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Oxygen release from hemoglobin has limited effects on mitochondrial respiration measured from red blood cells. Reply to the Comment on "Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress"

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Oxygen release from hemoglobin has limited effects on mitochondrial respiration measured from red blood cells. Reply to the Comment on "*Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress*"

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Disclosures

No conflicts of interest to disclose

Contributions

AS collected and analyzed the data, and wrote the manuscript with input from PC, SE, EN and DR.

Main text

The measurement of mitochondrial aerobic metabolism from blood cells has gained in popularity over the past decade due to its low invasiveness [1,2]. In non-mammalian vertebrates, red blood cells are nucleated and possess functional mitochondria[3], which enables the assessment of mitochondrial respiration from small blood samples (as low as 20 µL of whole-blood[4]). Recently, we have showed that human mature sickle red blood cells retain some functional mitochondria, which was associated with increased sickling tendency, hemolysis and oxidative stress[5]. Willis et al.[6] recently questioned the methodology used to demonstrate the functionality of the mitochondria retained in sickle red blood cells, since hemoglobin-O_2 dissociation could influence the oxygen consumption rate (JO_2) measured with high-resolution respirometry.

As rightly noted by Willis et al.[6], the PO_2 within the *in-vitro* chamber declines over time due to oxygen consumption by the cells. Such a decline in PO_2 leads to the potential release of O_2 by hemoglobin, to an extent depending mostly on: 1. the change in PO_2, 2. the HbO_2 binding parameters, and 3. the amount of hemoglobin in the chamber. Willis et al.[6] state that because the PO_2 is progressively falling, the errors will confound not only the absolute rates but also the relative differences between respiratory states (*i.e.* the proof of mitochondrial functionality we used in[5]). Such statement assumes that O_2 dissociation from hemoglobin will be higher at lower PO_2. However, using the same protocol as in previous study on avian red blood cells[7] Fig. 1 shows that it would not be the case since non-mitochondrial JO_2 (after antimycin A addition) does not vary over a broad range of PO_2 (see also electronic supplementary material (ESM) S1 for raw data). At low PO_2, O_2 release from hemoglobin could have been anticipated as assumed by Willis et al.[6], which would have led to decreased or even negative (*i.e.* O_2 release > O_2 consumed) JO_2.

Actually, the release of O_2 from hemoglobin will not directly depend on the PO_2, but on the instantaneous rate of change in PO_2 linked to oxygen consumption (*i.e.* JO_2 in a given state), and on the position in terms of absolute PO_2 on the hemoglobin-O_2 dissociation curve. As supposed by Willis et al.[6] based on Stier et al.[1] and Esperti et al.[5], our measurements on both avian species and human were conducted at high PO_2 (Fig 2A, see raw data in ESM S2), enabling to remain within the linear and almost flat portion of the hemoglobin-O_2 dissociation curve (Fig. 2B). Extracting the data from Abdu et al. (2008)[8] and

Powell (2015)[9] for human and birds (Fig. 2B, ESM S3), respectively, enabled to calculate the change in hemoglobin saturation (%) linked to JO_2-induced changes in PO_2, and the associated release of O_2 for each respiratory state (see ESM S4 and S5 for calculations). Based on these calculations, we can note that the release of O_2 is relatively minor (difference between JO_2 and JO_2 corrected in Fig. 2C and 2D). While absolute respiration rates are affected to a minor extent (Fig. 2C & 2D), the relative differences between respiratory states are not, as demonstrated for instance by the similarity between OXPHOS coupling efficiencies calculated from raw *vs.* corrected JO_2 values (*i.e.* for Japanese quail: raw = 0.731 ± 0.013 *vs.* corrected = 0.730 ± 0.013; for human sickle red blood cells: raw = 0.437 ± 0.065 *vs.* corrected = 0.424 ± 0.061). Since O_2 release is proportional to JO_2 and influenced by hemoglobin content (that does not vary between the different respiratory states), it is not surprising from our perspective that the relative differences between respiratory states are not influenced by O_2 release from hemoglobin, as long as the assay is conducted within the linear and almost flat part of the Hb-O_2 dissociation curve (Fig. 2B).

As rightly pointed out by Willis et al.[6], issues can arise when two groups have different Hb contents and/or O_2 binding kinetics. This is however unlikely to confound the results presented in Esperti et al.[5] because O_2 binding kinetics does not vary between healthy and sickle red blood cells in normoxic conditions[8] (> 92.5% saturation, PO_2 > 65 mm Hg), and Hb content (and thus potential O_2 release) is lower in sickle cell patients than healthy individuals[10].

Willis et al.[6] also rightly questioned the choice of Mir05 as a respiratory medium and the lack of exogenous substrate (*i.e.* glucose) when assessing mitochondrial respiration of 'intact' red blood cells. Respiration of intact blood cells can for instance be conducted in PBS or plasma[11], but our own experience with avian blood cells shows that mitochondria loose functionality along the assay with PBS, as evidenced by a FCCP-induced respiration being lower than the endogenous respiration, which does not happen when using Mir05. Mir05 also enables to first measure the endogenous respiration and then to permeabilize the red blood cells for more detailed investigation[12]. From our perspective, refraining from using exogenous substrates enables the measurement of mitochondrial respiration rates being more closely related to the *in-vivo* physiology, where substrates are usually not at saturating levels. Using the subject's own plasma[11], whenever possible, is likely the best way to obtain the more meaningful information about *in-vivo* mitochondrial metabolism. Regarding the

amount of red blood cells used in respirometry assays, Willis et al.[6] also question the precision of pipetting packed red blood cells, which we have questioned before (see[1]). Counting red blood cells is likely the best approach possible (as done in[4,12]).

Around 75% of the patients included in our study[5] were under hydroxyurea therapy, and all patients from the mitochondrial respiration experiments received this treatment. Hydroxyurea has recently been shown to promote erythroid differentiation by accelerating maturation processes[13], which may impact the degree of mitochondrial retention into mature red blood cells. To answer this question, a study would be needed to test the degree of mitochondrial retention into mature red blood cells and their functionality before and after hydroxyurea therapy.

In light of the excellent comment by Willis et al.[6], here are some recommendations that should be useful to ensure the best accuracy when measuring mitochondrial respiration from red blood cells: 1. provide the range of PO_2 at which JO_2 measurements are conducted; 2. ensure that measurements are conducted at a PO_2 being within a linear and almost flat part of the Hb-O_2 dissociation curve; 3. check for potential bias between experimental groups in terms of Hb content or O_2 binding kinetics; 4. measure Hb in individual samples to correct JO_2 if deemed necessary based on theoretical expectations (*e.g.* if Hb content is high and variable between samples) or if a difference exists between groups in terms of Hb content.

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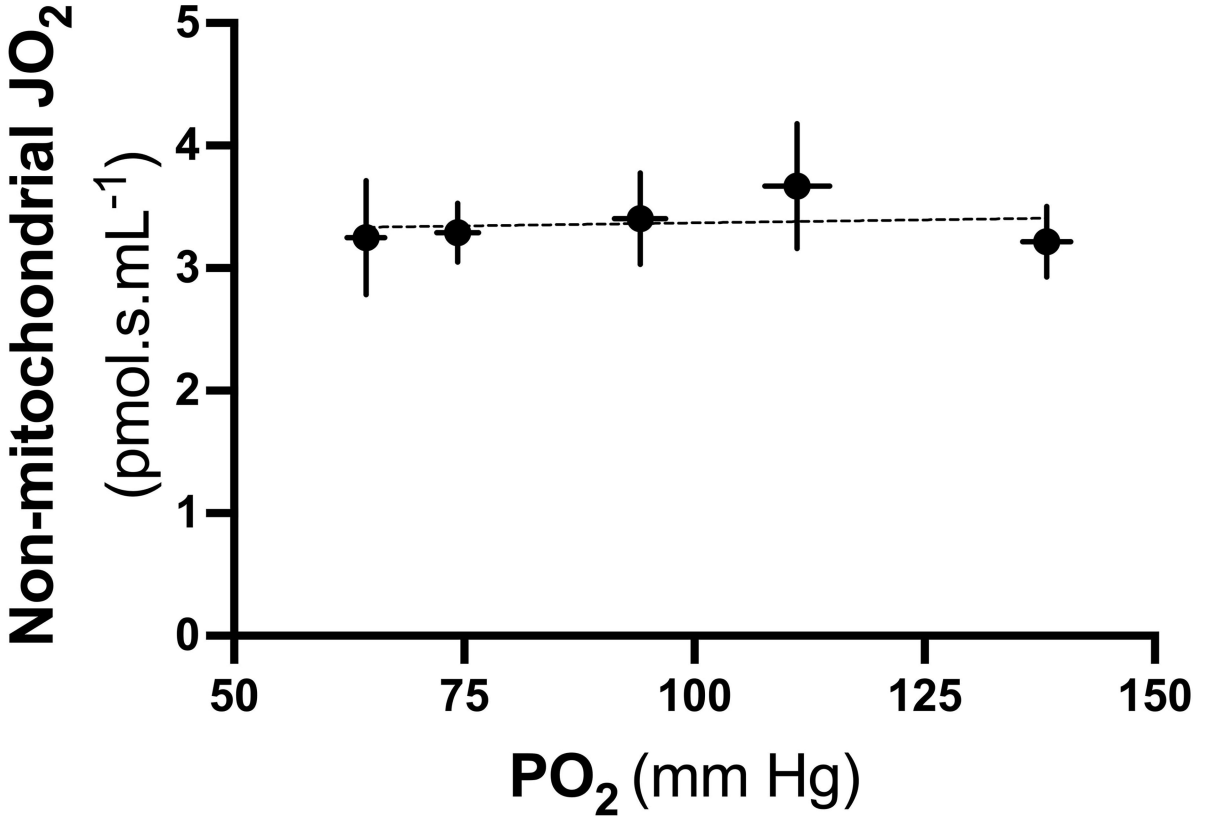
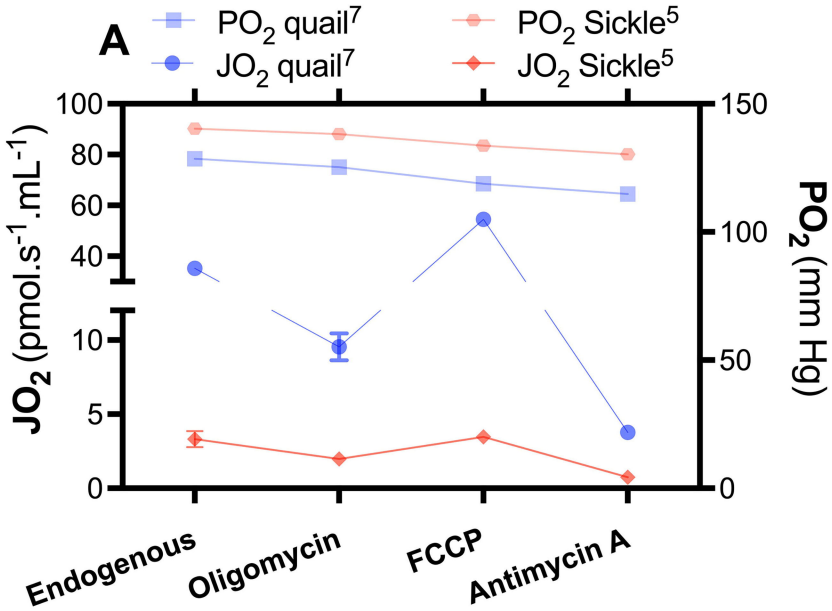
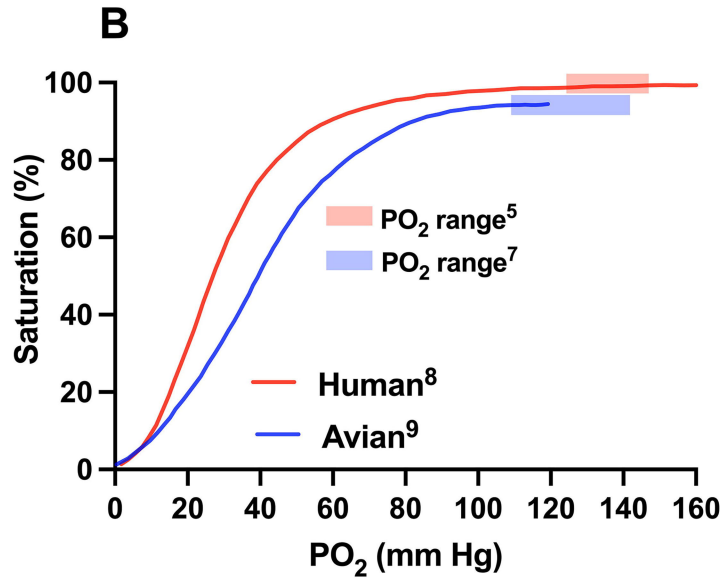
Figure legends

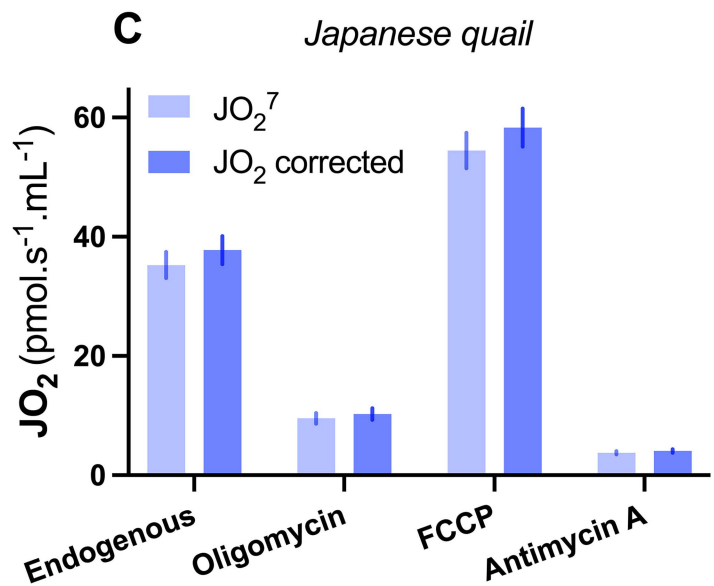
Fig. 1: Relationship between non-mitochondrial JO_2 and PO_2 in Japanese quail (*Coturnix japonica*) red blood cells measured *in-vitro* with high-resolution respirometry (N = 2 biological replicates per PO_2; mean ± SD). Measurements were conducted according to the methodology described in Stier et al.[7], and the various PO_2 at which non-mitochondrial JO_2 was measured were achieved by letting intact red blood cells consume more or less O_2 within the chamber before adding antimycin A.

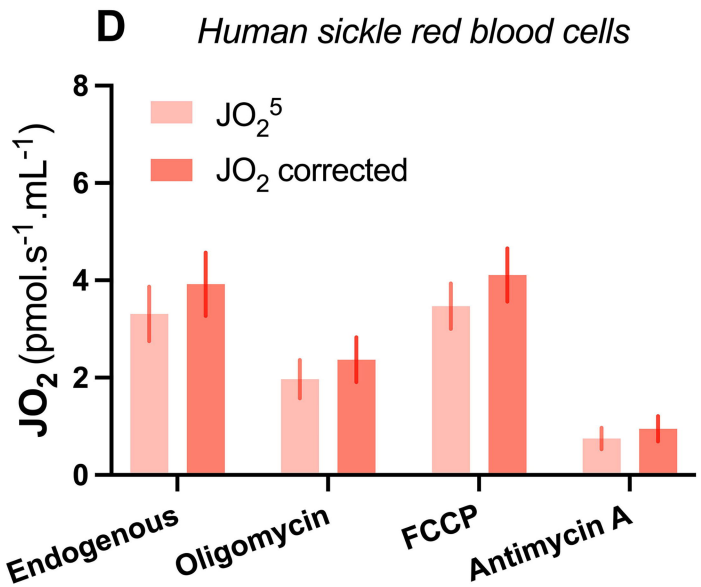
Fig. 2: Relationship between PO_2 and JO_2 in red blood cells of Japanese quail (blue) and human with sickle cell disease (red): **A. Raw JO_2 measurements and the associated PO_2 during measurement**; **B. Hemoglobin-oxygen dissociation curves,** redrawn from Powell[9] and Abdu et al.[8]**; C. Comparison of raw JO_2 and JO_2 corrected for O_2 release by hemoglobin for Japanese quails; D**. C**omparison of raw JO_2 and JO_2 corrected for O_2 release by hemoglobin for human sickle red blood cells.** N = 8 biological replicates for each species, data from Stier et al.[7] and Esperti et al.[5] re-analyzed (mean ± SE).


Non-mitochondrial JO_2
(pmol.s.mL^{-1})
5
4
3
2
1
0
50
75
100
125
150
PO_2 (mm Hg)


A
PO2 quail7
JO2 quail7
PO2 Sickle5
JO2 Sickle5
JO2 (pmol.s-1.mL-1)
PO2 (mm Hg)
Endogenous
Oligomycin
FCCP
Antimycin A


B
Saturation (%)
PO2 range5
PO2 range7
Human8
Avian9
PO2 (mm Hg)


C
Japanese quail
JO2 7
JO2 corrected
JO2 (pmol.s-1.mL-1)
Endogenous
Oligomycin
FCCP
Antimycin A


D
Human sickle red blood cells
JO2 5
JO2 corrected
JO2 (pmol.s-1.mL-1)
Endogenous
Oligomycin
FCCP
Antimycin A

Electronic Supplementary Material of Stier et al. 2024, *Haematologica*:

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ALL TABLES AS EXCEL FILES ONLY

Table S1: Data of Fig. 1 from Japanese quail (*Coturnix japonica*) on non-mitochondrial oxygen consumption (JO_2) by red-blood cells in response to variation in PO_2 in-vitro. The variation in PO_2 was achieved by letting the cells consume more or less oxygen before adding antimycin A to inhibit mitochondrial respiration.

Table S2: Data of Fig. 2A on oxygen consumption (JO_2) by red blood cells of both Japanese quail and human with sickle cell disease in response to a standard high-resolution respirometry protocol. JO2 data were already published in Stier et al. 2022 and Esperti et al. 2023, and are here plotted along the respective PO_2 at which they have been measured *in-vitro*

Table S3: Data of Fig. 2B on hemoglobin-O2 dissociation curves extracted from the literature for birds (Powell 2015) and human (Abdu et al. 2008). Slopes of the PO_2 - % Hb saturation for the range of PO_2 encountered in Stier et al. 2022 and Esperti et al. 2023 (highlighted in blue and green respectively) have been calculated.

Table S4: Calculations and Data of Fig. 2C on the contribution of O_2 release by hemoglobin to JO_2 in Japanese quail red blood cells assessed *in-vitro* using high-resolution respirometry

Table S5: Calculations and Data of Fig. 2D on the contribution of O_2 release by hemoglobin to JO_2 in human sickle red blood cells assessed *in-vitro* using high-resolution respirometry