In vivo high-resolution magic angle spinning proton NMR spectroscopy of *Drosophila melanogaster* flies as a model system to investigate mitochondrial dysfunction in *Drosophila GST2* mutants

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Received July 15, 2013; Accepted September 3, 2013

DOI: 10.3892/ijmm.2014.1757

Abstract. *In vivo* nuclear magnetic resonance spectroscopy (NMR), a non-destructive biochemical tool used for investigating live organisms, has recently been performed in studies of the fruit fly *Drosophila melanogaster*, a useful model organism for investigating genetics and physiology. We used a novel high-resolution magic angle-spinning (HRMAS) NMR method to investigate live *Drosophila GST2* mutants using a conventional 14.1-T NMR spectrometer equipped with an HRMAS probe. The results showed that, compared to wild-type (wt) controls, the *GST2* mutants had a 48% greater (CH_2)n lipid signal at 1.33 ppm, which is an insulin resistance biomarker in *Drosophila* skeletal muscle (P=0.0444). The mutants also had a 57% greater CH_2C= lipid signal at 2.02 ppm (P=0.0276) and a 100% greater -CH=CH- signal at 5.33 ppm (P=0.0251). Since the -CH=CH- signal encompasses protons from ceramide, this latter difference is consistent with the hypothesis that the *GST2* mutation is associated with insulin resistance and apoptosis. The findings of this study corroborate our previous results, support the hypothesis that the *GST2* mutation is associated with insulin signaling and suggest that the IMCL level may be a biomarker of insulin resistance. Furthermore, direct links between *GST2* mutation (the *Drosophila* ortholog of the *GSTA4* gene in mammals) and insulin resistance, as suggested in this study, have not been made previously. These findings may thus be directly relevant to a wide range of metabolically disruptive conditions, such as trauma, aging and immune system deficiencies, that lead to increased susceptibility to infection.

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Abbreviations: Ac, acetate; *akhr*, adipokinetic hormone receptor; β-Ala, β-alanine; CPMG, Carr-Purcell-Meiboom-Gill; Cho, choline; EMCLs, extramyocellular lipids; FFA, free fatty acids; HRMAS, high-resolution magic angle spinning; IMCLs, intramyocellular lipids; Lip, lipids; PUFA, poly-unsaturated fatty acid; TGA, triglycerides; wt, wild-type

Key words: magnetic resonance spectroscopy, high-resolution magic angle spinning, *Drosophila melanogaster*, *GST2* mutation, biomarkers, insulin signaling, insulin resistance, apoptosis

Introduction

High-resolution magic angle spinning (HRMAS) nuclear proton magnetic resonance spectroscopy (^{1}H NMR) is a novel non-destructive technique that substantially improves spectral line-widths and allows high-resolution spectra to be obtained from intact cells, cultured tissues (1,2) and unprocessed tissues (3-7). HRMAS ^{1}H NMR enables us to investigate relationships between metabolites and cell processes. For example, choline (Cho)-containing compounds involved in phospholipid metabolism and lipids, such as triglycerides, that are involved in apoptosis have been studied (8-11). Nevertheless, HRMAS ^{1}H-NMR has only been performed *ex vivo* thus far.

Studies combining *in vivo* ^{1}H NMR with *ex vivo* HRMAS ^{1}H NMR have demonstrated an important functional role of intramyocellular lipids (IMCLs) in rodent burn biology (11,12), while other *ex vivo* HRMAS ^{1}H NMR studies have focused on lipid metabolism (13). Szczepaniak *et al* demonstrated that IMCL stores could be quantified accurately in a clinical setting by *in vivo* ^{1}H NMR (14). In a recently published ^{1}H

NMR study, Van der Graaf *et al* found an inverse correlation between IMCL content in human calf muscle and local glycogen synthesis rate (15). Jacob *et al* emphasized the importance of these resonances as biomarkers of insulin resistance in type-2 diabetes patients and their offspring (16). Additionally, IMCL content was found to be increased in the soleus muscle of insulin-resistant elderly patients, providing support for the hypothesis that an age-associated decline in mitochondrial function contributes to insulin resistance (17).

I*n vivo* HRMAS ^{1}H NMR is a potentially useful tool in *Drosophila* since *in vitro* NMR studies have shown the metabolic effects of hypoxia (18) and temperature stress (19) in flies. Although *Drosophila* is a distinctively useful model organism that can be employed to investigate genetics, physiology, and metabolism (20), with the exception of a recent feasibility report (21), *in vivo* NMR studies in *Drosophila* are lacking. Thus, we attempted to implement an *in vivo* HRMAS ^{1}H NMR method that we developed in *Drosophila* (22), with the aim of investigating the metabolism of *Drosophila* mutants. Such a study would be particularly useful for assessing the biomarkers of pathophysiology with the long-term goal of providing critical information that may direct novel therapeutic development.

State-of-the art, *in vivo* NMR techniques are used to elucidate metabolic patterns in *Drosophila melanogaster* as a model organism of interest owing to the notable parallels in the metabolism between *Drosophila* and mammals (23,24). Indeed, the study of *Drosophila* metabolism is an emerging field that can potentially elucidate conserved metabolic mechanisms. Furthermore, the powerful genetic tools available in *Drosophila* research render the fruit fly a particularly tractable model organism in which to probe metabolic pathways and lead to a better understanding of human metabolic disorders.

Drosophila melanogaster glutathione S-transferase (GST2, also known as DmGSTS1-1) was recognized originally as an indirect flight muscle-associated protein with no known catalytic properties. In relation to mammalian GSTs, *Drosophila* GST2 is most similar to the sigma class of GSTs, and the mammalian *GSTA4* gene is an ortholog of *Drosophila GST2*. In the present study, we investigated mutant flies that do not express the *GST2* gene in skeletal muscle. We examined the feasibility of a novel, *in vivo* HRMAS ^{1}H NMR approach towards the investigation of the metabolic derangements in these *GST2* mutant flies and compared them to isogenic control flies.

Materials and methods

Drosophila flies. Male *Gst2* gene deletion flies (25), designated as GstS1M38 were used, and compared to male wild-type (wt) isogenic strain C5 flies. The two strains were kindly provided by Helen Benes (University of Arkansas). At the time of the experiments, all flies were 5-8 days of age and weighed 0.7-1.0 mg (n=6 per group). Prior to insertion in the spectrometer, each fly was anesthetized by being placed on ice for <1 min. Flies were kept at 4˚C while in the spectrometer.

In vivo HRMAS ^{1}H NMR spectroscopy. All HRMAS ^{1}H NMR experiments were performed on a wide-bore Bruker Bio-Spin Avance NMR spectrometer (600.13 MHz) using a 4-mm triple resonance (^{1}H, ^{13}C, ^{2}H) HRMAS probe (Bruker, Billerica, MA, USA). The flies were placed into a zirconium oxide rotor tube (4 mm diameter, 50 μl), and 8 μl of external standard trimethylsilyl-propionic-2,2,3,3-d4 acid (TSP) (molecular mass = 172 Da, d = 0.00 ppm, 50 mM in D_2O) was introduced. TSP functioned as a reference for both resonance chemical shift and quantification. Each fly was placed in the rotor using the insert, which was sealed with a screw and covered with parafilm to prevent contact between the fly and the TSP/D_2O (Fig. 1). The samples were secured and tightened in the rotors with a top cap (Bruker). The HRMAS ^{1}H NMR was performed at 4˚C with 2 kHz MAS.

One dimensional (1D) water-suppressed spin-echo Carr-Purcell-Meiboom-Gill (CPMG) pulse sequencing [90˚ - (τ - 180˚ -τ)$_n$ - acquisition] (26) was performed on single flies. CPMG is a methodological improvement of particular interest in developing *ex vivo* 1D HRMAS of intact tissue samples since it suppresses broad signals that destroy the linear baseline in typical Free Induction Decay (FID) spectra. Thus, the CPMG proton NMR spectra are free from the broad component that contributes to the baseline of simple FID spectra. The CPMG sequence has also been applied to two-dimensional sequences for the same reason.

Additional parameters for the CPMG sequence included an inter-pulse delay of $\tau = 2\pi/\omega_r = 250$ msec, a total spin-echo delay of 30 msec, two total 180˚ cycles, 256 transients, a spectral width of 7.2 kHz, 32,768 (32k) data points, and a 3-sec relaxation time. A spin-echo delay of 30 msec was chosen based on the observation that at this echo time, line broadening without loss of signal from triglycerides was avoided. When the spin-echo delay was increased, all the lipid signals were affected, but not in favor of other metabolites.

In vivo ^{1}H HRMAS NMR data processing. MR spectra of specimens were analyzed using MestReC software (Mestrelab Research, www.mestrec.com). A 0.5-Hz line-broadening apodization function was applied to CPMG HRMAS ^{1}H FIDs prior to Fourier transformation. MR spectra were referenced with respect to TSP at δ = 0.0 ppm (external standard), manually phased, and a Whittaker baseline estimator was applied to subtract the broad components of the baseline.

Quantification of metabolites from 1D ^{1}H CPMG HRMAS spectra. For metabolite quantification from 1D ^{1}H CPMG HRMAS spectra, we used the highly accurate 'external standard' technique. Metabolite concentrations were calculated using MestReC software. An automated fitting routine based on the Levenberg-Marquardt (27,28) algorithm was applied after manual peak selection; peak positions, intensities, line widths, and Lorentzian/Gaussian ratios were adjusted until the residual spectrum was minimized. Metabolite concentration (mol/kg) was calculated using the equation (29): $mass_{TSP}/PM_{TSP}$ x $Met_{(area)}/TSP_{(area)}$ x N_{TSP}/N_{Met} x $1/wt_{(sample)}$, where $mass_{TSP}$ was constant (0.069 mg), PM_{TSP} was the molar mass of TSP (172.23 g/mol), Met signifies metabolites, N_{TSP} was the TSP proton number (9 ^{1}H), N_{Met} was the metabolite proton number, and $wt_{(sample)}$ was the sample weight in mg (29).

Statistical analysis. Group data were compared with the Student's t-test. A P-value of 0.05 (corrected) was accepted as significant and all P-values are reported to two significant digits. Calculations were performed using SPSS (SPSS 12, SPSS Inc).

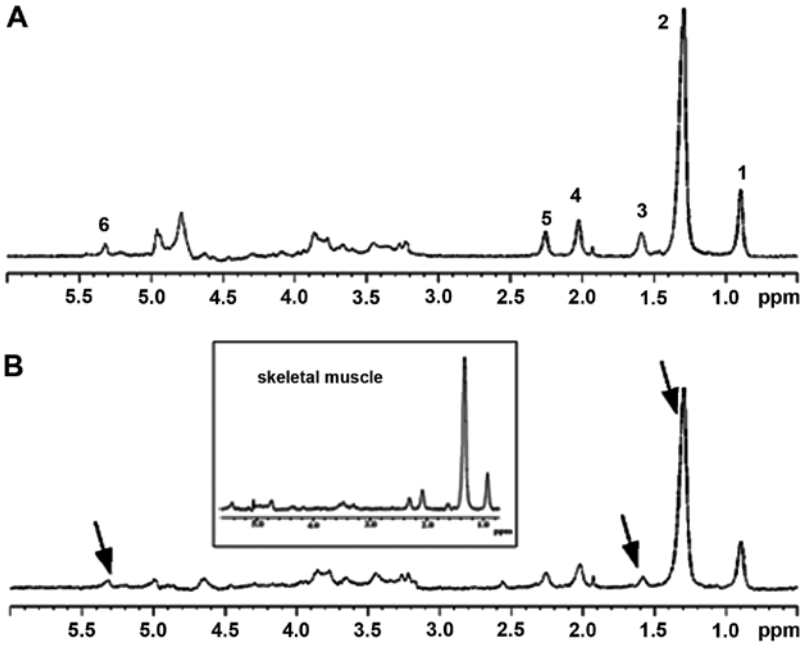

Figure 1. *In vivo* 1D HRMAS ^{1}H CPMG spectra of: (A) *GST2* and (B) young wt flies. Lipid components were: 1, CH_3 (0.89 ppm); 2, (CH_2)n (1.33 ppm, putative IMCLs); 3, CH_2C-CO (1.58 ppm, putative EMCLs), acetate (Ac, 1.92 ppm); 4, CH_2C=C (2.02 ppm); 5, CH_2C=O (2.24 ppm); and 6, CH=CH (5.33 ppm). Other spectral components included: β-alanine (β-Ala, 2.55 ppm), phosphocholine (PC, 3.22 ppm), phosphoethanolamine (PE, 3.22 ppm), and glycerol (4.10, 4.30 ppm 1,3-CH; 5.22 ppm 2-CH_2). The spectra in the inset are from the thorax of dissected flies and thus represent primarily skeletal muscle. Note the similarity of spectra for the dissected and whole flies. The spectra shown were normalized to TSP at each echo time and therefore do not exhibit a T2 decay. HRMAS, high-resolution magic angle spinning; wt, wild-type; IMCLs, intramyocellular lipids; EMCLs, extramyocellular lipids.

Results and Discussion

In the present study, we detected and quantified lipids and small metabolites in live *Drosophila* using ^{1}H HRMAS NMR at 14.1 T (Fig. 1) (30). All the flies survived the procedure, which was completed in ~45 min per fly. Our results confirmed our expectations in that we were able to reduce acquisition time, and thereby achieve zero mortality. We employed a novel *in vivo* HRMAS ^{1}H NMR approach in *Drosophila* to examine the hypothesis that the *GST2* mutation results in insulin resistance, due to a phylogenetically conserved pathway for the regulation of glucose and lipid metabolism between flies and mammals (31,32).

Drosophila was utilized in this study because, relative to other animal models, flies are inexpensive and easy to maintain and manipulate, and they have a well-known genome as well as numerous available mutants. These characteristics make *Drosophila* an ideal genetically amenable model organism with which to investigate the physiology of biomedical paradigms. To this end, invertebrate *Drosophila* models have already provided powerful experimental systems for muscle developmental biology investigations (33-35), age-related decline in function (36), such as neurodegeneration (37) and loss of immune (38,39) and cardiac (40) functions, and specifically, regarding muscle degeneration, for the investigation of protein synthesis (41,42), sarcomere integrity (43-45), apoptosis (46), mitochondrial function and morphology (44,45,47-50), stress response (48,51), glycogen content (45), muscle function and morphology (52,53), flight ability (54) (flight) myofiber stiffness and power (44), and protein modifications (55,56) and related transcriptional changes (48,57,58). The conservation of insulin signaling between flies and mammals (31) renders *Drosophila* a particularly interesting model organism for metabolism studies. The focus of the present study on *Drosophila* as a model organism distinguishes this study from traditional metabolism experiments. The findings of this study are supported by findings in mammals showing evidence of insulin resistance and mitochondrial dysfunction in *mGsta4* null mice (59).

The *in vivo* fly spectra (see representative spectra in Fig. 1) of this study compare well to other published *in vivo* skeletal muscle spectra (11,60,61). All of these studies have shown high amounts of lipids in skeletal muscle, particularly triglycerides. Other HRMAS reports involving skeletal muscle showed spectra with more metabolites than those of the present study (8,62). The samples and set conditions in our experiments differed from those of prior studies in that we had a smaller quantity of sample (0.6-1.1 mg) and we performed the experiment with a lower spin rate, which may have limited spectral resolution. The NMR-visible non-lipid components are expected to contribute only a small percentage in the total signal from sample flies, which are of extremely small size (0.7-0.8 mg total body weight), with concomitantly low sensitivity of detection. Even spectra from the thorax of dissected flies, which is highly enriched in skeletal muscle, are similar to whole fly spectra (inset of Fig. 1). Nevertheless, we were still able to detect certain metabolites from the 1D experiment (Fig. 1).

From a biomedical perspective, the principal finding of our experiments was that the *GST2* mutation was associated with an accumulation of mobile lipids in muscle tissue. The quantitative data of selected components (triglycerides) detected in live *Drosophila* with HRMAS ^{1}H NMR are summarized in Table I. Fig. 2 (30) shows a bar graph of the amounts of the same selected components. There was a marked and signifi-

Table I. Quantity of selected lipid components in live *Drosophila* according to ^{1}H HRMAS NMR (n=6/group).

		Mean quantity ± SE (μmol/g)			
Peak no. (ppm)[a]	Lipid	wt	*GST2*	% difference	P-value
1 (0.89)	CH_3	0.12±0.02	0.17±0.03	+41.7	0.1342
2 (1.33)	$(CH_2)_n$	0.67±0.09	0.99±0.13	+47.7	0.0444[b]
3 (1.58)	CH_2CCO	0.03±0.01	0.07±0.02	+133.3	0.0748
4 (2.02)	$CH_2C=C$	0.07±0.01	0.11±0.01	+57.1	0.0276[b]
5 (2.24)	$CH_2CO=O$	0.05±0.01	0.05±0.02	0	0.4676
6 (5.33)	CH=CH	0.02±0.004	0.04±0.005	+100	0.0251[b]

^{1}H HRMAS NMR, ^{1}H high resolution magic angle spinning nuclear magnetic resonance spectroscopy; [a]Chemical shifts are in parts per million (ppm). [b]Statistically significant comparison. P-value was determined using the Student's t-test; wt, wild-type.

cant increase in (CH_2)n (1.33 ppm) in the mutants relative to the wt controls. Additionally, we observed a trend towards more CH_2C-CO lipids (1.58 ppm) in the mutants (Table I).

Although determining the source of these accumulated lipids is beyond the scope of this study, it should be considered that extramyocellular lipids (EMCLs), IMCLs, and triglycerides can all contribute to cellular lipid peaks (14,63,64). Specifically, EMCLs and IMCLs can be distinguished by *in vivo* NMR by differences in their bulk magnetic susceptibilities and geometric arrangements (65), with 1.33 ppm lipids, (CH_2)n, being attributed to IMCLs and 1.58 ppm lipids, CH_2C-CO, being attributed to EMCLs. However, discrimination is not likely in the present study. Spinning a sample at the magic angle (HRMAS) with respect to the static field direction averages the second-order tensors of the anisotropic chemical shift, the dipolar interaction, and the susceptibility variations in heterogeneous samples (66-68). Garroway (67) indicated that MAS eliminates the broadening effect produced by magnetic susceptibility, and eliminates the shift itself. In their study, Chen *et al* (69) clarified that, irrespective of system geometry, MAS eliminates only the anisotropic contribution of bulk susceptibility inside a homogeneous susceptibility region. Inspecting the isotropic part of the susceptibility tensors available for IMCLs and EMCLs (63,70), we can deduce that IMCLs and EMCLs have an identical chemical shift under MAS conditions due to bulk susceptibility.

IMCLs probably serve as an energy substrate for oxidative metabolism (71), and can be mobilized and utilized with turnover times in the range of several hours (72). In insects, triglycerides are located in body fat. Triglycerides in insect body fat (73-75) are used for storage of both energy and fatty acid precursors, such as transported lipids, phospholipids (membrane structure), hydrocarbons, and wax esters (that minimize water loss from the cuticle due to evaporation) (76). In our study, mobility of fat body contents may have been affected by trauma or immune status, leading to strong IMCL and EMCL signals (77). However, this suggestion is only hypothetical as the intracellular signaling cascade mediating mobilization of triglycerides has not been as fully elucidated in insects as it has in mammals (78). Nevertheless, we suggest that there was mobilization of triglycerides in the *GST2*$^{-/-}$ flies because the peaks indicative of triglycerides at 1.33 and 1.58 ppm were increased (79). The significantly greater triglyceride signals

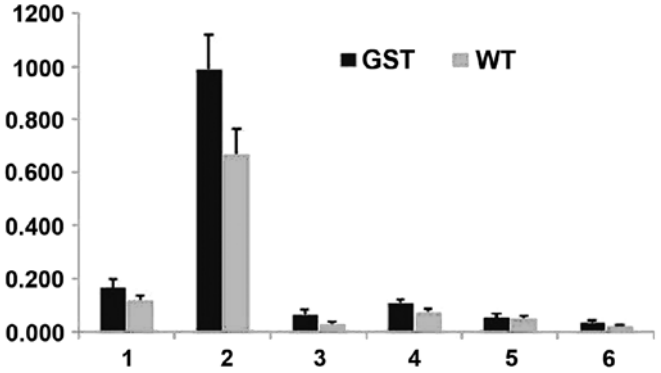

Figure 2. Lipid quantities calculated from *in vivo* 1D HRMAS ^{1}H CPMG spectra of young wt flies (light gray) and *GST2* mutant flies (black). 1, CH_3 (0.89 ppm); 2, (CH_2)n (1.33 ppm) or IMCLs; 3, CH_2C-CO (1.58 ppm) or EMCLs; 4, CH_2C=C (2.02 ppm); 5, CH_2C=O (2.24 ppm); 6, CH=CH (5.33 ppm). HRMAS, high-resolution magic angle spinning; wt, wild-type; IMCLs, intramyocellular lipids; EMCLs, extramyocellular lipids.

(both IMCLs and EMCLs) detected in the *GST2*$^{-/-}$ mutants vs. wt controls (Figs. 1 and 2, Table I) resembles the metabolic profile of *akhr* flies with an obese phenotype and abnormal accumulation of both lipids and carbohydrates (79). Specifically, elevated IMCL levels are associated with insulin resistance, a major metabolic dysfunction of diabetes, aging (80,81), burn trauma (82,83) and obesity (84-87).

Moreover, our observations of increased peaks indicative of triglycerides at 1.33 ppm in *GST2*$^{-/-}$ flies agree with prior findings in *chico* flies (79) with disrupted insulin signaling. *Chico* flies have a mutated insulin receptor substrate (IRS) gene, a *Drosophila* homolog of vertebrate IRS1-4. *Chico* flies have a small stature and show abnormally high triglyceride levels (88,89) that are attributable to a dysfunctional mutated insulin signaling pathway (31), resulting in insulin resistance. The high 1.33 ppm peak in *chico* flies is clearly due to IMCLs and not to EMCLs since these flies are not obese. Accordingly, *chico* flies do not exhibit significantly increased 1.58 ppm peaks, which are frequently attributed to EMCLs (79). Thus, despite the theoretical considerations of HRMAS, it remains likely that the lipids that produce the peak at 1.33 ppm are primarily IMCLs, whereas the lipids that yield a peak at

1.58 ppm are primarily EMCLs. Thus, *chico* flies are a suitable comparison strain for *GST2* flies, which also exhibit increased triglycerides, evidently due to increased IMCLs since they are not obese, and thus not expected to have increased EMCLs. Conversely, *akhr* flies exhibit a metabolic profile with significantly increased peaks in all assigned lipids, agrees with their obese phenotype (79).

Another principal finding of our experiments was that peaks 4 ($CH_2C=C$ at 2.02 ppm) and 6 (CH=CH at 5.33 ppm), which includes protons from ceramide, were also significantly increased in the mutant flies compared to wt controls (Table I and Fig. 2). Ceramide accumulation decreases insulin-stimulated GLUT4 translocation to the plasma membrane and, consequently, reduces glucose transport (90), resulting in insulin resistance. Paumen and co-workers demonstrated that saturated fatty acids such as palmitoleic acid at 2.02 ppm in our study, induce *de novo* synthesis of ceramide and programmed cell death (90). They suggested that inhibition of carnitine palmitoyltransferase I activity induces both sphingolipid synthesis and palmitate-induced cell death. Ruddock *et al* (91) suggested that long-chain saturated fatty acids (palmitoleic acid C16:0) attenuate insulin signal transduction in hepatoma cell lines. Their study suggests that an increase in palmitoleic acid signifies insulin resistance. If this is the case, then the signal at 2.02 ppm in our study may also be a biomarker of insulin resistance; this peak was elevated in our *GST2*$^{-/-}$ flies ($CH_2C=C$ at 2.02 ppm, peak 4 in Figs. 1 and 2) and in *chico* flies (79).

From a biomedical perspective, the findings of this study support the hypothesis that the *GST2* mutation is associated with insulin signaling and suggest that the IMCL level may be a biomarker of insulin resistance in *GST2*$^{-/-}$ flies. However, whether IMCLs are directly involved in the development of insulin resistance simply serve as an indirect marker is currently a topic of debate (92). Insulin resistance has not been demonstrated previously in flies with currently available assays. Furthermore, direct links between *GST2* mutation (the *Drosophila* ortholog of the *GSTA4* gene in mammals) and insulin resistance, as suggested in this study, have not been made previously. The common characteristics shared among innate immunity activation, obesity, and insulin resistance, as recently described (79), support the findings of this study.

In conclusion, findings of the present study have demonstrated that a novel solid-state HRMAS NMR method is a sensitive tool for the molecular characterization of metabolic perturbations in *Drosophila*. We observed increased levels of triglycerides in *GST2*$^{-/-}$ *Drosophila* mutant that may be indicative of insulin resistance. These findings may thus be directly relevant to the mitochondrial dysfunction that occurs in a wide range of metabolically disruptive conditions, such as trauma, aging, and immune system deficiencies, that lead to elevated susceptibility to infection. Our findings advance the development of novel *in vivo* non-destructive research approaches in *Drosophila* strains, offers biomarkers to investigate biomedical paradigms, and thus may direct novel therapeutic development.

Acknowledgements

This study was supported in part by a grant from DM103014 of the Defense Medical Research and Development Program (DMRDP) to Laurence G. Rahme (A. Aria Tzika, co-investigator). We would like to thank Professor Helen Benes of the University of Arkansas for Medical Sciences for providing the *Drosophilas* used in this study. We would also like to thank Dr Ann Power Smith (Write Science Right, Las Vegas, NV, USA) for editorial assistance.

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