The mitochondrial activity of leukocytes from *Artibeus jamaicensis* bats remains unaltered after several weeks of flying restriction

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Abstract

Bats are the only flying mammals known. They have longer lifespan than other mammals of similar size and weight and can resist high loads of many pathogens, mostly viruses, with no signs of disease. These distinctive characteristics have been attributed to their metabolic rate that is thought to be the result of their flying lifestyle. Compared with non-flying mammals, bats have lower production of reactive oxygen species (ROS), and high levels of antioxidant enzymes such as superoxide dismutase. This anti-oxidative vs. oxidative profile may help to explain bat´s longer than expected lifespans.

The aim of this study was to assess the effect that a significant reduction in flying has on bat leukocyte mitochondrial activity. This was assessed using samples of lymphoid and myeloid cells from peripheral blood from *Artibeus jamaicensis* bats shortly after capture and up to six weeks after flying deprivation. Mitochondrial membrane potential ($\Delta\psi_m$), mitochondrial calcium (mCa^{2+}), and mitochondrial ROS (mROS) were used as key indicators of mitochondrial activity, while total ROS and glucose uptake were used as additional indicators of cell metabolism.

Results showed that total ROS and glucose uptake were statistically significantly lower at six weeks of flying deprivation ($p<0.05$), in both lymphoid and myeloid cells, however no significant changes in mitochondrial activity associated with flying deprivation was observed ($p>0.05$).

These results suggest that bat mitochondria are stable to sudden changes in physical activity, at least up to six weeks of flying deprivation. However, decrease in total ROS and glucose uptake in myeloid cells after six weeks of captivity

suggest a compensatory mechanism due to the lack of the highly metabolic demands associated with flying.

Key terms: bats, wildlife, captivity, lifespan, leukocytes, mitochondria

Running title: mitochondrial activity in bat leukocytes

1. Introduction

Bats are classified within the order Chiroptera, the only order of flying mammals (Maina et al., 2000). They serve as reservoirs of several human pathogens, including SARS-CoV-2 (Li et al., 2005; Brook and Dobson, 2014; Zhou et al., 2020), and can carry high loads of certain intracellular pathogens, notably viruses, without showing any sign of disease (Brook and Dobson, 2015). As a cosequence, the bat´s immune system has become a subject of intensive research. Their ability to cope with viral infections has been attributed to their flying lifestyle (Zhang et al., 2013; Gorbuniva et al., 2020) and also to immune adaptations that mostly dampen, rather than activate, the immune response (Baker et al. 2013; O´Shea et al., 2014; Stockmaier et al. 2015; Gorbuniva et al., 2020).

There is increasing evidence of a close correlation between metabolism and immune response (Pearce et al., 2018; Jung et al., 2019). Bats have one of the highest basal metabolic rates amongst mammals, which further increases during flying (Speakman et al., 2003). Paradoxically, the production of reactive oxygen species (ROS) in relation to oxygen consumption is lower in bats than in other mammalian species with similar metabolic rates, and bats also live longer

compared to other mammals of a similar size and weight (Brunet-Rossinni et al., 2004). Studies aimed at understanding this long lifespan have included the analyses of reactive oxygen species (ROS) and anti-oxidants production, as well as mitochondrial proton leak, and their ability to control inflammation (Wilhelm et al., 2007; Gorbunova et al., 2020). Compared with non-flying mammals, bats exhibit a lower production of ROS and a lower mitochondrial membrane potential ($\Delta\psi_m$) (Brown et al., 2009), and higher levels of antioxidants such as superoxide dismutase (Wilhelm et al., 2007).

Since mitochondria are key components of metabolism and immunity (Mehta et al., 2017; Angajala et al., 2018; Breda et al., 2019), comparing mitochondrial function in immune cells shortly after capture and following flying deprivation, will help to reveal whether mitochondria play a role in bats unique resistance to pathogens and longer lifespan.

Here we evaluated mitochondrial membrane potential ($\Delta\psi_m$), mitochondrial ROS (mROS) and mitochondrial calcium (mCa^{2+}), as key indicators of mitochondrial function (Duchen, 2000; Murphy, 2009; Görlach et al., 2015; Zorova et al., 2018), and total ROS and glucose uptake as additional metabolic indicators, from lymphoid and myeloid cells obtained from the peripheral blood of *Artibeus jamaicensis* bats shortly after capture and up to six weeks of flying restriction.

2. Materials and methods

2.1 Animals and blood samples

An initial experiment was conducted with five fruit-eating bats (*Artibeus jamaicensis*) captured in Oaxtepec, Morelos, Mexico (latitude: 18° 54' 20.99" N; longitude: -98° 58' 50.56" W) with the aid of a 12-meter mist net, set up at sunset.

In a second experiment six *Artibeus jamaicensis* bats were captured in Cocoyoc, Morelos, Mexico (latitude: 18° 53' 3.01" N; longitude: -98° 58' 54.98" W) (Fig. 1). Blood samples (100-200 μl) were obtained from the cephalic vein, immediately after capture and two weeks later in the first experiment and, at two, four and six weeks of captivity in the second experiment. Blood samples were added to Alsever gelatin containing micro-tubes (200 μl) and left at room temperature for 20-30 min to allow red blood cells separation from the leukocyte rich plasma (LRP). LRP was separated into several tubes individually containing probes for the assessment of mitochondrial membrane potential ($\Delta\psi_m$), mitochondrial calcium (mCa^{2+}), mitochondrial ROS (mROS), total ROS, or glucose uptake, as indicated below. All animals were kept in two cages (40 x 40 x 55 cm), and during the studies bats consumed a diverse array of fruits *ad libitum*, in addition to water, in the absence of any flight, in order to assess how bat´s mitochondria deal with a surplus of energy. Of note, animals did not show any appreciable change in their behavior, they ate well, had normal resting periods, and remained healthy for the duration of the experiments. The project had the approval of SEMARNAT Mexico (SGPA/DGVS/08986/18).

2.2. Mitochondrial function analysis

Mitochondrial function was assessed with the use of three probes that measure mitochondrial membrane potential ($\Delta\psi_m$), mitochondrial calcium (mCa^{2+}), and mitochondrial ROS (mROS). The experimental procedure was identical for all three mitochondrial function indicators except for the molecular probe. Briefly, cells from leukocyte rich plasma were labeled with the $\Delta\psi_m$–sensitive MitoTracker Red CMXRos (Thermo Fisher Scientific Inc., Walthman, MA, USA.) or Tetramethyl rhodamine, methyl ester (TMRM) (Thermo Fisher Scientific Inc)

molecular probes, at a final concentration of 5 µM for 20 minutes at room temperature, before being washed with PBS and then fixed with 4% paraformaldehyde in PBS. $\Delta\psi_m$ was assessed by flow cytometry (FACScan, BD Biosciences, San José, CA. USA). Forward scatter (FSC) and side scatter (SSC) parameters were used to distinguish between lymphoid cells and myeloid cells. The mean fluorescence intensity (MFI), as an indication of $\Delta\psi_m$, was retrieved from the gated cells (lymphoid cells or myeloid cells). Raw data was analyzed with the CellQuest program (BD Biosciences) and expressed as F_1/F_0, where F_1 is the MFI of MitoTracker Red CMXRos- (first experiment), or TMRM-labelled cells (second experiment), and F_0 is the MFI due to cells auto-fluorescence.

For mCa^{2+}, cells were labelled with the mitochondrial calcium indicator Rhod-2/AM (Thermo Fisher Scientific Inc.) at a final concentration of 10 µM, and for mitochondrial ROS (superoxide anion), cells were labeled with the mitochondrial superoxide indicator MitoSOX™ Red (Thermo Fisher Scientific Inc.) at a final concentration of 5 µM. Raw data was treated in the same way as for $\Delta\psi_m$ assessment.

2.3. Total ROS

Total reactive oxygen species (ROS) was assessed in a similar way as mitochondrial function assessment, except that the molecular probe used for this functional parameter was CM-H_2DCFDA (Thermo Fisher Scientific, Inc.), an indicator of general oxidative stress, at 5 µM final concentration, followed by flow cytometric analyses as previously described.

2.4. Glucose uptake

Leukocyte rich plasma obtained from the peripheral blood was added to a tube containing the fluorescent glucose analogue 2-(N-(7-nitrobenzen-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) (Thermo Fisher Scientific, Inc.), at a final concentration of 250 μM. Cells were incubated at room temperature for 20 minutes and then washed with PBS, followed by fixation with 4% paraformaldehyde, and flow cytometric analyses, again as previously described.

2.5. Statistical analyses

Mean Fluorescence Intensity (MFI) numerical values for each metabolic parameter were compared between sample groups using a Mann-Whitney test, ANOVA one-way non-parametric Kruskal Wallis test, and Turkey post hoc using Graph Pad Prism Software (Graphpad, La Jolla, CA).

3. Results

3.1. Mitochondrial activity of leukocytes from the fruit-eating bat *Artibeus jamaicensis* remains unchanged up to six weeks of flying restriction

Based exclusively on size (forward light scatter, FSC) and cell complexity (side light scatter, SSC), the flow cytometric analyses allowed us to distinguish between lymphoid- and myeloid-type cells. This enabled assessment of the mitochondrial activity ($\Delta\psi_m$, mCa^{2+}, and mROS) in these two cells populations. The first experiment (using bats captured in Oaxtepec, Morelos) showed an apparent increase in $\Delta\psi_m$, and mCa^{2+}, at two weeks of flying deprivation

compared with recently captured bats, especially in lymphoid cells (Fig. 2). However, these differences were not statistically significant. In a second experiment (bats captured in Cocoyoc, Morelos) where longer flying restriction was tested (2, 4, and 6 weeks) showed no statistically significant differences in $\Delta\psi_m$, mCa^{2+}, or mROS (Fig. 3).

3.2. Total reactive oxygen species diminishes at later time points of bats flying restriction in lymphoid and myeloid cells

In the first experiment no difference in mROS was observed between time of capture and 2 weeks of flying restriction (Fig. 2). In the second experiment total ROS in addition to mROS were assessed. No differences in mROS were observed. However, total ROS was significantly lower in lymphoid and myeloid cells at 6 weeks compared with 2 and 4 weeks ($p<0.05$) of flying deprivation (Fig. 4).

3.3. Glucose-uptake by Bat´s lymphoid and myeloid cells varies during flying restriction

In the first experiment (Oaxtepec) glucose uptake by bat´s myeloid-type cells was significant higher two weeks after capture compared with recently captured bats ($p=0.03$) (Fig. 2). In the second experiment (Cocoyoc), glucose uptake by both lymphoid- and myeloyd- type cells was significantly lower after six weeks of flying restriction compared with four weeks of flying restriction ($p<0.05$) (Fig. 4).

4. Discussion

The main finding of this study is that bat leukocytes take up less glucose while keeping mitochondrial function un-altered, as a way of adapting to several weeks of flying restriction.

Three specific characteristics distinguish bats from non-flying mammals of similar weight and size: i) higher metabolic rate, ii) longer than expected lifespan, and iii) resistance or tolerance to viral infections (Speakman et al., 2003; Subudhi et al., 2019; Gorbunova et al., 2020). Flight is among the most energy consuming physiological activities and it is thought that adaptive evolution of energy metabolism is at the core of flight development in bats. Shen et al found that bat mitochondrial and nuclear encoded oxidative phosphorylation genes display evidence of adaptive evolution that supports the high metabolic demands of flying (Shen et al., 2010; Subudhi et al., 2019), and Pollard et al identified differences in the proteome and lipidome of bats mitochondria, compared with those of mice (Pollard et al., 2019).

Mitochondria are key components of metabolism, lifespan and resistance to viral infections (Mills et al., 2017; Sharma et al., 2018), we therefore wanted to test the effect that flying restriction has on bat leukocyte mitochondrial activity. Results showed that $\Delta\psi_m$, mCa^{2+} and mROS remained un-altered in bat peripheral blood lymphoid and myeloid cells after 6 weeks of flying restrictions, in spite of the fact that during their daily flights, bats metabolic rate increases about 15-fold compared with their resting metabolic rate (Speakman and Thomas, 2003; O´Shea et al., 2014).

$\Delta\psi m$ is instrumental for aerobic energy production, and the driving force behind other mitochondria physiological processes, such as Ca^{2+} uptake and heat production by brown fat (Gerencser et al., 2012). During flight, a bats body temperature may reach 41^{o}C or more (Morrison et al., 1967) making them highly dependent on $\Delta\psi m$ maintenance. $\Delta\psi m$ also drives the generation of NADPH, which constitutes an antioxidant mechanism (Brand et al., 1995; Nicholls et al., 2004). In spite of 6 weeks feeding on a diverse array of fruits *ad libitum,* and in the absence of any flight, the bats $\Delta\psi m$ remained un-altered allowing, amongst other functions, mitochondrial Ca^{2+} uptake. Mitochondrial Ca^{2+} activates Krebs cycle dehydrogenases such as pyruvate dehydrogenase, isocitrate dehydrogenase, and oxoglutarate dehydrogenase, favouring ATP synthesis (Takeuchi et al., 2015). Calcium influx into mitochondria also increases the electron transport chain flux, and thus $\Delta\psi_m$ and mROS production (Brookes et al., 2004), thus providing a link between mitochondrial calcium transport and the regulation of cellular bioenergetics (Yi et al., 2004; Walsh et al., 2009; McKenzie et al., 2016).

Mitochondrial ROS (mROS) production is related to the respiration rate, and while low levels of mROS are required for normal cell function (Sena and Chandel, 2012), such as innate immune responses (West et al., 2011), increased production of mROS may lead to oxidative stress, cell death, and disease (Wilking et al., 2013). In spite of their high metabolic rate, bats produce low amounts of ROS and may also be more resistant to oxidative stress (Brunet-Rossinni et al., 2004). A mild depolarization of the $\Delta\psi_m$ by tethering of ATP-consuming kinases to mitochondrial membranes may account for the limited production of mROS in mammals. Interestingly, this mechanism is lost in aging

mice but remains active in aging bats species (Vyssokikh et al., 2020), providing yet another possible mechanism for the longer lifespan of bats. Our results showed that after 6 weeks of flying restriction bat´s leukocytes mitochondria maintain unaltered $\Delta\psi_m$ and mROS. Blood leukocytes are good sensors of their metabolic microenvironment~~,~~ as they can sense metabolic stress and modulate their mitochondrial energetics in response. They have been dubbed as “the canary in the coal mine” for bioenergetics dysfunction (Kramer et al., 2014). Our results showing that $\Delta\psi_m$, mCa^{2+}, and mROS remain un-altered after six weeks of flying restriction (Fig. 3) suggesting that bat mitochondria are well adapted to sudden changes in their usual and highly energy-demanding activity of flying.

Bats usually fly at the onset of their activity period, fuelled by limited fat reserves (Voigt et al., 2010). They therefore need to rapidly fuel metabolism through the ingestion of simple carbohydrates as otherwise their high metabolic rates and small body size would place them at risk of starvation if sufficient food is not found (Welch et al., 2016; O´Mara et al., 2017). During this study bats were allowed to consume a diverse array of fruits *ad libitum* (in the absence of any flight) in order to assess how their mitochondria deal with a surplus of energy. In the first experiment (Oaxtepec) a significant increase in glucose uptake in myeloid-type cells but not in lymphoid-type cells was measured after two weeks of flying restriction (Fig. 2). However, in the second experiment (Cocoyoc) glucose uptake was significant lower at six weeks of flying restriction, alongside a significantly lower production of total ROS (Fig. 4), and an apparent but non-statistically significant reduction in mROS production in both lymphoid and myeloid cells (Fig. 3). While the increase in glucose uptake by myeloid cells at 2 weeks of flying restriction is intriguing, the results showing that bat myeloid and lymphoid cells

take up less glucose at a later time point of flying restriction (six weeks) suggests a way of adapting to flying restrictions, and thus reduced energy requirements, so as to avoid oxidative damage, but keeping mitochondrial function un-altered.

Acknowledgements

The authors thank the Immunology postgraduate core lab for the use of facilities. This work was supported by SIP-IPN (20210917) grant. MMBM-A and FJS-G are COFAA/EDI/SNI fellows, AA-S is an SNI fellow.

Conflict of Interest.

All authors of this manuscript declare no conflict of interest.

Author contributions

Conceptualization: MMBM-A, FJS-G, JAA-S; Field work: JAA-S, MMBM-A, FJS-G; Performed experiments: MMBM-A, FJS-G; Formal analysis MMBM-A, FJS-G, ER-F, CAP-H; Wrote the manuscript: MMBM-A, FJS-G, SEK; Reviewed the manuscript: SEK, AC, MMBM-A; Resources: MMBM-A. All authors read, edited and approved the final manuscript.

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Figure legends

Fig. 1. Oaxtepec and Cocoyoc geographical location.

Oaxtepec (latitude: 18° 54' 20.99" N; longitude: -98° 58' 50.56" W) and Cocoyoc (latitude: 18° 53' 3.01" N; longitude: -98° 58' 54.98" W) linear distance form one another is approximately 3 km. Both are within the Morelos state in Mexico, and *Artibeus jamaicensis* as well as other bat species are widely distributed.

Fig. 2 Glucose uptake by peripheral blood myeloid-type cells increased after two weeks in captivity. In the first experiment (Oaxtepec), bats peripheral blood was taken shortly after capture and then at two weeks of flying restriction. Mitochondrial membrane potential ($\Delta\psi_m$), mitochondrial ROS (mROS), mitochondrial calcium (mCa^{2+}), and glucose uptake were assessed by flow cytometry in both myeloid-type and lymphoid-type cells as described in the materials and methods section. Data was analysed using an ANOVA one-way non-parametric Kruskal-Wallis test and Tukey post hoc with statistical significance considered as $p<0.05$ (n=5).

Fig. 3. Mitochondrial membrane potential ($\Delta\psi m$), mitochondrial calcium (mCa^{2+}) and mitochondrial ROS (mROS) remains unchanged after six weeks of flying restriction. In the second experiment (Cocoyoc), *A. jamaicensis* bats were kept under flying restriction for up to six weeks. $\Delta\psi_m$, mROS), and mCa^{2+} were assessed by flow cytometry in lymphoid-, and myeloid-type cells. Data was analysed using ANOVA, one-way non-parametric Kruskal-Wallis test and Tukey post hoc with statistical significance considered as $p<0.05$. No statistically significant differences were observed (n=6).

Fig. 4. Glucose-uptake and total ROS production of *A. jamaicensis* lymphoid and myeloid cells decreases by 6 weeks of flying restriction. In the second experiment (Cocoyoc), glucose uptake and total ROS production in lymphoid- and myeloid-type cells were assessed by flow cytometry. Data was analysed using an ANOVA one-way non-parametric Kruskal-Wallis test and Tukey post hoc with statistical significance considered as $p<0.05$ (n=6).

Fig. 1


N
W
E
S
Oaxtepec
(Experiment 1)
Cocoyoc
(Experiment 2)
500m
Morelos state

Fig. 2

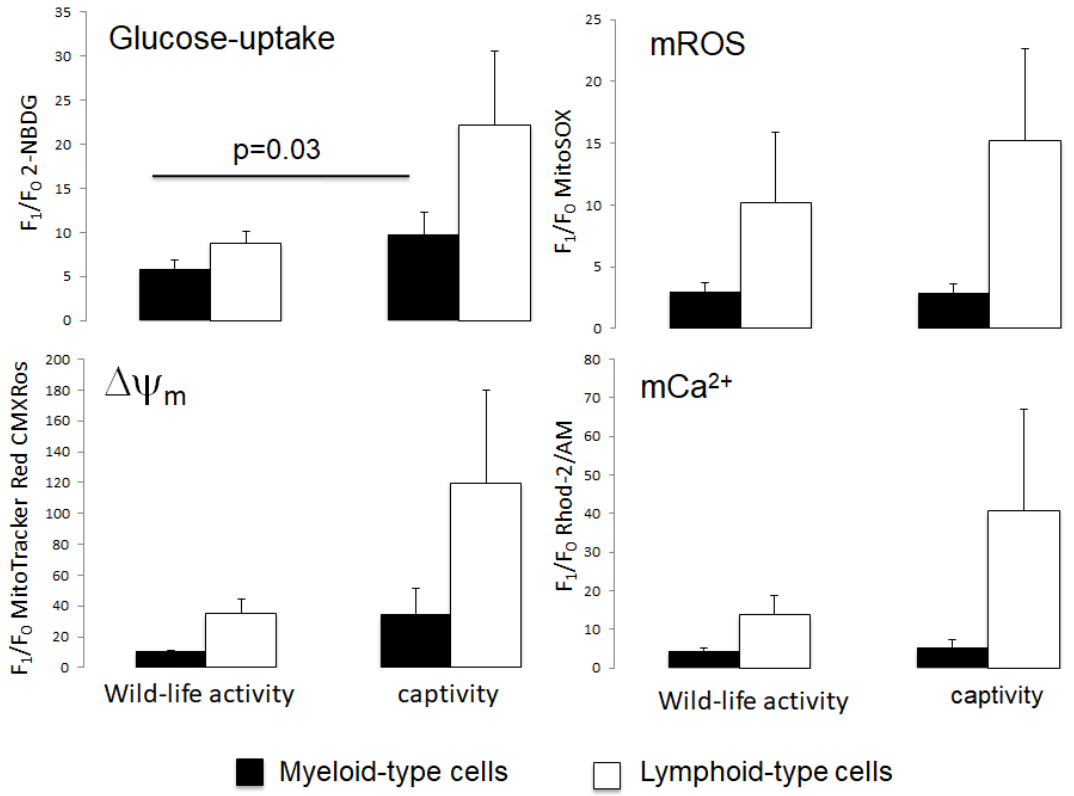

Glucose-uptake
p=0.03
F1/F0 2-NBDG
mROS
F1/F0 MitoSOX
Δψm
F1/F0 MitoTracker Red CMXRos
mCa2+
F1/F0 Rhod-2/AM
Wild-life activity
captivity
Wild-life activity
captivity
Myeloid-type cells
Lymphoid-type cells

Fig.3

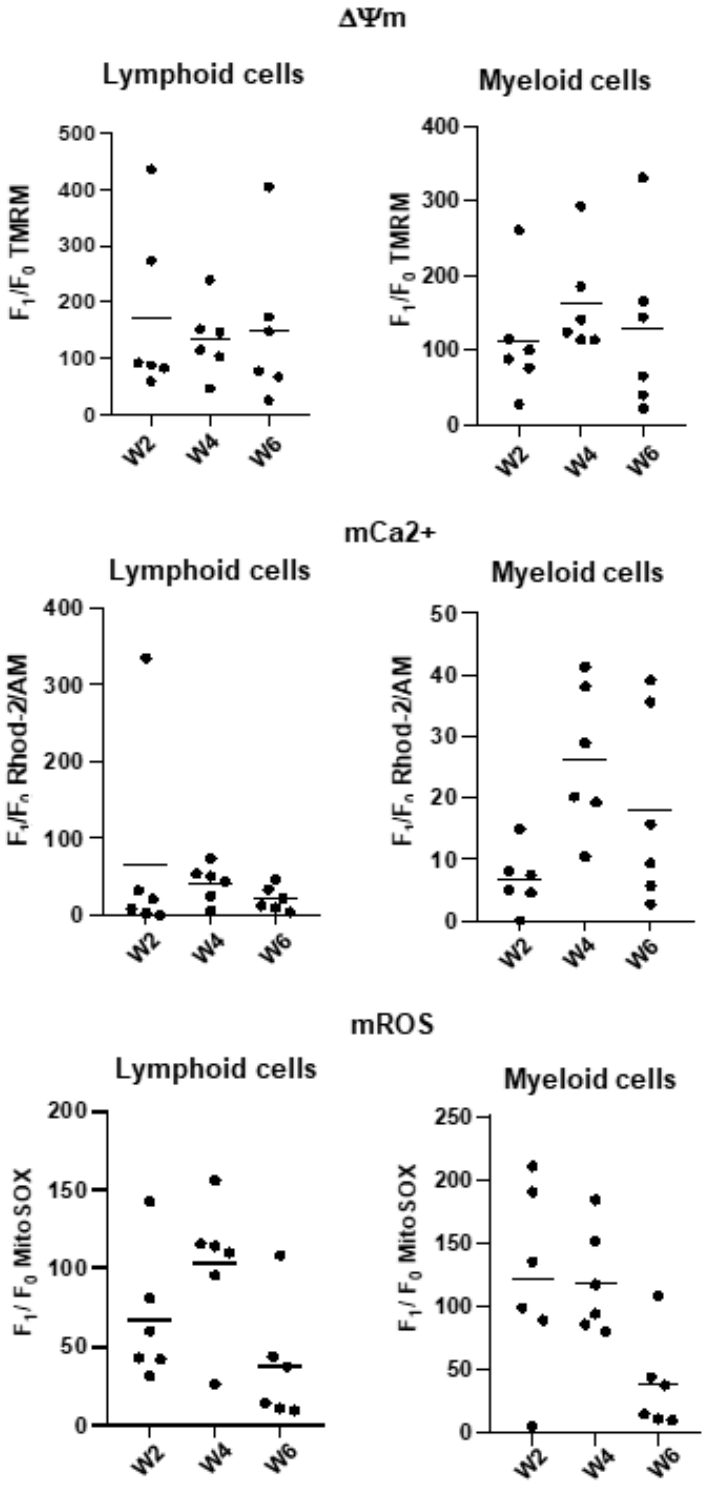
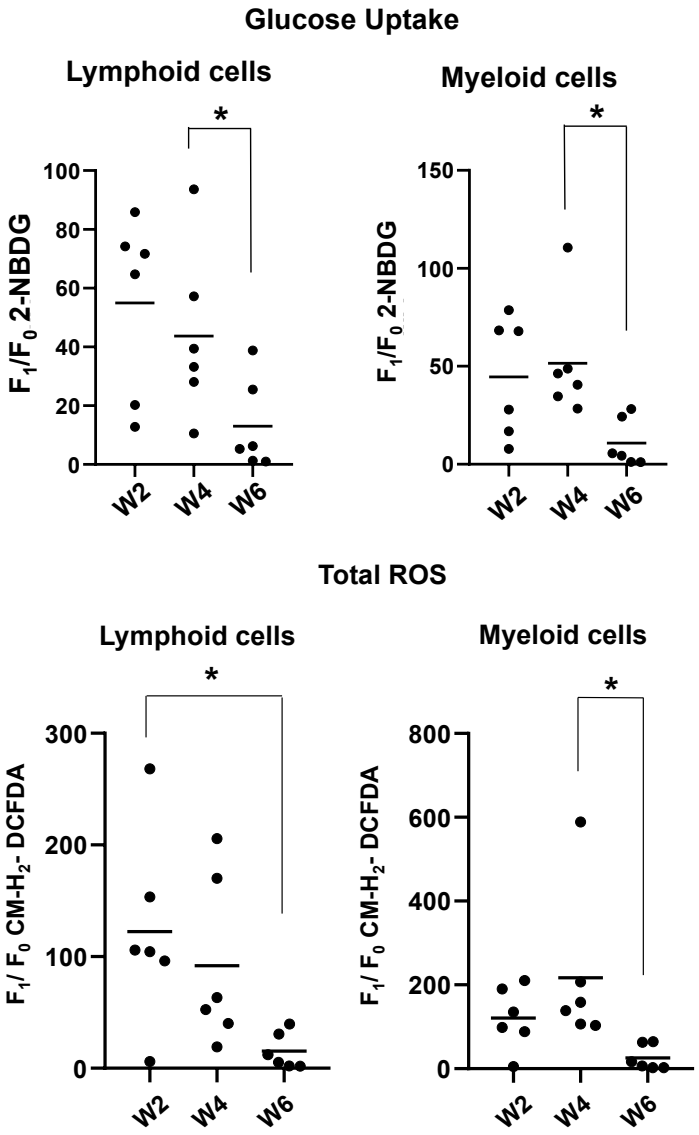

ΔΨm
Lymphoid cells
Myeloid cells
F1/F0 TMRM
500
400
300
200
100
0
400
300
200
100
0
W2
W4
W6
mCa2+
Lymphoid cells
Myeloid cells
F1/Fn Rhod-2/AM
400
300
200
100
0
50
40
30
20
10
0
W2
W4
W6
mROS
Lymphoid cells
Myeloid cells
F1/ F0 MitoSOX
200
150
100
50
0
250
200
150
100
50
0
W2
W4
W6

Fig. 4


Glucose Uptake
Lymphoid cells
Myeloid cells
F_1/F_0 2-NBDG
100
80
60
40
20
0
150
100
50
W2
W4
W6
*
Total ROS
Lymphoid cells
Myeloid cells
F_1/F_0 CM-H_2- DCFDA
300
200
100
800
600
400