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"

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 shown 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 (Hb)-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 oxygen tension (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: i) the change in PO_2, ii) the HbO_2 binding parameters, and iii) the amount of Hb 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 a statement assumes that O_2 dissociation from Hb will be higher at lower PO_2. However, using the same protocol as in a previous study on avian red blood cells[7] Figure 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 *Online Supplementary Material (ESM) S1* for raw data). At low PO_2, O_2 release from Hb could have been anticipated as assumed by Willis *et al.*,[6] which would have led to decreased or even negative JO_2 (i.e., O_2 release >O_2 consumed).

Actually, the release of O_2 from Hb 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 Hb-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 (Figure 2A, see raw data in *ESM S2*), enabling to remain within the linear and almost flat portion of the Hb-O_2 dissociation curve (Figure 2B). Extracting the data from Abdu *et al.* (2008)[8] and Powell (2015)[9] for human and birds (Figure 2B; *ESM S3*), respectively, enabled us to calculate the change in Hb 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 corrected JO_2 in Figure 2C, D). While absolute respiration rates are affected to a minor extent (Figure 2C, D), the relative differences between respiratory states are not, as demonstrated for instance by the similarity between OXPHOS coupling efficiencies calculated from raw *versus* 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

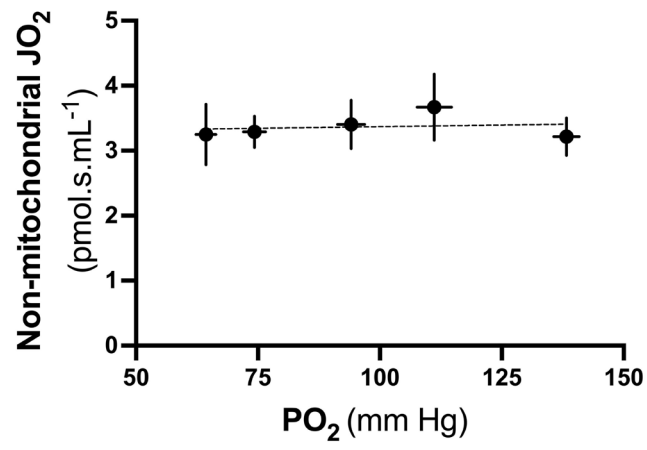

Figure 1. Relationship between non-mitochondrial JO_2 and PO_2 in Japanese quail (*Coturnix japonica*) red blood cells measured *in vitro* with high-resolution respirometry. Measurements were conducted according to the methodology described in Stier *et al.*,[7] and the various oxygen tension (PO_2) at which non-mitochondrial oxygen consumption rate (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. N=2 biological replicates per PO_2; mean ± standard deviation.

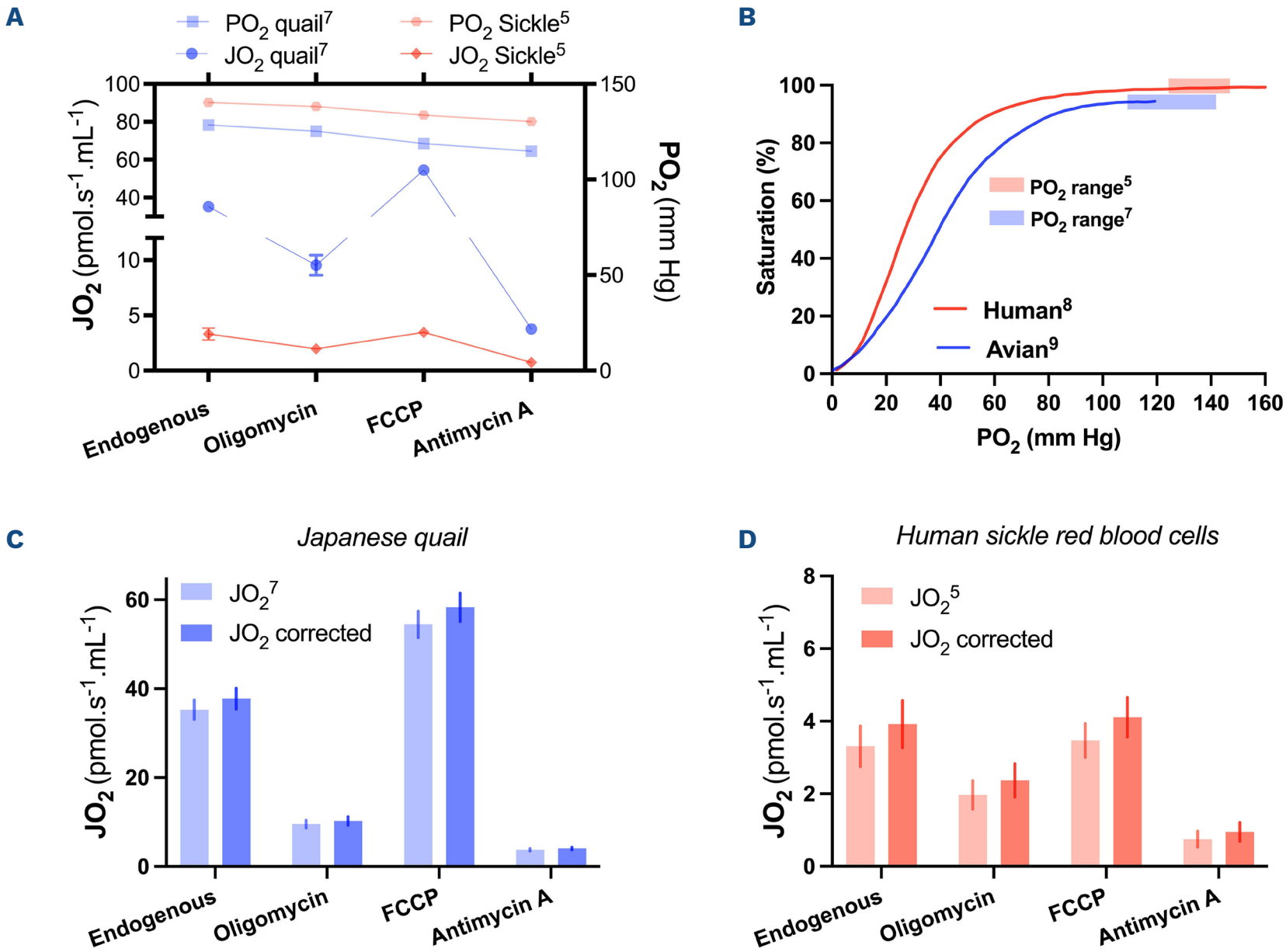

Figure 2. Relationship between PO_2 and JO_2 in red blood cells of Japanese quail (blue) and human with sickle cell disease (red). (A) Raw oxygen consumption rate (JO_2) measurements and the associated oxygen tension (PO_2) during measurement. (B) Hemoglobin (Hb)-oxygen dissociation curves, redrawn from Powell[9] and Abdu *et al.*.[8] (C) Comparison of raw JO_2 and JO_2 corrected for O_2 release by Hb for Japanese quails. (D) Comparison of raw JO_2 and JO_2 corrected for O_2 release by Hb 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 ± standard error).

to JO_2 and influenced by Hb 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 Hb, as long as the assay is conducted within the linear and almost flat part of the Hb-O_2 dissociation curve (Figure 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: i) provide the range of PO_2 at which JO_2 measurements are conducted; ii) ensure that measurements are conducted at a PO_2 being within a linear and almost flat part of the Hb-O_2 dissociation curve; iiii) check for potential bias between experimental groups in terms of Hb content or O_2 binding kinetics; iv) 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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Contributions

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

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Data-sharing statement

The data used in this study is publicly available in the *Online Supplementary Appendix*.

References

1. Stier A, Romestaing C, Schull Q, et al. How to measure mitochondrial function in birds using red blood cells: a case study in the king penguin and perspectives in ecology and evolution. Methods Ecol Evol. 2017;8(10):1172-1182.
2. Braganza A, Annarapu GK, Shiva S. Blood-based bioenergetics: an emerging translational and clinical tool. Mol Aspects Med. 2020;71:100835.
3. Stier A, Bize P, Schull Q, et al. Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. Front Zool. 2013;10(1):33.
4. Cossin-Sevrin N, Hsu B-Y, Marciau C, Viblanc VA, Ruuskanen S, Stier A. Effect of prenatal glucocorticoids and thyroid hormones on developmental plasticity of mitochondrial aerobic metabolism, growth and survival: an experimental test in wild great tits. J Exp Biol. 2022;225(9):jeb243414.
5. Esperti S, Nader E, Stier A, et al. Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress. Haematologica. 2023;108(11):3086-3094.
6. Willis WT, Berry AC, Gladden LB. Does hemoglobin affect measures of mitochondrial respiration in red blood cells? Haematologica. 2024 Aug 1. doi: 10.3324/haematol.2024.286135. [Epub ahead of print]
7. Stier A, Monaghan P, Metcalfe NB. Experimental demonstration of prenatal programming of mitochondrial aerobic metabolism lasting until adulthood. Proc Royal Soc B. 2022;289(1970):20212679.
8. Abdu A, Gómez-Márquez J, Aldrich TK. The oxygen affinity of sickle hemoglobin. Respir Physiol Neurobiol. 2008;161(1):92-94.

9. Powell FL. Ch. 13: Respiration. In: Sturkie's Avian Physiology 6th Edition. Academic Press. 2017; p301-330.
10. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. Lancet. 2010;376(9757):2018-2031.
11. Sjövall F, Ehinger JKH, Marelsson SE, et al. Mitochondrial respiration in human viable platelets-Methodology and influence of gender, age and storage. Mitochondrion. 2013;13(1):7-14.
12. Stier A, Bize P, Hsu B-Y, Ruuskanen S. Plastic but repeatable: rapid adjustments of mitochondrial function and density during reproduction in a wild bird species. Biol Lett. 2019;15(11):20190536.
13. Kuznicki L, Laurance S, Claer L, Kim CLV. Hydroxyurea's impact on erythropoiesis, cell death and HbF expression in the Hudep-2 model. Blood. 2023;142(Suppl 1):2493.