CORRESPONDENCE

Commentary on Thoral et al. (2024) 'The relationship between mitochondrial respiration, resting metabolic rate and blood cell count in great tits'

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In a recent study, Thoral et al. (2024) explored the link between whole-organism resting metabolic rate (RMR) and mitochondrial respiration in red blood cells of wild great tits (*Parus major*). They captured great tits in winter to measure their night-time RMR with traditional respirometry, at thermoneutrality (20°C) for the first 4 h, and at 8°C for the remaining 4 h of measurements. Immediately after RMR measurements birds were blood-sampled and mitochondrial respiration states assessed in red blood cells at 41°C with high-resolution respirometry. RMR at 20°C and mitochondrial respiration (ROUTINE) did not show the expected positive relationship in intact cells, in which mitochondrial function relies on endogenous substrates. Yet, they identified a positive relationship between RMR at 20°C and phosphorylating respiration (OXPHOS CI+CII) in permeabilized cells, which are (non-viable) cells with modified plasma membranes that permit the entry of respiratory substrates, supplied at saturating amounts. However, RMR at 8°C and mitochondrial respiration in intact cells (ROUTINE) tended to be positively related (P=0.065, results provided in the supplemental materials) and it also again covaried positively with OXPHOS CI and OXPHOS CI+CII in permeabilized cells (P=0.037 and P=0.023, respectively). The authors concluded that a relationship between RMR and blood mitochondrial respiration traits exists, but that it remains hidden when measuring intact cells and only emerges in permeabilized cells. These results are in contrast to two recent studies, also on great tits (Malkoc et al., 2021) and on European starlings (*Sturnus vulgaris*; Casagrande et al., 2023), which found positive associations between RMR and mitochondrial respiration in intact cells. We fully agree with the call by Thoral et al. for more validations of the use of mitochondrial respiration as a proxy for whole-organism metabolic rate. This commentary is meant to highlight potential reasons for the divergent findings in the three studies, particularly on the correlation between RMR and mitochondrial respiration in intact blood cells, and to develop best practices for the use of this exciting and promising technique.

i) *Using intact versus permeabilized cells*. Measuring mitochondrial metabolism in living, intact cells, which rely on their natural reserves and maintain processes of membrane transport as in their tissue of origin, holds a considerable potential for ecological studies. For example, in Thoral et al. ROUTINE and RMR tended to be positively correlated when RMR was measured at 8°C, and not at 20°C. Indeed, not only does a temperature of 8°C better reflect the natural ecological conditions experienced by the wintering great tits used in the study, but also, RMR at this temperature was measured in closer proximity to blood sampling, which ensured that individual's physiological status was more similar between the two measures. Conversely, permeabilization and substrate saturation removes effects of resource limitation and membrane transport dynamics, representing a more artificial situation (which has its merits, see below). Cell permeabilization represents a state never experienced by cells *in vivo*, unless their cell membrane is damaged. Such artificial conditions may lead ecologists to question the ecological relevance of mitochondrial measures in permeabilized cells. For example, the use of substrates should be validated to test whether malate and pyruvate alone are used by erythrocytes' complex I in wintering great tits, and not other substrates like glutamate or even β-oxidation products like palmitoyl or carnitine, as recently shown in muscles of migratory birds (Rhodes et al., 2024). Exploring the relationship between ROUTINE and OXPHOS could prove crucial to establish how much mitochondrial function is affected by the cell's condition. For instance, Thoral et al. (2024) could have investigated the correlation between their measures of ROUTINE (quantified when cells were still intact) and of OXPHOS (quantified after permeabilizing the cells).

ii) *Effects of temperature on metabolic rates*. While all three studies used similar *in vitro* temperatures for measuring mitochondrial metabolic rate (40-41°C), the temperatures to which the birds were acclimatized prior to the experiment and those they experienced during RMR measurements differed substantially (Casagrande et al., 2023; Malkoc et al., 2021; Thoral et al., 2024)[1], resulting in different temperature dynamics before and after the blood sampling. Similarly to RMR, which increases with greater thermoregulatory costs (Swanson and Olmstead, 1999), mitochondrial respiration is strongly affected by changes in environmental temperature (Seebacher, 2009).

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1. Acclimatized to winter temperatures versus RMR being measured at 20 and 8°C in Thoral et al.; acclimatized to winter temperatures that were maintained during RMR measurements in Malkoc et al.; and acclimatized to autumn temperatures versus RMR being measured at 25°C in Casagrande et al.

However, it seems likely that the divergent temperature regimes in the three studies have affected organismal and cellular respiration rates differently, as well as their relationship. Therefore, in future validation studies, considerable research effort should be directed to investigate how environmental temperature, acclimatization state, body and assay temperatures affect RMR and mitochondrial metabolic rate.

iii) *Effects of stressors on metabolic rates*. Malkoc et al. (2021) showed that high, stress-induced corticosterone concentrations resulting from the experimental procedure (i.e. catching, handling and confinement in metabolic chambers), can reverse the positive relationship between metabolic rate measured at the organismal and cell levels. This could be attributed to the regulatory role of elevated corticosterone by which non-immediately essential tissues, including blood, are prevented access to blood substrates. Indeed, in this case, 'feeding' blood cells with exogenous substrates (as done in permeabilization protocols) could be a valuable way to test for the effects of tissue-specific resource availability. However, care should be taken when using mitochondrial substrates, as species can differ in their utilization, necessitating proper validation to ensure ecologically meaningful results (Metcalfe et al., 2023). Measuring plasma glucocorticoid concentrations from the same blood sample from which mitochondrial metabolism is quantified (within the species-specific time after which glucocorticoids begin to rise, for example within the 3 min following the removal from the metabolic chamber; Romero and Reed, 2005) is especially important when mitochondrial respiration is measured in laboratory conditions where study individuals are typically exposed to a series of stressful events. This measurement would have been useful in Thoral et al. to account for (a) differences in stress status among individuals at the time of sampling[2], (b) its (potentially divergent) effects on RMR and ROUTINE and (c) to facilitate comparison with other studies[3] to increase our understanding of mitochondrial bioenergetics and its links with stress.

We appreciate Thoral et al. (2024) for advancing our understanding of blood mitochondrial bioenergetics, and emphasize that their study marks a step forward in the field. Indeed, their documented relationship between the oxygen consumption by permeabilized cells and the organism as a whole, pinpoints substrate saturation as an experimental procedure that may allow us to address mechanistic questions that do not require a cell to be in its natural state or to overcome context-dependent nutrient limitation. However, for increasing our understanding of the metabolic challenges that animals face in the wild, knowing how intact cells regulate their energy metabolism may be most relevant.

2. In Thoral et al, up to 3 h could elapse daily between catching and the start of respirometry. Consequently, birds caught at the beginning of the catching session likely experienced stressful conditions for a much longer duration than those caught at the end.

3. For example, the cumulative stress experienced by the birds at the time of blood sampling differed among the three studies due to a different duration of pre-respirometry experimental procedures. While in Malkoc et al. and Casagrande et al., respectively, 40 and 20 minutes passed between catching and the start of respirometry, in Thoral et al. these procedures could take up to 3 h.

Competing interests
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Response to 'Commentary on Thoral et al. (2024) The relationship between mitochondrial respiration, resting metabolic rate and blood cell count in great tits'

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The commentary by Malkoc et al. highlights possible arguments explaining divergent findings of how mitochondrial metabolism in blood cells is related to whole-organism resting metabolic rate (RMR) in birds across the three studies that have addressed this relationship (Casagrande et al., 2023; Malkoc et al., 2021; Thoral et al., 2024). The authors argue that the lack of correlation between ROUTINE respiration in blood and RMR in Thoral et al. (2024), but the presence of such in their previous papers (Casagrande et al., 2023; Malkoc et al., 2021), can be explained by methodology (intact versus permeabilized blood cells), acclimation temperature, or the stress status of the individuals. In our response, we argue why the concerns expressed by Malkoc et al. should be interpreted with caution, and comment on the conclusions that can be drawn from studying both individual and mitochondrial metabolisms.

Firstly, we agree that permeabilization status can explain the absence or presence of a relationship between mitochondrial respiration traits in blood cells and RMR. However, this alone cannot explain why ROUTINE respiration was uncorrelated to RMR in Thoral et al. (2024), but correlated to RMR in Malkoc et al. (2021) and Casagrande et al. (2023), since blood cells are *not* permeabilized when ROUTINE is measured (Gnaiger, 2020). We therefore disagree that a relationship between ROUTINE respiration in intact blood cells and RMR is a general phenomenon and that the absence of such reflects neglectful experimentation. In line with this, a study on Japanese quail (*Coturnix japonica*) in our laboratory found no relationships between these traits (Fig. 1). We suggest that permeabilized experiments may bypass any rate-limiting steps influencing mitochondrial function in intact cells (Djafarzadeh and Jakob, 2017), such as substrate availability (Kyriazis et al., 2022; Leverve, 2007), transmembrane transport rate (Osellame et al., 2012), or any interactions between mitochondria and other cell components (Boldogh and Pon, 2007; Wieckowski et al., 2009). Thus, even though the measurement conditions in intact cell experiments may be more similar to the *in vivo* situation, permeabilization could be necessary to reveal a link between cellular and organismal metabolic rate. We propose that future studies apply complete SUIT protocols (substrate – uncoupler – inhibitor titration protocols; Gnaiger, 2020) in both intact and permeabilized samples from the same individual. The resultant improved understanding of mechanisms underlying phenotypic changes observed at the individual level would provide a more comprehensive ecological context.

Secondly, the authors argue that acclimation temperature may affect metabolism at different biological scales, altering the relationship between RMR and mitochondrial respiration. However, since the effect of environmental temperature on RMR is linear below the lower critical temperature (Scholander et al., 1950), it can be argued that an acute organismal metabolic response would only affect the intercept of the regression, not the slope. In support of this, the relationship between organismal metabolic rate and mitochondrial respiration parameters were largely identical when RMR was measured at 7.5°C and 20°C in our study [Fig. S4 in Thoral et al. (2024)]. Even if the timeline of changes to cellular and organismal metabolic rate differ [e.g. if acclimation time could be too short or too stochastic to cause either intrinsic changes in mitochondrial functioning or increased presence of a new blood cell phenotype (Voss et al., 2010)], it seems unlikely that variation in the temporal response would completely negate a potential underlying biological relationship. However, we agree with the incentive that "*…effort should be directed to investigate how environmental temperature, acclimatization state, body and assay temperatures affect RMR and mitochondrial metabolic rate*". Such a study has already been performed: García-Díaz et al. (2023) exposed winter-acclimated great tits to −15°C, +5°C or +25°C for 13-15 h overnight whilst measuring RMR and body temperature, and mitochondrial respiration in intact blood cells at a normothermic (41°C) and a hypothermic (35°C) assay temperature the morning after. While RMR was nearly twice as high in −15°C compared to +25°C, all mitochondrial respiration traits were unaffected by acclimation temperature. Moreover, while ROUTINE and LEAK respirations, and associated flux control ratios, were strongly affected by assay temperature, body temperature was unrelated to all mitochondrial respiration traits but the E-R control efficiency (an index of respiratory reserve capacity; Gnaiger, 2020; García-Díaz et al., 2023).

Thirdly, Malkoc et al. argue that the stress status of individuals can affect both individual and mitochondrial metabolism, potentially changing the relationship between the two. While consideration of glucocorticoid levels may sometimes reveal biologically interesting relationships otherwise not apparent (Malkoc et al., 2021), other studies report that circulating stress hormone levels are unrelated to both organismal and blood cell respiration (Casagrande et al., 2023).

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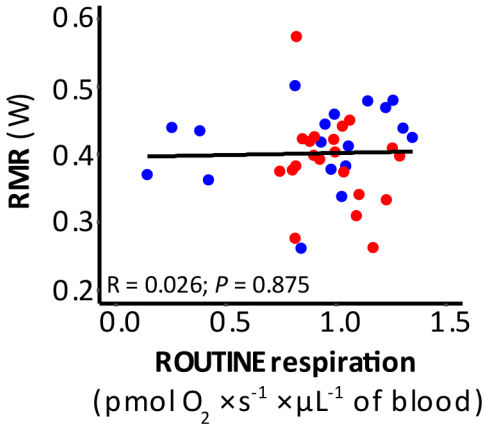

Fig. 1. Relationship between RMR and ROUTINE respiration obtained in intact blood cells from Japanese quail (*Coturnix japonica*). Half of the birds were acclimated at 10°C (blue dots) while the other half was acclimated at 30°C (red dots) for 11 weeks. RMR was measured at 30°C, which is thermoneutral in Japanese quail, and blood respiration was measured at 41°C. A Pearson correlation test on pooled data revealed no significant association between RMR and ROUTINE. Nor was this relationship significant within groups (cold birds: R=0.255, *P*=0.306; warm birds: R=−0.250, *P*=0.275).

Hence, while we agree that the physiological status of individuals, including affective state, may impact metabolism, such relationships are arguably of limited effect compared to the main issue of relating metabolic rate at different biological scales.

At any point in time, organismal metabolism can be considered a sum of mitochondrial respiration across the body (Salin et al., 2016), with some oxidative tissues contributing more heavily to the energy expenditure than others (Casagrande et al., 2023). However, even if mitochondrial metabolism affects organismal performance (Koch et al., 2021) and can thus be related to fitness (Hood et al., 2018), mitochondrial respiration differs between tissues (Casagrande et al., 2023; Salin et al., 2016, 2019), and subpopulations of distinct mitochondrial phenotypes may be present within single tissues (Scott et al., 2018). Moreover, mitochondrial metabolism is affected by multiple biotic and abiotic factors (Sokolova, 2018), and the conditions under which mitochondrial metabolism is measured, whether with intact or permeabilized cells, are always far from the physiological conditions experienced by cells within organisms (i.e. hormonal status, connection with the other tissues, substrate availability, supra-physiological oxygen concentrations, etc.). Organismal metabolic rate is equally labile when extrinsic and intrinsic conditions change (Versteegh et al., 2008; Norin and Malte, 2011; White et al., 2013; Welcker et al., 2015; Burton et al., 2011). Thus, the relationship between organismal metabolic rate and blood cell respiration remains a physiological parameter that must be interpreted with caution as prediction of RMR from mitochondrial respiration in specific tissues may be imprecise (Thoral et al., 2024).

To uncover the ecological and mechanistic explanations for why there is a relationship between blood cell respiration and RMR, we believe that future studies would benefit from addressing: 1) the relative aerobic level of bird blood, by estimating the proportion of ATP produced via the oxidative and glycolytic pathways compared to the situation in other tissues; and 2), as already initiated by Casagrande et al. (2023), partitioning the total contributions of mitochondrial respiration across key metabolic tissues towards RMR, when substrate availability, environmental conditions, and affective state of individuals vary; and 3) determining whether the *response* to thermal acclimation over short and long time periods is comparable between blood cells and tissues with more clearly defined roles in energy metabolism and thermogenesis.

Competing interests

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