigeons recently captured and kept in outdoor aviaries (15 h:9 h light: dark cycle; 13–23 °C). Rats were killed under isoflurane-induced general anaesthesia, whereas pigeons were killed by cervical dislocation. Fresh skeletal muscle samples were obtained and used for the mitochondria isolation.

### 2.2. Tissue preparation and mitochondrial isolation

Pectoralis muscles from pigeons and hind-limb muscles (gastrocnemius, quadriceps) from rats were excised and used for mitochondrial preparation. Mixed populations of skeletal muscle mitochondria were isolated in an ice-cold isolation buffer (100 mM sucrose, 50 mM KCl, 5 mM EDTA, and 50 mM Tris-base, pH 7.4), following a standard extraction protocol, using a Potter–Elvehjem homogenizer, protease digestion, and differential centrifugation, all steps at 4 °C (Boel et al., 2019). Briefly, 2–3 g of skeletal muscle were cleared of adipose tissue, finely cut up, homogenized with a Potter-Elvehjem homogenizer (five passages), and centrifuged at 1000×*g* for 10 min. The supernatant containing subsarcolemmal mitochondria was maintained in an ice bath and the pellet containing intermyofibrillar mitochondria was suspended in an isolation buffer and treated with protease from *Bacillus licheniformis* (1 mg $g^{-1}$ muscle wet mass) for 5 min in an ice bath. The homogenate was diluted (1:2) using the supernatant from the first centrifugation and the mixture was centrifuged at 1000×*g* for 10 min. The resulting supernatant was filtered and centrifuged at 8700×*g* for 10 min to pellet mitochondria. The mitochondrial pellet was resuspended in an isolation buffer and centrifuged at 8700×*g* for 10 min. This step was carried out a second time. Mitochondrial protein concentrations were determined using the biuret method with bovine serum albumin as standard.

### 2.3. Mitochondrial respiration and oxidative parameters

The mitochondrial respiration was assessed at 37 °C, 40 °C, and 43 °C by measuring oxygen consumption rates (Clark-type oxygen electrode, Rank Brothers Ltd, UK). The air-saturated medium was assumed to contain 406, 393, and 380 nmol of O/ml at 37 °C, 40 °C, and 43 °C respectively, according to Reynafarje et al. (1985). Isolated mitochondria were incubated in the respiratory buffer (120 mM KCl, 5 mM $KH_2PO_4$, 2 mM $MgCl_2$, 1 mM EGTA, 3 mM HEPES, and 0.3% BSA, pH 7.4) supplemented with 20 mM glucose and 2 $U.ml^{-1}$ hexokinase. Respiration was initiated by adding either 5 mM pyruvate/2.5 mM malate or 40 μM palmitoyl-$_{DL}$-carnitine/2.5 mM malate. The rate of phosphorylating oxygen consumption was increased step-by-step by the sequential addition of 5, 10, 20, 100, and 500 μM ADP. Thus, intermediate steady-state rates of oxidative phosphorylation were recorded at the final concentration of 5, 15, 35, 135, and 635 μM ADP.

The basal non-phosphorylating respiration rates were measured in the presence of respiratory substrate alone in the absence of added ADP (state 2). The phosphorylating respiration (OXPHOS, P) was the maximal value of respiration rate measured in the presence of ADP.

The fuel selection index was calculated by dividing the maximal phosphorylating respiration rate associated with the oxidation of palmitoyl-carnitine/malate over that measured during the oxidation of pyruvate/malate ($OXPHOS_{PCM}/OXPHOS_{PM}$ ratio). This ratio reflects the relative mitochondrial oxidation of lipid-over carbohydrate-derived substrate (Kuzmiak et al., 2012).

The control efficiency, the OXPHOS capacity (P) corrected for the basal non-phosphorylating respiration (Basal) was calculated as 1-(Basal/OXPHOS). This parameter describes the flux control of ADP on substrate oxidation, which can viewed as an indicator of the OXPHOS coupling efficiency: a value approaching 1 indicates a maximally coupled respiration, and a value of 0 indicates a non-ADP controlled respiration (Gnaiger et al., 2020).

### 2.4. Statistical analysis

All analyses were performed in R version 4.2.1 (R Core Team, 2022). A paired two-tailed Wilcoxon test was performed to determine whether there was a difference in the body mass of the pigeons and rats, a requirement for the application of this study. The effect of species (pigeon or rat), respiratory substrates (Pyruvate/Malate, and Palmitoyl-carnitine/Malate), and assay temperature (37, 40 or 43 °C) on mitochondrial bioenergetics were evaluated using linear mixed models (LMMs, "lmer" function of the lme4 package) by fixing "individuals" as a random factor for repeated measure analysis (effect of temperature within species). A stepwise procedure was applied to remove the non-significant interaction from the model involving the species effect. Data were tested for normality of the residuals (Shapiro-Wilk test), and Levene's test to assess the homoscedasticity, followed by Tukey's post hoc tests ("glht" function of the multcomp package). We assessed the magnitude of the quantitative relationship between variables to validate the observed differences and because we lacked statistical power due to small sample sizes. Thus, we estimated the effect sizes using *Cohen's d* (Cohen, 2013; Nakagawa and Cuthill, 2007) and the *eta*$^2$ ($\eta^2$ *and* generalized $\eta g^2$ for dependent variables) with their 95% CIs with "effsize" library (Torchiano, 2020) and "effectsize" library (Ben-Shachar et al., 2020) in R. To assess the magnitude of the effect, the following thresholds were used: "negligible" ($|d| < 0.2$ or $|\eta^2| < 0.10$); "small" ($|d| < 0.5$ or $|\eta^2| < 0.25$); "medium" ($|d| < 0.8$ or $|\eta^2| < 0.40$) and "large" beyond these values. The threshold for statistical significance for all analyses was $p < 0.05$ and all data are presented as means ± s. e.m.

## 3. Results and discussion

### 3.1. Effect of acute hyperthermia on mitochondrial oxidative activity

Following exposure to increasing assay temperature (37 °C, 40 °C, and 43 °C), isolated skeletal muscle mitochondria of both species showed distinct respiration patterns regarding the nature of the substrate (Fig. 1). Mitochondrial respiration in rats and pigeons for the pyruvate/malate did not display a significant dependence on increasing temperature (Fig. 1A; Pigeon $\chi^2 = 0.9141$, df = 2, $p = 0.6331$ with $\eta g^2 = 0.03$, 95% CI [0.0, 1.00], and Fig. 1B; Rat $\chi^2 = 3.0911$, df = 2, $p = 0.2132$ with $\eta g^2 = 0.09$, 95% CI [0.0, 1.00]), except for the rate of basal non-phosphorylating respiration (Basal) in rat (Table 1; $\chi^2 = 9.2301$, df = 2, $p < 0.01$ with $\eta g^2 = 0.18$, 95% CI [0.0, 1.00]). Nonetheless, there was a large difference in the OXPHOS shift from the lowest temperature (37 °C) to the highest temperature (43 °C) between the two species, being nearly null in pigeons but 35% higher in rats (Table 1). The intensity to which OXPHOS and basal respiration increased with

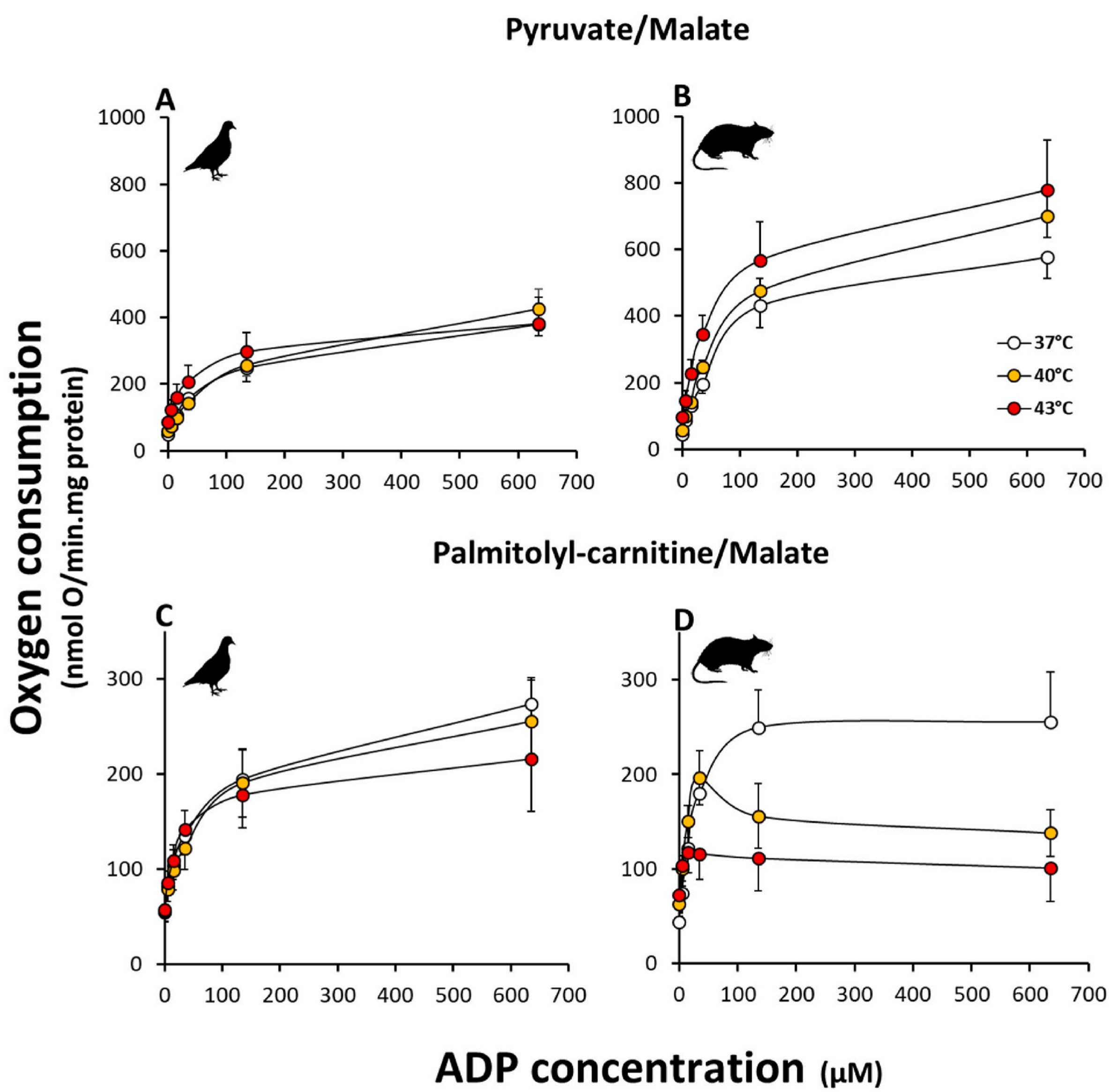


**Fig. 1. Dependence of oxygen consumption rate on ADP concentration in isolated skeletal muscle mitochondria.** Mitochondria were isolated from the skeletal muscle of pigeons (panels A, C) or rats (panels B, D). Mitochondrial respiration was measured at 37 °C, 40 °C, and 43 °C in the presence of 5 mM pyruvate/2.5 mM malate (panels A, B) or 40 µM palmitoyl-carnitine/2.5 mM malate (panels C, D). Values are means ± s. e.m. from n = 8 independent mitochondrial preparations, excepted n = 6 at 43 °C for panels C and D.

**Table 1**
**Effects of temperature on mitochondrial respiration rates.** Respiration rates were determined at 37, 40, and 43 °C for pigeons and rats in the presence of 5 mM pyruvate/2.5 mM malate or 40 μM palmitoyl-L-carnitine/2.5 mM malate. OXPHOS is the maximal value of respiration rate measured in the presence of ADP, and basal non-phosphorylating rate (Basal) measured in the presence of substrate alone. Values are means ± s.e.m for n = 8 animals; *n = 6 at 43 °C for mitochondria from pigeon and rat respiring on palmitoyl-carnitine/malate. Different lower-case letters denote significant differences ($p < 0.05$) within the same species. †p < 0.05 significantly different from pigeon within the same assay temperature.

| Substrates | Respiration rates | Species | Assay temperature (°C) | | |
|---|---|---|---|---|---|
| | | | 37 | 40 | 43 |
| Pyruvate/Malate | OXPHOS | Pigeon | 379 ± 35 | 426 ± 59 | 383 ± 79 |
| | | Rat | 577 ± 64 | 700 ± 64 | 778 ± 151[†] |
| | Basal | Pigeon | 50.5 ± 7.7 | 58.3 ± 6.9 | 85.3 ± 23.8 |
| | | Rat | 46.4 ± 7.4[a] | 57.4 ± 6.6[ab] | 96.7 ± 22.5[b] |
| Palmitoyl-Carnitine/Malate* | OXPHOS | Pigeon | 273 ± 28 | 256 ± 43 | 242 ± 52 |
| | | Rat | 284 ± 51[a] | 222 ± 30[ab] | 133 ± 32[b] |
| | Basal | Pigeon | 54.6 ± 7.6 | 55.7 ± 11.3 | 57.5 ± 12.4 |
| | | Rat | 43.9 ± 6.7[a] | 63.5 ± 10.2[b] | 72.3 ± 17.1[b] |

increasing temperatures in rats is similar to previously reported results in skeletal muscle mitochondria respiring on pyruvate/malate (Brooks et al., 1971b; Jarmuszkiewicz et al., 2015; Zoladz et al., 2016) With a lipid-derived respiratory substrate, no significant effect of temperature on the ADP dependence of oxygen consumption appeared in pigeon mitochondria (Fig. 1C). The rates of OXPHOS ($\chi^2 = 2.8993$, df = 2, $p = 0.2347$ with $\eta g^2 = 0.11$, 95% CI [0.0, 1.00]), and basal respiration ($\chi^2 = 0.9406$, df = 2, $p = 0.6248$ with $\eta g^2 = 0.04$, 95% CI [0.0, 1.00]) were not significantly different between assay temperatures in pigeon mitochondria oxidizing palmitoyl-carnitine/malate (Table 1). Surprisingly, the kinetic pattern of lipid-derived oxidation was different in rats compared with pigeons (Fig. 1C and D). In rats, the rates of phosphorylating respiration were inhibited by the highest concentrations of ADP (135 and 635 μM) at 40 °C and 43 °C. It results that high temperatures had a significant negative impact on OXPHOS rates in rat mitochondria oxidizing palmitoyl-carnitine/malate ($\chi^2 = 6.8722$, df = 2, $p < 0.05$ with $\eta g^2 = 0.21$, 95% CI [0.00, 1.00]), which was 2-fold lower at 43 °C compared to 37 °C (Table 1). This result contrasts with the study of (Zoladz et al., 2017) who reported an increased oxidative activity at high temperatures in rat skeletal muscle mitochondria respiring on palmitoyl-carnitine. The reason for this discrepancy is unknown, but it may be linked to methodological differences: i) the assay temperatures (35 and 42 °C in Zoladz et al., 2017 *versus* 37 and 43 °C in the present study); ii) the combination of the respiratory substrates (500 μM palmitoyl-carnitine with no malate in Zoladz et al., 2017 *versus* 40 μM palmitoyl-carnitine with 2.5 mM malate in the present study); and iii) the lower ADP concentration used to initiate OXPHOS (150 μM in Zoladz et al., 2017 *versus* 635 μM for the highest concentration in the present study). The effect of hyperthermia on the oxidation of lipid-derived substrate in skeletal muscle needs further investigation to be clarified.

In contrast, the oxidative activity in pigeon mitochondria was not affected at high temperatures. Yet, it has been reported in rat that the rates of OXPHOS in heart mitochondria were also not affected by increasing temperature, at least up to 43 °C (Power et al., 2014; Zukiene et al., 2010). These data suggest that some mitochondrial phenotypes can be more or less sensitive to acute changes in temperature. The proportion of the long-chain polyunsaturated docosahexaenoic acid (C22:6 n-3) of the inner membrane, which is positively correlated to mitochondrial membrane proton conductance (Brookes et al., 1998) and OXPHOS activity (Piquet et al., 2004), might be among characteristics acting on the temperature sensitivity of mitochondria. The rationale is that the higher contents of unsaturation would trigger a more rapid transition from molecular order to hyperfluidity (Park et al., 2005). The fact that the mitochondrial membrane of skeletal muscles in birds and the heart in mammals exhibit a lower C22:6 n-3 content than skeletal muscle mitochondria of rats (Montgomery et al., 2011) could explain part of the differential sensitivity of these mitochondria to hyperthermia. Although this hypothesis has yet to be tested, it remains that the molecular mechanisms of mitochondrial response to hyperthermia are obviously more complex, involving interactions between OXPHOS proteins and physical membrane properties.

### 3.2. Mitochondrial fuel preference

Mitochondria isolated from rats' skeletal muscle exhibited a greater preference for carbohydrate-derived substrates than skeletal muscle mitochondria from pigeons (Fig. 1). On the whole, the $OXPHOS_{PM}$ rates of mitochondria respiring on pyruvate/malate were 50–60% higher in rats than in pigeons at 37 °C and 40 °C, becoming significantly different between the two species only at the highest temperature (Table 1; ($\chi^2 = 9.9544$, df = 1, $p < 0.01$ with $\eta^2 = 0.42$, 95% CI [0.09, 1.00]). Similarly, mitochondrial pyruvate/malate oxidation has been reported to be 47% higher in rat biceps than in pigeon pectoralis (Rasmussen et al., 2004). These mitochondrial oxidative differences between rats and pigeons were less pronounced when succinate was used as a respiratory substrate (Montgomery et al., 2011; Rasmussen et al., 2004). The rates of $OXPHOS_{PCM}$ (i.e., measured in the presence of palmitoyl-carnitine/malate) were not significantly different between the two species (Table 1; $\chi^2 = 0.3588$, df = 1, $p = 0.54918$ with $\eta^2 = 0.03$, 95% CI [0.00, 1.00]). This result contrasts with those reported by Kuzmiak and colleagues, who found a higher mitochondrial rate of lipid oxidation in sparrows than in rats (Kuzmiak et al., 2012). However, the authors compared a small bird (the house sparrow weighing 25 g on average) to rats 10 times heavier, weighing 250–300 g. Since the activity of mitochondrial oxidative phosphorylation in skeletal muscles correlates negatively with body mass (Boel et al., 2019), the reported differences in Kuzmiak et al. (2012) might be partly ascribed to an allometric effect. This is supported by the comparative study of Rasmussen et al. (2004) who reported a slightly higher, although not significant, rate of maximal phosphorylating respiration in muscle mitochondria respiring on palmitoyl-carnitine/malate between pigeons (pectoralis) and rats (soleus or biceps).

Notwithstanding the body mass, lipid oxidation preference can be calculated relative to pyruvate oxidation (the end product of carbohydrate catabolism), and it has been reported to be higher in birds than in mammals (Kuzmiak et al., 2012; Kuzmiak-Glancy and Willis, 2014; Rasmussen et al., 2004). In the present study, the relative oxidation of lipid-derived substrates over that of carbohydrate-derived substrates was significantly higher in pigeons than in rats (Fig. 2; $\chi^2 = 41.38$, df = 1, $p < 0.001$ with $\eta^2 = 0.53$, 95% CI [0.34, 1.00]). Fig. 2 also shows that this ratio greatly dropped with increasing temperatures in rats ($\chi^2 = 17.695$, df = 2, $p < 0.001$ with $\eta g^2 = 0.45$, 95% CI [0.04, 1.00]), an effect that results from the combination of a significant downregulation of fatty acid oxidation and a substantial increase in the oxidation of carbohydrate-derived substrates at high temperatures (Table 1). On the contrary, these ratios were not significantly affected by temperature in pigeons (Fig. 2; $\chi^2 = 4.0874$, df = 2, $p = 0.1296$ with $\eta g^2 = 0.18$, 95% CI [0.00, 1.00]). These data thus indicate that, relative to the capacity of skeletal muscle mitochondria to oxidize carbohydrate-derived substrates, pigeon mitochondria had a higher capacity to oxidize lipid-derived substrates than rat mitochondria, a "capacity for lipid oxidation" that is maintained at high temperature.

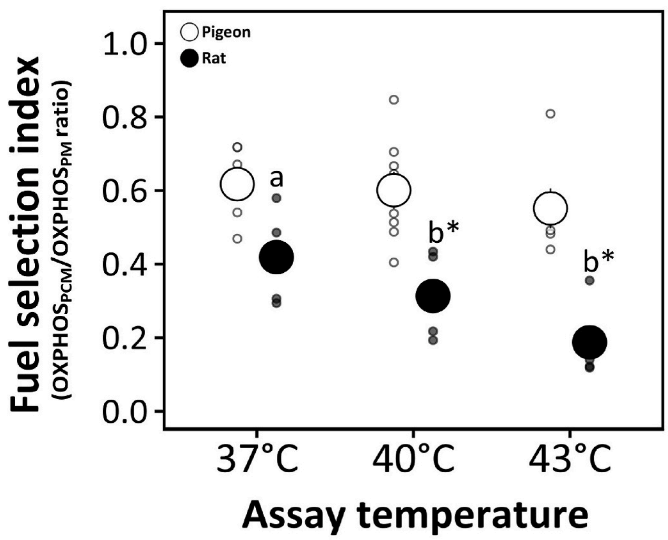


**Fig. 2. Fuel selection index of skeletal muscle mitochondria from pigeon and rat**. The fuel selection index refers to the relative oxidative phosphorylation capacity of skeletal muscle mitochondria for palmitoyl-carnitine/malate ($OXPHOS_{PCM}$) over that for pyruvate/malate ($OXPHOS_{PM}$). This ratio was calculated in pigeon (white bars) and rat (black bars) by dividing the maximal OXPHOS rate of mitochondria respiring on palmitoyl-carnitine/malate by the corresponding rate measured in the presence of pyruvate/malate ($OXPHOS_{PCM}$/$OXPHOS_{PM}$). See "Materials and Methods" section for more details. Values are means ± s. e.m. from n = 8 independent mitochondrial preparations, excepted n = 6 at 43 °C. Different lower-case letters denote significant differences ($p < 0.05$) within the same species. *$p < 0.05$, significantly different from pigeons within the same temperature assay.

### 3.3. Mitochondrial coupling efficiency

Fig. 3 shows the flux control efficiency of ADP on pyruvate/malate-supported respiration (Fig. 3A), and palmitoyl-carnitine/malate-supported respiration (Fig. 3B). The values for carbohydrate-derived substrate were higher in rat than in pigeon, at the highest assay temperature (Fig. 3A; $\chi^2 = 17.545$, df = 1, $p < 0.001$ with $\eta^2 = 0.56$, 95% CI [0.23, 1.00]). The flux control efficiencies on pyruvate/malate-supported respiration were slightly affected by temperature in rats ($\chi^2 = 7.7025$, df = 2, $p < 0.05$ with $\eta g^2 = 0.21$, 95% CI [0.00, 1.00]), and decreased significantly by 10% with increasing temperatures in pigeons (Fig. 3A; $\chi^2 = 7.7025$, df = 2, $p < 0.05$ *with* $\eta g^2 = 0.27$, 95% CI [0.00, 1.00]). On the contrary, the flux control efficiencies on palmitoyl-carnitine/malate respiration were not affected by temperature in pigeons ($\chi^2 = 3.8253$, df = 2, $p = 0.1477$ with $\eta g^2 = 0.16$, 95% CI [0.00, 1.00]), but decreased significantly with increasing temperature in rats ($\chi^2 = 18.425$, df = 2, $p < 0.001$ with $\eta g^2 = 0.49$, 95% CI [0.08, 1.00]), becoming significantly lower in rats than in pigeons at 43 °C (Fig. 3B; $\chi^2 = 10.288$, df = 1, $p < 0.01$ with $\eta^2 = 0.47$, 95% CI [0.15, 1.00]). This lower flux control efficiency of ADP on pyruvate/malate-supported respiration reported at the highest temperature is explained by a disproportionate increase in basal respiration (+110% in rats and +69% in pigeons compared with values at 37 °C) compared with changes in OXPHOS respiration (Table 1). This is in line with the well-known primary effect of hyperthermia on mitochondrial respiration, which is the pronounced increase in inner mitochondrial membrane permeability, leak respiration, and subsequent decrease in coupling efficiency (Brooks et al., 1971b; Jarmuszkiewicz et al., 2015; Power et al., 2014). In contrast, the 2-fold decrease in the flux control efficiency of ADP at 43 °C reported in rat mitochondria respiring on lipid-derived substrate results from the combination of the increase in basal respiration and the decrease in OXPHOS respiration. Altogether, these results clearly highlight that the mitochondrial activity and efficiency, and thus the ATP synthesis capacity, are compromised at 43 °C in rat muscle mitochondria respiring on lipid-derived substrates but not in pigeons.

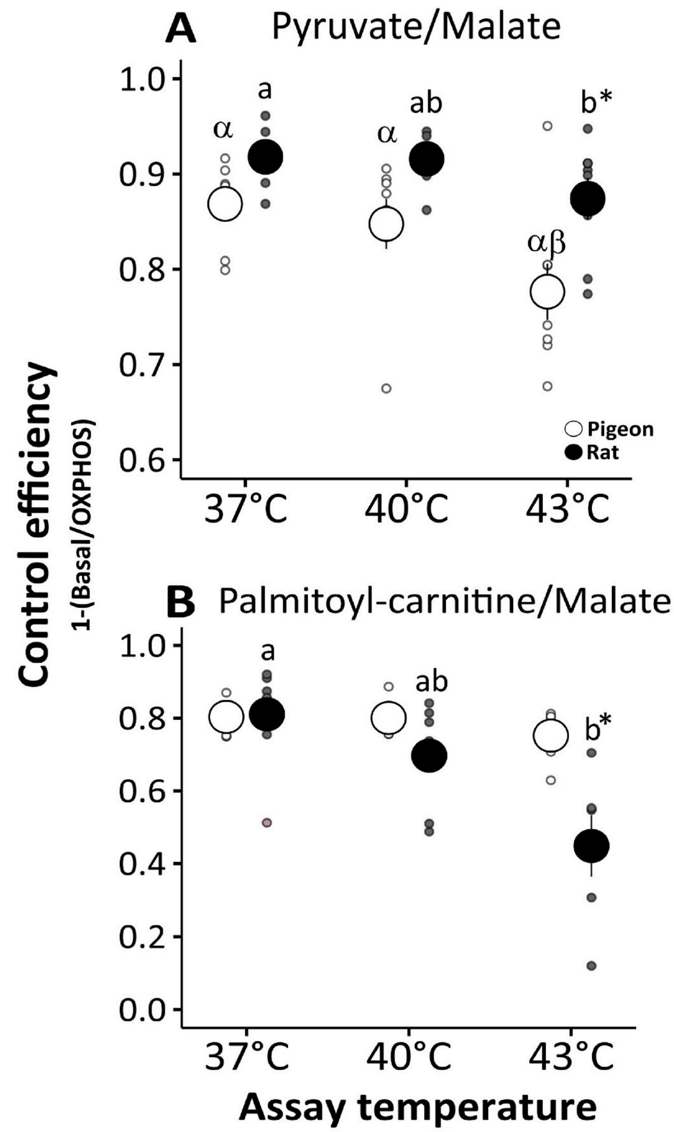


**Fig. 3. Flux control efficiency of skeletal muscle mitochondria.** Flux control efficiency of ADP on A) pyruvate/malate- and B) palmitoyl-carnitine/malate-supported respiration were calculated in pigeons (white bars) and rats (black bars). Values are means ± s. e.m. from n = 8 independent mitochondrial preparations, excepted n = 6 at 43 °C for panel B. Different lower-case letters denote significant differences ($p < 0.05$) within the same species. *$p < 0.05$, significantly different from pigeons within the same temperature assay.

## 4. Conclusion

The fundamental finding of the present study is that skeletal muscle fuel selection occurs at the mitochondrial level during experimentally induced hyperthermia *in vitro*. Pigeon mitochondria maintain high lipid oxidation capacities relative to carbohydrate-derived substrates, whereas hyperthermia negatively impacts the capacity of mammalian mitochondria to oxidize fatty acids. In contrast, mammalian mitochondria primarily oxidize carbohydrate-derived substrates, maintaining this preference for pyruvate oxidation during hyperthermia.

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## CRediT authorship contribution statement

**Jessica Barbe:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – review & editing. **Damien Roussel:** Conceptualization, Methodology, Data curation, Validation, Supervision, Writing – original draft. **Yann Voituron:** Conceptualization, Writing – review & editing, Supervision.

## Declaration of competing interest

The authors declare that they have no competing interests.

## Data availability

The data and code source of this study are openly available in Zenodo at: https://doi.org/10.5281/zenodo.8164745.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2023.103719.

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