Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research

Graphical Abstract

Highlights

- Tetracyclines promote mitonuclear protein imbalance and mitochondrial dysfunction
- The effects of tetracyclines are conserved across eukaryotic kingdoms
- Use of tetracyclines in research can confound the experimental outcome
- Tetracyclines delay plant growth and may pose an environmental hazard

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In Brief
The bacterial origins of mitochondria explain why they may be vulnerable to antibiotics such as the tetracyclines. Moullan et al. demonstrate that low concentrations of tetracyclines inhibit mitochondrial function across different species. This effect of tetracyclines can potentially confound experimental outcomes, and the data suggest a potential negative impact on the environment and health that should be further explored.
Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research

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http://dx.doi.org/10.1016/j.celrep.2015.02.034
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SUMMARY

In recent years, tetracyclines, such as doxycycline, have become broadly used to control gene expression by virtue of the Tet-on/Tet-off systems. However, the wide range of direct effects of tetracycline use has not been fully appreciated. We show here that these antibiotics induce a mitonuclear protein imbalance through their effects on mitochondrial translation, an effect that likely reflects the evolutionary relationship between mitochondria and prokaryotes. Even at low concentrations, tetracyclines induce mitochondrial proteotoxic stress, leading to changes in nuclear gene expression and altered mitochondrial dynamics and function in commonly used cell types, as well as worms, flies, mice, and plants. Given that tetracyclines are so widely applied in research, scientists should be aware of their potentially confounding effects on experimental results. Furthermore, these results caution against extensive use of tetracyclines in livestock due to potential downstream impacts on the environment and human health.

INTRODUCTION

Advances in the mechanistic understanding of gene function are often based on the characterization of gain-of-function (GOF) and loss-of-function (LOF) mutations in cells and model organisms. Constitutive GOF and LOF studies have now become an essential part of the post-genomic biomedical toolkit (Argmann et al., 2005; Branda and Dymecki, 2004). Since many genes are essential for cellular function and/or animal development (i.e., they are lethal if knocked out from the embryonic state), conditional systems have been developed in which gene expression can be spatially or temporally controlled. In mammalian systems, cell-specific promoters are often used in genetic strategies to spatially control GOF and LOF. For example, tissue- or cell-type-specific expression of the Cre recombinase is commonly employed to restrict recombination at LoxP sites, introduced at specific locations in the genomic DNA, to a given cell type and/or tissue (Utomo et al., 1999). Temporal control often requires responsiveness to an exogenously added inducer. Two prototypical examples of such temporal control are the use of chimeric Cre recombinase proteins (Utomo et al., 1999) and the Tet-On/Tet-Off system (Gossen and Bujard, 1992) (reviewed in Argmann et al., 2005 and Ryding et al., 2001). The best characterized chimeric Cre recombinase is the Cre-ER² protein, where recombinase activity is gated by a mutated version of the ligand binding domain of the estrogen receptor (ER), modified to be only responsive to the synthetic ER antagonist tamoxifen, which does not occur naturally (Feil et al., 1996). Similar chimeric Cre proteins have been developed using the affinity of the progesterone or ecdysone receptor ligand binding domains for RU-486 or ecdysone, respectively (Minamino et al., 2001; No et al., 1996). Long-lasting side effects of the use of these nuclear receptor ligands have been described (Lelliott et al., 2005; López et al., 2006), which have to be factored in as potential confounders in functional genomic studies. The Tet-On/Tet-Off system employs a tetracycline, doxycycline, to activate or inactivate the tetracycline-responsive promoter (Gossen and Bujard, 1992). In Tet-On systems, doxycycline binds the tetracycline transactivator protein and thereby allows binding to a tetracycline response element and transcriptional activation to occur (Gossen et al., 1995). In Tet-Off systems, doxycycline binding to a slightly modified tetracycline transactivator protein impairs its ability to activate the responsive promoter, thus preventing transcriptional activation (Gossen and Bujard, 1992). Although the Tet-On/Tet-Off system provides exquisite flexibility to study gene function, few researchers consider the potential detrimental effects of the use of tetracyclines themselves, although prolonged antibiotic use is known to cause adverse effects in the clinic (Brummett and Fox, 1989; Mingeot-Leclercq and Tulkens, 1999; Selimoglu, 2007).

Work in the 1960s described that tetracyclines, as well as chloramphenicol, inhibit translation of proteins encoded by mitochondrial DNA (mtDNA), but not by nuclear DNA (nDNA)
(Clark-Walker and Linnane, 1966). We recently showed that this selective inhibition of mitochondrial protein translation by both types of antibiotics leads to a state of so-called mitonuclear protein imbalance, which disturbs mitochondrial proteostasis (Houtkooper et al., 2013). Mitonuclear protein imbalance ensues when protein synthesis from mtDNA is not matched by protein synthesis from nDNA. This abnormal mitochondrial proteostasis robustly induces the mitochondrial unfolded protein response (UPR™), leading to a pronounced lifespan extension in the worm and marked metabolic and molecular changes in cells and mice (Houtkooper et al., 2013). Since tetracyclines are widely applied to control gene expression in cells and a large panel of model systems—we found over 18,000 hits in a Google Scholar search (using Tet-On OR Tet-Off OR “tet inducible” OR “tet-induced” OR “doxycycline inducible” OR “tetracycline inducible”)—we explored here in detail whether these antibiotics also interfere with mitochondrial function in these model organisms.

**RESULTS**

**Disturbed Mitochondrial Proteostasis and Function after Doxycycline Treatment**

We first treated four commonly used cell lines originating from distinct tissues with increasing concentrations of doxycycline: (1) the human embryonic kidney-derived HEK293 cell line, (2) the human cervical carcinoma HeLa cell line, (3) the mouse hepatoma Hepa 1-6 cell line, and (4) the mouse hypothalamus cell line GT1-7 (Figure 1). We determined mitochondrial proteostasis or mitonuclear protein imbalance (Houtkooper et al., 2013) by analyzing the ratio between an mtDNA-encoded OXPHOS subunit (cytochrome c oxidase subunit I or MTCO1) and an nDNA-encoded OXPHOS subunit (succinate dehydrogenase A or SDHA). Indeed, in all four cell lines, doxycycline dose dependently induced mitonuclear protein imbalance (Figures 1A–1D). These effects occurred at doses that are typically...
used in Tet-On/Tet-Off experiments, i.e., in the low \( \mu \text{g/ml} \) range. In order to demonstrate that these concentrations are indeed relevant, we exposed the Tet-On cell line A549 to increasing concentration of doxycycline. This cell line expresses Tet-controlled luciferase, which was activated at 5–10 \( \mu \text{g/ml} \) after 24-hr treatment (Figure S1A). At the same concentrations, these cells displayed marked mitonuclear protein imbalance, comparing the expression of mtDNA-encoded (MTCO1 and MTCO2) and nDNA-encoded (SDHA and SDHB) OXPHOS subunits (Figures S1B and S1C). Furthermore, when we extended the time of treatment to 48 hr, we observed the same mitonuclear protein imbalance at a concentration of 1 \( \mu \text{g/ml} \) (Figures S1D and S1E).

The mitonuclear protein imbalance in all these cellular systems was accompanied by a strong decrease in cellular respiration, indicative for severely impaired mitochondrial activity (Figures 1E–1H). Even though the inhibition of respiration was dose dependent with an EC\(_{50}\) at 6.3 \( \mu \text{g/ml} \) (Figure S1F), it was reversible when doxycycline was removed from the medium (Figure S1G). As the effects of doxycycline could also represent a general effect of antibiotic exposure, we tested the same parameters in the presence of the antibiotic amoxicillin, which is from the penicillin family and disrupts bacterial cell wall synthesis rather than translation (Cole et al., 1972). Consistent with its mode of action in bacteria, amoxicillin did not affect the mitonuclear protein imbalance (Figures 1I–1L) and had no impact on mitochondrial function as reflected by the unchanged cellular respiration (Figures 1M–1P).

One key factor in the regulation of mitochondrial function is mitochondrial dynamics (Gao et al., 2014). Healthy mitochondrial dynamics consist of repeated cycles of mitochondrial fusion and fission and are coupled to mitochondrial breakdown through mitophagy (Andreux et al., 2013; Gao et al., 2014; Liesa and Shirihai, 2013; Youle and van der Bliek, 2012). We previously showed that inhibiting mitochondrial translation promotes mitochondrial fission in worms (Houtkooper et al., 2013). Similarly, short-term exposure of Hepa 1-6, HeLa, or GT1-7 cell lines to doxycycline induced a striking balance toward mitochondrial fission and a more fragmented mitochondrial appearance, as reflected by TOM40 staining (Figures 2A–2C).

**Doxycycline Disrupts Global Cellular Transcriptional Profiles**

To examine how doxycycline impairs mitochondrial function and ignites global adaptive responses, we analyzed gene expression datasets generated following treatment of the human bladder cancer cell line RT112 with 1 \( \mu \text{g/ml} \) of doxycycline (Du et al., 2012). Overall, doxycycline treatment in the RT112 cell line altered the expression of 2181 genes (9.5% of total genes), including 142 mitochondrial protein-coding genes (8.9% of mitochondrial genes under GO accession: 0005739), of which 65.5% were significantly downregulated by doxycycline treatment (Figure 3A). We next performed a targeted gene set enrichment analysis (GSEA) focused on mitochondrial gene sets to explore the influence of doxycycline on mitochondrial pathways (Figures 3B and S2). Pathways that were robustly downregulated included mitochondrial transport, mitochondrial protein synthesis, mitochondrial membrane potential, ATP synthesis, and electron transport chain (Figures 3B–3F and S2). Of note, from the 124 mitochondria-related gene sets in analysis, many more mitochondrial gene sets were downregulated (55 of 124) than upregulated (0 of 124) in these human RT112 cells (Figure S2). Altogether, these results indicate that doxycycline leads to global repression of mitochondrial protein synthesis and function.
Doxycycline Impairs Development and Mitochondrial Function in Worms and Flies

To assess the longer term effect of doxycycline in intact organisms, we switched to the nematode C. elegans and the fruit fly D. melanogaster, commonly used models for functional genomic studies. In worms, we measured oxygen consumption using the Seahorse XF96 respirometer as a physiological proxy for metabolic activity (Houtkooper et al., 2013). Indeed, supplementing doxycycline to worms during their development from egg to adulthood (4 days) led to a dose-dependent reduction of oxygen consumption (Figure 4A). These doses also delayed worm development, leading to a higher percentage of worms staying in larval phases after 4 days of treatment (Figure 4B), and an overall smaller appearance (Figure 4C). Despite the lowered oxygen consumption, doxycycline-treated worms moved more than untreated worms (Figure 4D). Even though both treated and untreated worms displayed decreasing mobility after the third day of adulthood, doxycycline-treated worms maintained significantly higher mobility throughout life (Figure 4D), which is consistent with our previous findings that inhibition of mitochondrial translation—using mps-5 RNAi—prevents age-related decline in physical fitness (Houtkooper et al., 2013).

In flies, doxycycline supplementation in the food led to a significant decrease in oxygen consumption, doxycycline-treated worms moved more than untreated worms (Figure 4D). Even though both treated and untreated worms showed similar decrease in oxygen consumption, doxycycline-treated worms moved more than untreated worms (Figure 4D). Even though both treated and untreated worms displayed decreasing mobility after the third day of adulthood, doxycycline-treated worms maintained significantly higher mobility throughout life (Figure 4D), which is consistent with our previous findings that inhibition of mitochondrial translation—using mps-5 RNAi—prevents age-related decline in physical fitness (Houtkooper et al., 2013).
Doses that are found in soil of agricultural fields (Bowman et al., 2011; Xie et al., 2011). This effect became even more apparent after transferring the doxycycline-treated plants into soil for 7 days and watering with 25 μg/ml of doxycycline (Figure 5B). Furthermore, a closer observation of the root hairs in the maturation region shows that gradually increasing the doxycycline concentration inhibits root hair elongation (Figure 5C). To examine whether plants share the same mechanisms as worms and flies in response to doxycycline treatment, we measured oxygen consumption in Arabidopsis seedlings. Leaves from doxycycline-treated seedlings displayed a marked reduction in oxygen consumption (Figure 5D), which was accompanied by changes in the expression of the stress response genes AOX1A (AT3G22370), CPN10X (AT1G23100), HSC70-5 (AT5G09590), and BCS1 (AT3G50930) (Figure 5E). AOX1A (alternative oxidase 1a) is a plant-specific quinol oxidase that is activated when complex IV function is inhibited and may play an antioxidant role in plant mitochondria by the reduction of oxygen into water without proton translocation (Jacoby et al., 2012). CPN10X (putative 10-kDa chaperonin), HSC70-5 (heat shock protein 70), and BCS1 (cytochrome B1 synthesis) are all molecular chaperones involved in mitochondrial stress response in plants (Van Aken et al., 2009). Similarly, at the protein level, plants that are exposed to doxycycline display marked mitochondrial protein imbalance (mtDNA-encoded CoxII versus nDNA-encoded AtpB) and activation of the UPRmt marker HSC70 (Figure 5F). Importantly, exposing plants to doxycycline did not apparent after transferring the doxycycline-treated plants into soil for 7 days and watering with 25 μg/ml of doxycycline (Figure 5B). Furthermore, a closer observation of the root hairs in the maturation region shows that gradually increasing the doxycycline concentration inhibits root hair elongation (Figure 5C). To examine whether plants share the same mechanisms as worms and flies in response to doxycycline treatment, we measured oxygen consumption in Arabidopsis seedlings. Leaves from doxycycline-treated seedlings displayed a marked reduction in oxygen consumption (Figure 5D), which was accompanied by changes in the expression of the stress response genes AOX1A (AT3G22370), CPN10X (AT1G23100), HSC70-5 (AT5G09590), and BCS1 (AT3G50930) (Figure 5E). AOX1A (alternative oxidase 1a) is a plant-specific quinol oxidase that is activated when complex IV function is inhibited and may play an antioxidant role in plant mitochondria by the reduction of oxygen into water without proton translocation (Jacoby et al., 2012). 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induce chloroplast-specific stress response, as the expression of chloroplast-specific chaperones PsaB and PsbA was unaltered upon doxycycline exposure (Figure 5G).

**Doxycycline Impairs Mitochondrial Proteostasis and Function in Mice**

We then tested whether the doxycycline effects are conserved in mice in vivo. We gave male C57BL/6J mice 50- or 500-mg/kg/day of doxycycline dissolved in their drinking water for 14 days. We used 50-mg/kg/day amoxicillin as a control since this antibiotic does not interfere with bacterial/mitochondrial translation but rather disrupts the bacterial cell wall. Similar to our results in cell lines, doxycycline dose dependently induced mitonuclear protein imbalance in different mouse tissues, including the liver, the heart, and the brain (Figures 6A–6C). We then performed a more detailed time course experiment to establish the dynamic induction of mitonuclear protein imbalance. While mitonuclear protein imbalance was apparent after 2 days of doxycycline treatment, the ratio between MTCO1 and SDHA became markedly increased at day 5 (Figure 6D).
Altogether, these experiments highlight the marked time- and dose-dependent regulation of mitochondrial proteostasis by doxycycline.

Finally, we analyzed the physiological consequences of doxycycline treatment in mice. The treatment did not affect body weight or food intake, suggesting no overt toxicity at these doses and within the treatment timeframe (Figures 7A and 7B). Nevertheless, respiration was reduced in mouse livers (Figure 7C), which was associated with a depletion of ATP levels (Figure 7D) and changes in expression of different mitochondrial genes (Figure 7E), together demonstrating inhibition of mitochondrial function. Of note and in support of the significant mitochondrial fragmentation (Figure 2), we observed a decrease in the expression of genes involved in fusion, such as Opa1 and Mfn2, with a concomitant increase in the expression of Fis1 involved in fission (Figure 7E).

At the whole-body level, these short-term treatments did not affect body composition in either lean or fat mass between the amoxicillin and doxycycline groups (Figures 7F and 7G). The decreased oxygen consumption of doxycycline-treated mice, indicative for reduced energy expenditure (Figure 7H), and the marked increase in physical activity (Figure 7I) are, however, clear indicators for altered physiological fitness.

**DISCUSSION**

**The Use of Tetracyclines to Control Gene Expression Confounds Research**

Tetracyclines are a group of antibiotics that have found their way in biomedical research for their use in tetracycline-responsive control of gene expression. Such Tet-On/Tet-Off systems offer experimental flexibility that is often looked for, especially in cases where temporal control of gene expression is necessary for cell survival, and are therefore now widely used. However, tetracycline use is seldom questioned, even though classical literature describes tetracyclines as mitochondrial inhibitors (Clark-Walker and Linnane, 1966). Similarly, we identified widespread effects of the tetracycline, doxycycline, on mitochondrial morphology and function in worms and mammalian cells (Houtkooper et al., 2013). Worms furthermore lived longer upon low-dose doxycycline exposure, which was dependent on changes in mitochondrial proteostasis (Houtkooper et al., 2013). Even though the physiological outcome in this study was beneficial to worms, the potential confounding effects of doxycycline treatment are obvious.

Our cross-species data confirm a strong inhibitory effect of doxycycline on mitochondria in common cell lines, invertebrate animal models, mice, and plants. The fact that these changes occur in cells derived from different tissues, and in organisms across kingdoms confirms that the mechanisms underlying these alterations are highly conserved. Also, these effects occurred at doses that are typically used in the literature to control gene expression. Admittedly, some experimentalists use a doxycycline-treated control groups, but even in this context, doxycycline may induce adverse effects on mitochondria. We here confirmed this effect in reanalyzing a global transcriptomics dataset (Du et al., 2012). Doxycycline treatment in the RT112 cell line altered the expression of almost 10% of genes. These changes, even if just considered a mild stress, can sensitize cells and animals to the Tet-On/Tet-Off, inducing a “two-hit” model, where the effect of Tet-controlled gene expression in the nucleus is confounded by and is occurring in a sensitized transcriptional milieu (Figure 7J). In fact, the stress inflicted by tetracyclines on the mitochondria induces a retrograde response—with signaling from the mitochondria to nucleus (Liu and Butow, 2006; Rhoads and Subbaiah, 2007). The phenotypic changes observed are hence the result of the synergistic actions of doxycycline and LOF or GOF of the gene of interest, rather than the singular effect of the gene of interest itself.

We observed a phenotypic imprint of doxycycline at several levels of organization. At the molecular and cellular level, doxycycline inhibits bacterial, but also mitochondrial translation. As a consequence, the 13 oxidative phosphorylation subunits encoded by mtDNA are not properly expressed. This likely leads to unstable oxidative phosphorylation complexes and an adaptive state we previously termed mitonuclear protein imbalance (Houtkooper et al., 2013). Exposure to doxycycline indeed induced mitonuclear protein imbalance in cultured cells but also in tissues from mice treated with this antibiotic. Mitonuclear protein imbalance is the trigger for the UPR mt, an adaptive stress response in mitochondria that involves the upregulation of mitochondrial chaperones and proteases (Jovaisaite et al., 2014). In fact, proper UPR mt activation is essential for the lifespan

**Figure 6. Doxycycline Inhibits Mitochondrial Function In Vivo in Mice**

(A–C) Doxycycline (at 50 and 500 mg/kg/day in the drinking water) induces mitonuclear protein imbalance in liver (A), heart (B), and brain (C), as shown by the reduced ratio between mtDNA-encoded MTCO1 and nDNA-encoded SDHA when compared with amoxicillin (at 50 mg/kg/day) after 14 days of treatment.

(D) Doxycycline at 500 mg/kg/day in the drinking water induces mitonuclear protein imbalance in the liver, as evaluated by the ratio of MTCO1/SDHA expression after 5 days. After 2 days of treatment, the effect is still discrete.

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extension observed in long-lived mitochondrial worm mutants (Durieux et al., 2011; Houtkooper et al., 2013). Concomitant with the induction of mitonuclear protein imbalance, we observed striking adaptive changes in global gene expression profiles of doxycycline-treated cells. Even though this state—mitonuclear protein imbalance and UPRmt—is adaptive and protective when induced early in life (Durieux et al., 2011; Houtkooper et al., 2013), we now show that prolonged or high-dose activation can in fact be maladaptive and harmful for homeostasis.

Tetracyclines Impair Organismal Physiology and Have a Major Environmental Impact

The effects of tetracyclines are also evident at the physiological level of the entire organism. In worms, flies, and plants, treatment with doxycycline led to a marked reduction and delay of growth, and oxygen consumption was severely impaired. The latter was also observed in mice, both using isolated livers and in vivo using indirect calorimetry in metabolic cages. Additionally, both flies and worms displayed reduced fertility. Interestingly, doxycycline treatment also caused increased mobility across organisms. Although the physiological reason for this change is not completely understood, research has previously shown that low-level mitochondrial stress and UPRmt were associated with improved muscle function and fitness in worms and flies (Houtkooper et al., 2013; Owusu-Ansah et al., 2013).

The results we obtained in plants are a reflection of one of the potential environmental challenges that tetracyclines pose at the ecological level. According to Food and Drug Administration (FDA) reports, tetracycline antibiotics account for about 41% of the ~13.74 million kg of antibiotics sold in 2011 in the United States for use in livestock (versus ~3.3 million kg of antibiotics used per year in humans in the United States) (FDA, 2011). More critically, a survey in 2007 estimated that almost half of the 210-million kg of antibiotics produced in...
China per year were used in animal feed and that these antibiotics are mostly tetracyclines (Hvistendahl, 2012). While plants are naturally away from tetracyclines, the increased use of these antibiotics in humans and especially livestock has led to widespread contamination of water and soil resources (Mathews and Reinhold, 2013), increasingly exposing plants to these toxins. As a consequence, tetracyclines accumulate in soils of agricultural fields at concentrations that are comparable to the concentrations we used in our study and that cause a major growth delay in A. thaliana (Bowman et al., 2011; Xie et al., 2011). Even though the extent of ecological disturbance is poorly understood, our data warrant further exploration in this direction.

Conclusions and Perspectives
Taken together, our results call for extreme caution when using tetracyclines for research purposes. This is particularly true if metabolism is the phenotype that is being studied. It is, however, not restricted to these applications since mitochondria have been implicated in a range of processes, including neurodegeneration, immunology, and cancer (Andreux et al., 2013). The widespread effects of antibiotics on mitochondrial function need also to be taken into account in clinical practice. Recently, much attention has been drawn to the relevance of microbiota in this respect, as the complete microbiome is estimated to be ten times larger than the number of bacterial cells in a human body (10^{14} bacteria versus 10^{13} cells), possibly explaining why early life exposure to antibiotics severely impacts metabolic traits through disruption of microbial homeostasis (Cho et al., 2012). One should keep in mind, however, that tetracyclines and some other antibiotic classes also inhibit the mitochondria—to be considered as bacteria within our cells—the population of which approximately exceeds the number of bacterial cells by a further order of magnitude (10^{15} mitochondria), thus providing a strong platform for adverse effects.

EXPERIMENTAL PROCEDURES

Cell Experiments
The human cervical carcinoma HeLa, the human embryonic kidney HEK293, and the mouse hepatoma Hepa 1-6 cell lines were obtained from ATCC. Mouse hypothalamic GT1-7 cell lines were kindly provided by Dr. Pamela Melon (UCSD) (Wetsel et al., 1991). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂/95% air in DMEM supplemented with nonessential amino acids and 10% fetal bovine serum without antibiotics. Doxycycline (Sigma-Aldrich) and amoxicillin (Mepha) were dissolved in water. Treatments were performed in DMEM complete medium without antibiotics, which was re-energized four times during 10 min at 37°C in the respiration chamber; values are normalized by the total number of cells in the chamber.

D. melanogaster Experiments
The Drosophila melanogaster strain w^{1} was provided by the Bloomington Drosophila Stock Center (University of Indiana). Flies were kept at 25°C, 60% humidity, and on a 12/12-hr light-dark cycle. The food was supplemented with doxycycline at the indicated concentration.

Developmental observation was performed with synchronized eggs. Two- to 3-day-old flies were placed in a grape-agar medium housing tube supplemented with yeast paste. After 24 hr, tubes containing the nonsynchronized eggs were replaced by new ones. Synchronized eggs were collected on the new tube after 22 hr. After washing in 1 x PBS, 220 eggs were placed in each tube. Pictures were taken from day 4 (third instar larvae) and following. The number of pupae were recorded.

C. elegans Experiments
C. elegans movement was recorded for 45 s at different times of adulthood using a Nikon DS-L2/DS-F1 camera and controller setup, attached to both a computer and a standard bright-field microscope. Five plates of worms, with ten worms per plate, were measured in each condition. Fly movement was recorded by placing flies in a sealed chamber, tapping the chamber, and recording their movement as they naturally climb toward the top. For the tapping test, flies at the corresponding ages were recorded using a standard SLR camera with a Leica macro objective. The experiment was performed three times for each cohort with 1-min recordings each, with a “tap” sending the flies to the bottom of the chamber every 10 s.

Using these video recordings, the movement traces of both worms and flies during all recording periods were calculated by following the organism centroids using a modified version of the Parallel Worm Tracker for MATLAB (Ramot et al., 2008). The distance covered during the recording periods was then averaged per plate and per condition.

A. Thaliana Experiments
Arabidopsis (Col-0) seedlings were cultured on 1/2 MS-agar medium (Caissonlabs) for 7 days. Doxycycline treatment was initiated by transferring the seedlings to 1/2 MS medium supplemented with different doxycycline concentrations of 0.5, 1, 5, 10, 15, and 25 μg/ml. Plants were grown in a growth chamber at 22°C with a 16/8-hr light-dark cycle, 100-μmol s⁻¹ m⁻² light intensity. Morphological phenotypes were recorded after 7 days of treatment. Root hair images were taken by using an Olympus AX70 microscope with a 4 x objective; other parameters include sensitivity of ISO200, manual exposure time of 1/128 s, and gray scale. Some of the seedlings were transferred into soil and watered with 25-μg/ml doxycycline three times per day to observe the longer effect of treatment.

Mouse Experiments
Male C57BL/6J mice at 8 weeks old were treated for 2, 5, and 14 days with 50- or 500-mg/kg/day doxycycline (Sigma-Aldrich) or 50-mg/kg/day amoxicillin (Mepha) in drinking water. As doxycycline is bitter, we supplemented the water for all the conditions (treatments and controls) with 50-g/l sucrose. Drinking water was changed every 48 hr. Indirect calorimetry to monitor O₂ consumption, CO₂ production, and measurement of activity was measured using Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments) (Lagouge et al., 2008). Mouse experiments were performed in accordance with Swiss law and institutional guidelines.

Western Blotting
Proteins were extracted from liver and cells in protein extraction buffer containing 25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS with added protease inhibitor cocktail (Roche). We used 20 μg of total protein lysate to detect mitochondrial protein imbalance. Antibodies against MITOF1, SDHA (both from Abcam), and β-tubulin (Santa Cruz) were used for immunoblotting.

Respiration Assays
For cultured cells, liver tissue homogenates, and flies, oxygen consumption was measured using the Oxygraph-2k (Oroboros Instruments). Cells are trypan-ized, counted, and resuspended in complete DMEM medium. Basal respiration was measured four times during 10 min at 37°C in the respiration chambers; values are normalized by the total number of cells in the chamber. Liver tissues and flies were homogenized with a pestle and mortar in the MIR05 respiration buffer (0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1-g/l BSA, 5 mM pyruvate, 2 mM malate, 10 mM glutamate, 2.5 mM ADP) at 4°C. Basal respiration was measured four times during 10 min at 37°C in the respiration buffer.
for worms, oxygen consumption was measured using the Seahorse XF96 (Seahorse Bioscience) as described (Mouchiroud et al., 2013). Typically, 100 animals were recovered from NGM plates with M9 medium, washed three times to eliminate residual bacteria, and resuspended in 200-μl M9 medium. Worms were transferred in 96-well Seahorse plates (ten worms per well), and oxygen consumption was measured six times. Respiration rates were normalized to the number of worms in each individual well. Doxycycline treatments were performed at the indicated concentration.

In the dark, leaf discs (10–15 mg) were first suspended in 2 ml of plant respiration buffer (Sew et al., 2013) and incubated in the dark for 30 min. Oxygen consumption rate (OCR) for 15 min after input was recorded. The OCR was calculated accordingly to the fresh weight (FW) of the leaf discs.

**Quantitative Real-Time PCR for mRNA**

For mouse tissues, total RNA was prepared using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA was treated with DNase, and 1 μg of RNA was used for reverse transcription (RT); 20x diluted cDNA was used for qRT-PCR reactions. The qRT-PCR reactions were performed using the Light-Cycler system (Roche) and a LightCycler QNtiTect reverse transcription kit (QIAGEN) with the indicated primers. Total RNA from plant samples was prepared using TRIzol as previously described (Connolly et al., 2006). 2 μg of RNA was used for RT using QuantiTect reverse transcription kit (QIAGEN). 20x diluted cDNA was used for qRT-PCR reactions using the Light-Cycler system (Roche) and a LightCycler 480 SYBR Green I Master mix (Roche). Ubiquitin gene UBQ10 (AT4G05320) was quantified by qRT-PCR reactions using the Light-Cycler system (Roche) and a qPCR Supermix (QIAGEN) with the indicated primers. Total RNA from plant samples was prepared using TRIzol as previously described (Connolly et al., 2006).

**ACKNOWLEDGMENTS**

We thank Professor Extranyat (EPHE) for helpful discussions. J.A. is the Nestlé Chair in Energy Metabolism. Work in the J.A. laboratory is supported by the École Polytechnique Fédérale de Lausanne, the NIH (R01AG043930), the Swiss National Science Foundation (31003A-124713), and Systems X (51RT00-151019). L.M. is supported by an FRM fellowship, and R.H.H. is supported by a VENI grant from ZonMW (#91613050).

**REFERENCES**


**AUTHOR CONTRIBUTIONS**

N.M., L.M., R.H.H., and J.A. conceived and designed the project. N.M. performed the cellular and mouse experiments. L.M. performed qRT-PCR experiments. X.W. performed the Arabidopsis experiments and Gene Set Enrichment Analysis, and M.V.F. performed and analyzed experiments. L.M., R.H.H., and J.A. wrote the manuscript with contributions from all other authors.


