Analysis of the effects of mitochondrial mutator system in *Drosophila melanogaster*

Mikael Parhiala

Advanced Thesis

University of Tampere

Institute of Biomedical Technology

Mitochondrial Disease Group

Tampereen Yliopisto

IBT

FinMIT, Howard T Jacobsin tutkimusryhmä

Joona Teo *Mikael* Parhiala: Mitokondriaalisen mutaattorin analysointi *drosophila melanogasterissa*.

Kirjallinen työ, s.20

Ohjaaja: Professori Howard T. Jacobs

Elokuu 2015

Avainsanat: Mitokondrio, banaanikärpänen, mutaatio, restriktioentsyymi, EcoBI

Mitokondrio on solujen aerobisen energian tuotannon kannalta tärkeä kaksi kalvoinen soluorganelli. Sen sisällä tapahtuva sitruunahappokierto tuottaa elektroneja elektronin siirtoketjuun, jota tarvitaan oksidatiiviseen fosforylaatioon. Nämä elektronit kulkevat mitokondrion sisäsolukalvolla tiettyjen proteiinikompleksien I, II and IV läpi joiden koentsyymeinä toimii ubikinoni ja sytokromi C. Tämä johtaa pelkistysreaktio sarjaan, jossa elektronien siirto aiheuttaa elektrokemiallisen protonigradientin solukalvojen väliseen tilaan. Gradientin potentiaalista energiaan käyttäen ATP-syntaasi proteiini tuottaa ATP:ta.

Pieni osa elektroneista vuotaa kompleksista I ja II ja reagoivat hapen kanssa muodostaen reaktiivisia happiradikaaleja, jotka reagoidessaan aiheuttavat kumulatiivisesti muun muassa mutaatioita mitokondrio DNA:han ja ovat osittain vastuussa vanhenemisesta. Nykyinen tieto näyttää, että vaikka happiradikaalit voivat vahingoittaa DNA:ta ja johtaa mutaatioihin, tämä ei ole verrannollinen happiradikaalien määrään. Tämä korostaa mitokondriaalisen DNA:n huollon merkitystä vanhenemisessa. Mitokondriaalinen DNA eroaa nukleaarisesta DNA:sta sen äidillisen periytyvyyden kautta ja sen suuresta kopiomäärästä per solu, joka voi johtaa tilanteeseen, jossa yhdessä solussa on mutaation kautta eri genomia sisältävää mitokondriaalista DNA:ta. Mitokondrion mutaatioilla on yhteyksiä moniin neurologisiin ja metabolisiin sairauksiin.

Ei ole olemassa hyvää työkalua muokata mitokondriaalista DNA:ta *in vivo*, joiden avulla voitaisiin ymmärtää mitokondriaalisia mutaatiota paremmin. Ryhmämme alkoi tutkimaan EcoBI tyypin 1 restriktioentsyymikompleksia, joka voi saada aikaan kahden ketjun katkoksia mitokondriaaliseen DNA:han. Mitokondrion korjaus mekanismin epätäydellisen luonteen vuoksi tämä altistaa mutaatioilla ja näin toimisi *in vivo* satunnaismutaattorina. EcoBI systeemiin on istutettu Drosophila melanogasterin (banaanikärpänen) mitokondrionaaliseen DNA:han ja risteytimme ne DJ-694-geeniaktivaattorin kanssa. Tämä promotoi EcoBI geeniä banaanikärpäsen lihaskudoksissa, johtaen lentokyvyn häviämiseen ja aikaiseen kuolemaan. Analysoimme kärpästen proteiinia westernblot-menetelmällä, DNA:n lukumäärää ja RNA:n expressioita kvantitatiivisella reaaliaikaisella PCR:llä. Kontrollina käytimme inaktiivista EcoBI R-alayksikköä omaavaa kärpästä.

Havaitsimme kompleksi I:n alayksikön määrän laskun naaras, että uros kärpäsillä, viitaten kompleksi I:n alentuneeseen määrään. Mitokondriaalisen DNA:n lukumäärässä havaitsimme selvän nousun. Mitokondrion geenieksprессiossa havaitsimme eroja useassa geenisssä, kontrolliryhmään verrattuna. Nämä ovat vasta alustavia vastauksia, jotka vahvistavat EcoBI:n kykyä vaikuttaa mitokondrion toimintaan ja ekspressioon.

Introduction

The mitochondrion is an energy ”power plant” present in nearly all eukaryotic organisms, which arose from a symbiotic relationship between a bacterium and a primitive eukaryotic cell. It is a double membrane organelle, with an intermembrane space and an inner space called the matrix. The inner membrane forms folded structures called cristae, giving the mitochondria a distinguishable structure visible under an electron microscope and increasing the surface area necessary for oxidative phosphorylation (OXPHOS). The mitochondria supplies the cell with adenosine triphosphate (ATP) by oxidative phosphorylation of adenosine diphosphate (ADP) through the action of the electron transport chain. This allows 15 times more ATP to be produced compared to anaerobic glycolysis per glucose molecule.

Glycolysis and inside the matrix the citric acid cycle provide electrons via coenzymes reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide $FADH_2$ for the electron flow required for OXPHOS. The citric acid cycle (also called tricarboxylic acid cycle or Krebs cycle) oxidates acetyl groups in acetyl coenzyme A through a series of reactions that involve transferring the acetyl group to oxaloacetate and forming citrate acid which is then oxidized in a series of steps back to oxaloacetate and CO_2. A complete cycle generates 3 NADH, $FADH_2$ and guanosine triphosphate (GTP) which transfers a phosphate group to ADP and forms ATP.

The acetyl groups are produced by catabolization of sugars through glycolysis, fatty acids via beta oxidation and amino acids. In the inner mitochondrial membrane the electrons from the citric acid cycle are passed through a series of protein complexes I, III and IV and electron transporters ubiquinone and cytochrome C. This results in a series of redox reactions using the flow of electrons to pump protons into the intermembrane space. Complex IV transports the electrons finally to oxygen. The pumping of protons creates an electrochemical proton gradient between the intermembrane space and mitochondrial matrix. This potential energy is used by the ATP synthase protein to form ATP from phosphate ions inside the matrix. (1, 2)

Small percentage of electrons do not follow the predisposed path through the electron transport chain (ETC) complexes and “leak” from complex I and complex III. They react with oxygen to form a superoxide radical, the first precursor molecule of highly reactive group of molecules called reactive oxygen species (ROS). The mitochondrial free radical theory of ageing claims that damage from mitochondrial ROS is the underlying cause of senescence in the living organisms (3, 4). The theory states that ROS damages accumulates throughout life span, causing among other things an

increase in mutations in mitochondrial DNA (mtDNA) which in turn will result in abnormal electron transport chain complexes with mutated subunits producing even higher amount of ROS, which leads to ageing.

Mitochondrion has an important role in the apoptosis of the cell, triggered by cell or DNA damage or signals from nearby cells. Apoptosis is from of programmed cell death (PCD) that provides a path for exterminating malign cells. Release of cytochrome C from matrix to cytosol triggers the downstream caspase cascade and activates PCD pathway. Mitochondria have also been implicated in tumorigenesis, where energy production is shifted in cancer cells from oxidative phosphorylation to glycolysis (Warburg effect) even in an aerobic environment, which might be caused by mtDNA damage. (5-8)

The mitochondrion respiratory chain consists of subunits mainly synthesized from nuclear DNA and some from mtDNA. Mitochondrial DNA defects can be linked to a number of diseases such as chronic progressive external ophthalmoplegia (CPEO), Kearns–Sayre syndrome, mitochondrial diabetes mellitus and mitochondrial deafness (9, 10). It also been proposed that preventation of mtDNA damage could be used for treatment in cardiovascular disease (10). In addition, because of its role as a nexus of metabolic regulation in cell, mitochondrial dysfunctions are linked to several other chronic and degenerative diseases (12, 13)

The mtDNA differs from nuclear DNA not only by its maternal inheritance but also by its copy number per cell (100-1000). This results in a phenomenon called heteroplasmy, where mutated mtDNA populations exist alongside wild-type mtDNA. In several cases the severity of symptoms of mitochondrial diseases are directly related to percentage of heteroplasmy – the higher the ratio of mutated mtDNA, the more severe the symptoms. Current data suggests that although ROS can damage DNA and lead to accumulation of mutations, the profile of mtDNA mutations accumulating during aging is not consistent with ROS damage. This suggests that mtDNA maintenance, not chemical damage by ROS, has a highly important role in damage accumulation during aging. For instance a mutated mouse model expressing mtDNA proof-reading deficient polymerase leads to increased point mutations and rapid ageing. (14) However this is not associated with increased ROS.

Therefore, proper maintenance of the mitochondrial genome is important to organismal homeostasis, ageing and a variety of disease states. *In vivo* modification of mtDNA would be an ideal tool to understand the process of mitochondrial dysfunction. However, no mtDNA mutagenesis system akin to approaches used to modify nuclear genome currently exists. Previous

attempts to change mtDNA in higher eukaryotes have not had a reliable delivery system, inefficient integration into mt genome and active negative selection against the modified mtDNA. Mitochondrial transformation *in vivo* has only been achieved for *Saccharomyces cerevisiae* (baking yeast), while delivery to mitochondria in mammalian cell cultures has been limited to nucleic acid analogues (15-17). Transformations by means of bacterial conjugation, electroporation and natural competency have been reported only to isolated organelles. (18-20).
A new approach has been explored for mtDNA manipulation wherein exogenous or mutated endogenous DNA-modifying enzymes are targeted to the mitochondrial matrix, allowing on-site manipulation of mtDNA within living organisms. Several Type II or tailored zinc-finger/TALEN nucleases have been used successfully to cleave mtDNA *in vivo*. (21, 22) However, such approach is limited by the fact that they cut at their recognition sequence only.

My project is associated with the research of postdoctoral fellow Priit Joers in Mitochondrial Disease and Gene Expression group titled "Design and development of a novel mitochondrial mutator system based on mitochondrially targeted EcoBI (mtEcoBI) restriction complex in *Drosophila melanogaster*." EcoBI is a type I restriction enzyme complex that can promote double strand breaks (DSB) at random positions throughout the Drosophila mitochondrial genome (contains three binding sites for EcoBI) due to its ability to translocate DNA template. It is proven that DSBs can stimulate recombination in mtDNA (23-25). The EcoBI multisubunit enzyme complex consists of three subunits (R, M and S) with stoichiometry of S1M2R2. The S subunit binds to the target site, the M methylates hemimethylated sites and the R is responsible of translocation and cleavage of DNA. Endogenous reapir mechanisms in *D.melanogaster* mitochondria can repair the EcoBI-inflicted double strand breaks to the mtDNA. However, due to the imperfect nature of repair process, spontaneous mutations will arise at these break points. We aim to generate an *in vivo* mitochondrial mutator system capable of random mutagenesis throughout the mitochondrial genome and utilize this system to advance our understanding of mitochondrial function.

The EcoBI system was targeted to the mitochondrial genome in the *D.melanogaster* by adding a mitochondrial targeting sequence from citrate synthase to original bacterial genes. The model organism *D.melanogaster* is ideal for our needs since it has a short lifespan, high reproduction rate and relatively cheap upkeep. Type I subunit genes were placed under the control of GAL4-

responsive expression system, which allowed us to have temporal and spatial control over the expression of EcoBI by using different GAL4-based Drosophila “drivers”. These are DNA constructs where GAL4 transcriptional activator is expressed from specific native promoters, thus activating EcoBI in the tissues and developmental stages where given promoter is functional A daGAL4 driver which gives a strong ubiquitous expression leads to the death of embryos and only a few larvae hatch but never develop into flies. So we chose DJ694-driver which becomes spontaneously activated in fly muscle at eclosion and in our case leads to the loss of flight ability and an early death before day 20. It reaches a peak by day 30 (26). The DJ694 is ideal since the muscle tissue has a lot of mitochondria and we are able to bypass lethality in larval stage and observe effects in fully developed postmitotic organism, therefore increasing the relevance to humans. We gathered our EcoBI expressing flies at different time points and analyzed there mtDNA amount and presence of complex I subunits on protein levels and expression of several mRNA-s of mitochondrial-encoded genes. We used a line expressing only the R subunit as a control for our studies.

Materials & methods

-*Drosophila melanogaster* husbandry

Fly food preparation and basic maintenance including the use of apparatus required for fly maintenance: the use CO_2 pads for narcosis and the microscope for handling and making new stocks of flies.

-RT-qPCR

I used *reverse-transcriptase quantitative polymerase chain reaction* (RT-qPCR) and *quantitative polymerase chain reaction* qPCR to analyze the expression of mtDNA and mtDNA copy number genes in the mtEcoBI expressing *D.melanogaster*. The qPCR amplifies and measures the DNA quantity at the same time using a SYBR Green dye that fluoresces when bound to the dsDNA. We extracted total DNA from flies and measured mtDNA copy number using primers for the mitochondrial gene cytochrome C oxidase (Complex IV) subunit II (COX2) and for nuclear RpL32 gene that served as a control. I had three biological replicates of each time point and ran the qPCR

using three experimental replicates. For the expression of mitochondrial genes measurements I used primer pairs for mitochondrial genes COX2, cytB, 16S and ND5.

The DNA was extracted and purified using a spin-column based method, where nucleic acids were separated from the rest of cellular constituents through binding to a silica membrane. I extracted the RNA using commercial TRI reagent designed for RNA isolation followed by alcohol precipitation. I then removed residual DNA by DNase treatment and extracted the RNA with phenol/chloroform. Finally I precipitated the RNA again using sodium acetate and ethanol, dissolving the RNA in RNase free water. To make RNase free water I mixed a 0.1% DEPC (Diethylpyrocarbonate) solution and let it stand overnight before autoclaving it.

RNA extraction protocol

1. Collect 30 females or 40 males into Eppendorf, put on ice
2. Add 200ul of Trizol, homogenize with plastic grinder
3. Add 800ul of Trizol, continue grinding
4. Incubate at room temperature for 5min, add 200ul of chloroform, shake for 15 sec and let stand at room temperature for 3min
5. Centrifuge 12.000 G at 4°C for 15min
6. Precipitate upper phase (0.6ml) with 0.5ml of ispropanol then mix
7. Incubate 10min RT°C and centrifuge 12.000G at 4°C for 10min
8. Wash pellet with 75% ethanol, spin 7500G for 5min and air dry for 5-10min.
9. Dissolve RNA pellet in 180ul of DEPC-treated water, keep 10min at 55-60°C, measure concentration spectrophotometrically at OD260

DNase treatment of RNA samples

1. Add 20ul of DNase buffer and 10 units of DNase I
2. Incubate 37°C for 1 hour
3. Extract RNA with 1 volume of phenol:chloroform:isoamyl alcohol
4. Invert the tube to mix well and centrifuge at max speed for 5min at 4°C
5. Add 1 volume of chloroform to upper phase and mix, spin as in step 4
6. Collect upper phase and precipitate RNA with follows:
    - Add 1/10 volume of 3M NaAc
    - Add 2.5 volume of 95% EtOh and incubate -20°C overnight
    - Recover RNA by centrifuging at 11.000 rpm for 10min at 4°C
    - Wash pellet with 500ul of 75% EtOH
    - Air dry pellet at RT°C for 5-10min
    - Re-dissolve the pellet in 100ul of DEPC water and measure concentration

– **Protein analysis**

I used Western blotting which involves protein separation in polyacrylamid gels following transfer to nitrocellulose membrane and probing with specific antibodies. The antibodies we chose were raised against NDUFS3, GAPDH and ATP5α, the latter two serving as controls. The NDUFS3 is a complex I nuclear subunit, there is no available mitochondrial subunit antibody for complex I in Drosophila but its expression should correlate with the expression of mitochondrial subunits, as nuclear subunits of ETC complexes roughly correlate with the available amount of mitochondrial

subunits. (27). Thus by following nuclear subunit levels we can draw conclusion on overall presence of complex I. The GAPDH is housekeeping control protein involved in glycolysis, which has been used extensively in molecular biology experiments as a cytosolic control. The ATP5a is an important subunit to the ATP-synthase aka. Complex V, it was used as marker for mitochondrial amount. We used secondary antibodies conjugated with peroxidase which will emit light through chemiluminescence reaction. The results were visualized using X-ray film. After the taking the X-ray film I used BioRad´s Quantity One software to quantify the western results. I had three biological replicates of each time point and ran two westerns using NDUFS3 and GAPDH antibodies and one copy of the ATP5α antibody.

Fly Western Protocal

1. Collect 35 flies into an eppendorf
2. Add 500ul of western homogenization buffer and crush with pestle
3. Incubate 30min on ice
4. Centrifuge at 1500 rpm at 4°C for 5min, collect the supernatant (~450ul)
5. Determine concentration with Bradford assay
6. Mix 100ul with 100ul 2xSDS loading buffer
7. Incubate at 95°C for 5min
8. Run gel (100V approximately 1 hour)
9. Dry blot and mark membrane with pencil
10. Shake for 1hr in Blocking solution
11. Add antibody 10 000 dilution with new Blocking solution
12. Probe with antibody at room temperature for 30min then overnight in 4°C and 30min at room temperature again.
13. Wash the membrane three times with buffer (TBS 0.05% Tween 20)
14. Shake the membrane for 15min three times changing the buffer every time
15. Add secondary antibody (10 000 dilution) and shake it for at least 1hour
16. Wash the membrane as in step 12 and 13
17. Add 6ml per membrane of ELC and wait for 5min before taking picture with x-ray film

Western homogenization buffer: 40ml

1.5% Triton X-100	600ul
Complete mini EDTA-free Protease Inhibitor	2 tablets
PBS	up to 40ml

2XSDS loading buffer:	20ml
130mM Tris-Cl, pH 8.0	2.6ml
20 % Glycerol	4ml
10% SDS	9.2ml

Bromophenol blue	2mg
2% DTT	400mg
dH20	4.2ml

10X Western Running Buffer 1l

0.25M Trizma base	30.3g
1.92M Glycine	144g
1% SDS	10g add last

Probing solution	250ml (For two membranes)
1xTBS	250ml
5% milk powder	12,5g
0.05% Tween 20	500ul (for 1litre)

Tris-buffered Saline (TBS) X10 1l

NaCl	80g
KCl	2g
Tris base	30g
Adjust pH to 7.4	

Western results

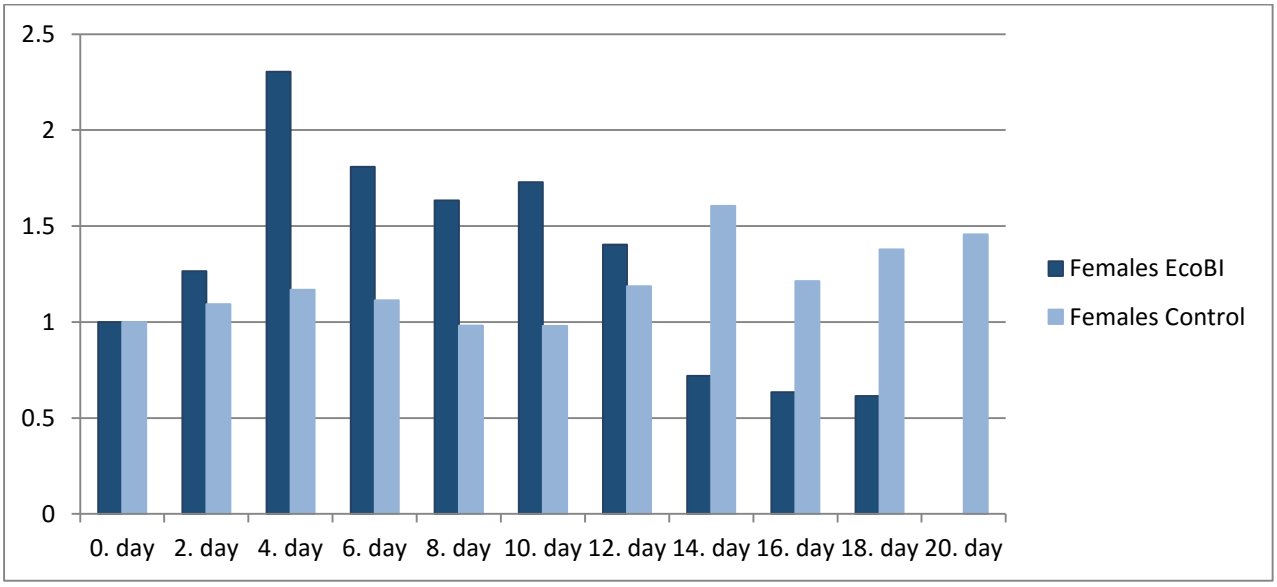

Fig.1 NDUFS3/ATP5α Western quantification females

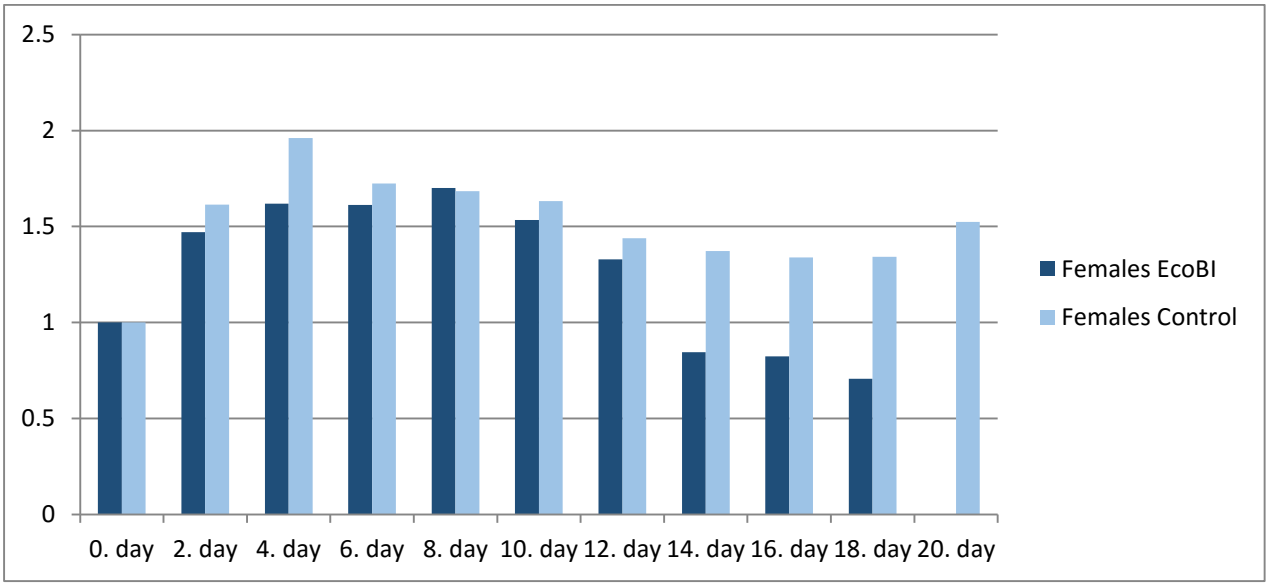

Fig. 2 NDUFS3/GAPDH Western quantification females

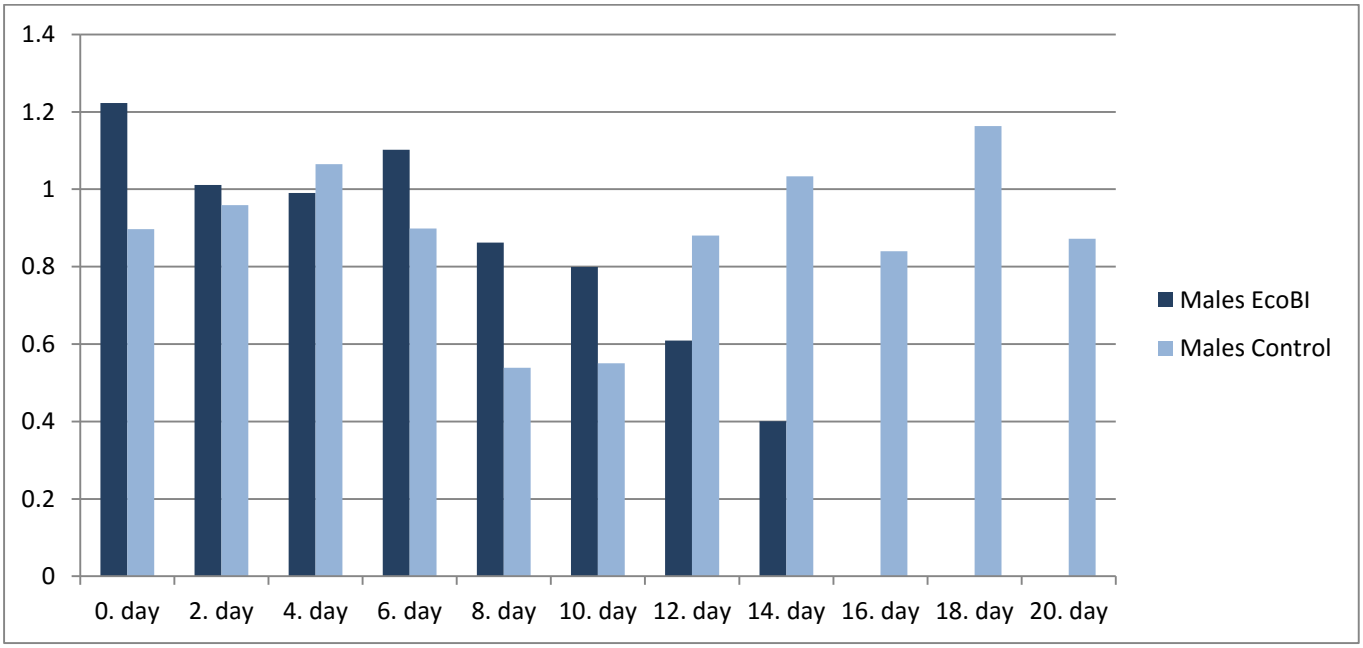

Fig.3 NDUFS3/ATP5α Western quantification males

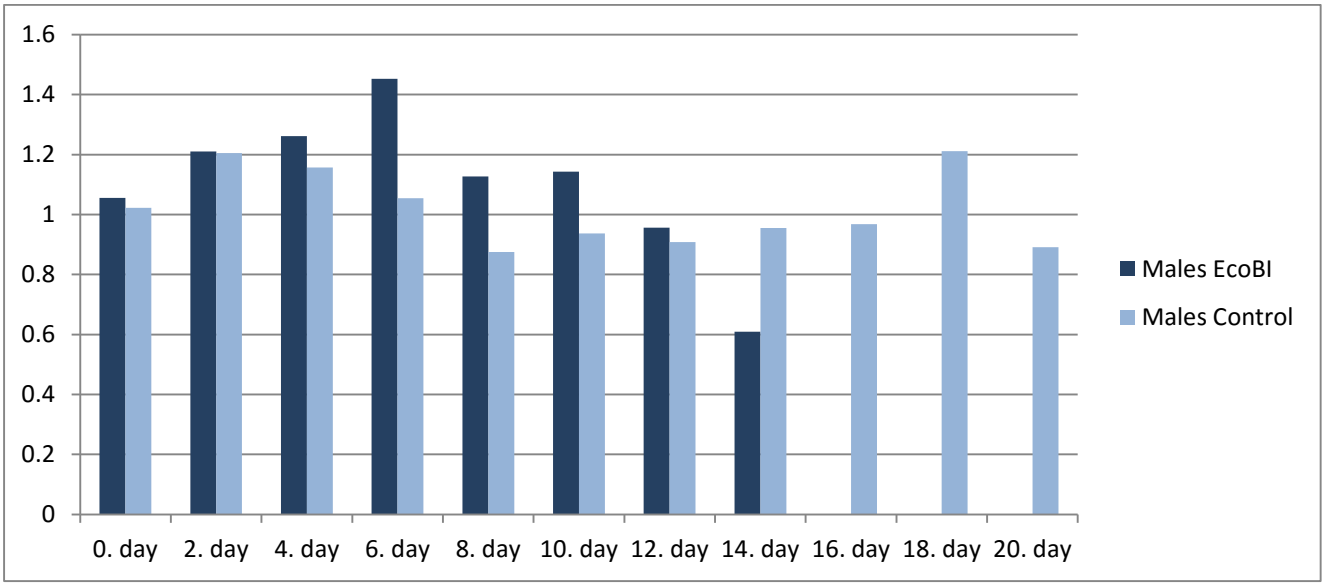

Fig. 4 NDUFS3/GAPDH Western quantification males

QPCR results

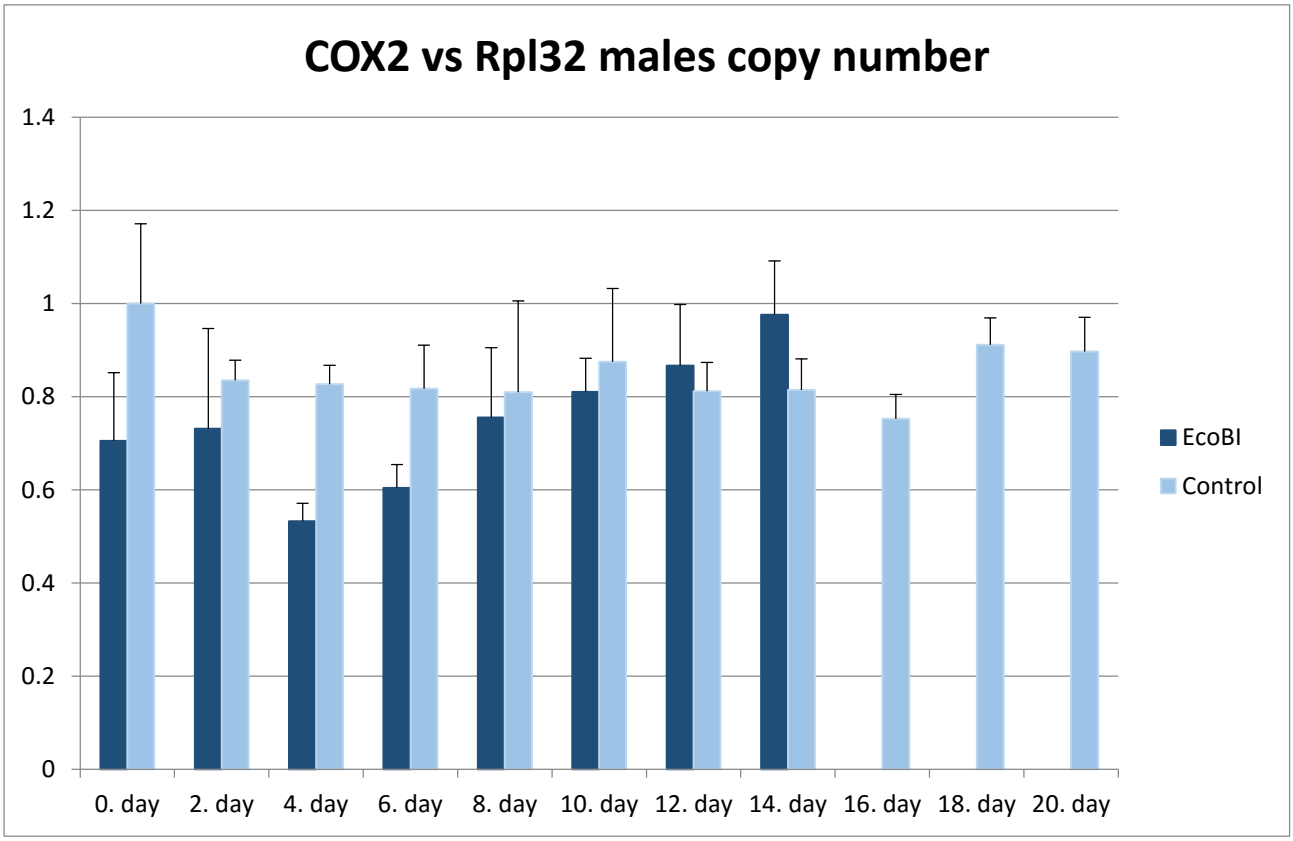

Fig. 5 DNA QPCR COX2 male copy number

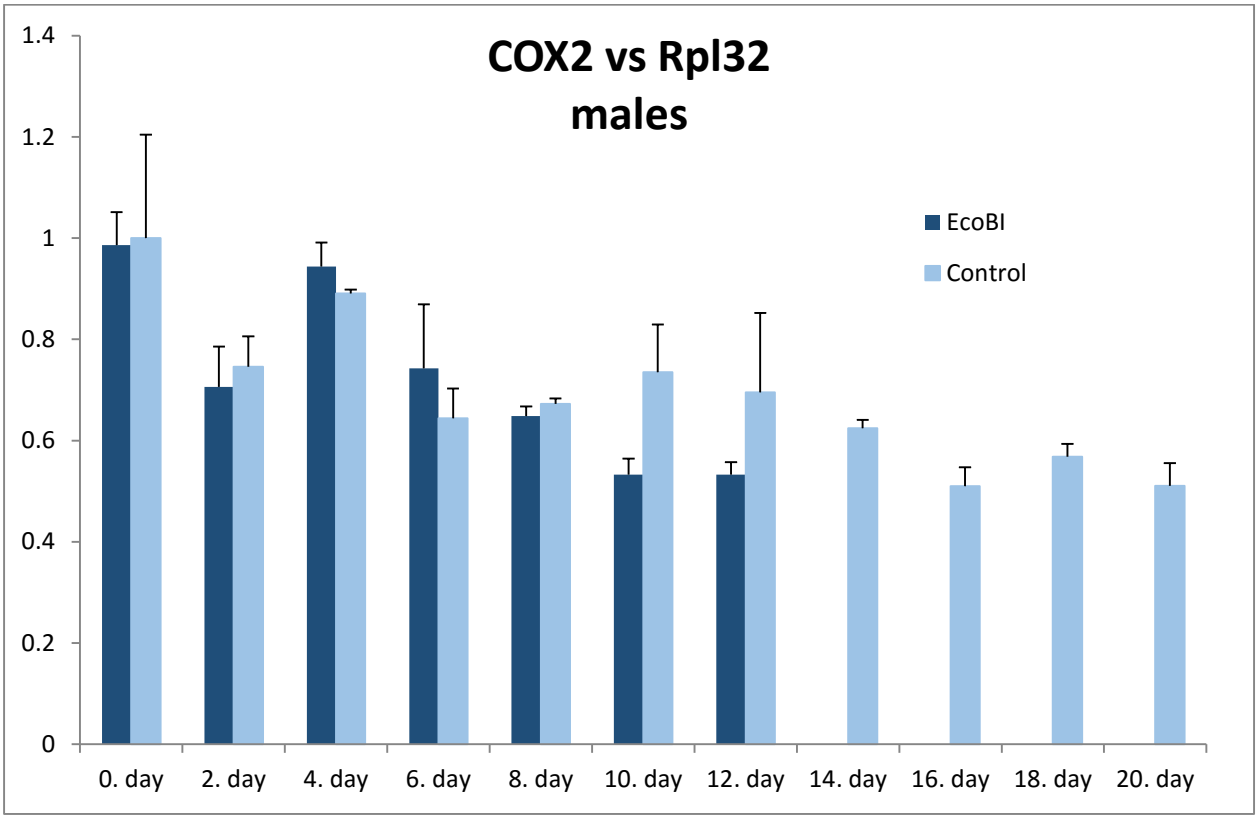

Fig.6 cDNA QPCR COX2 male expression

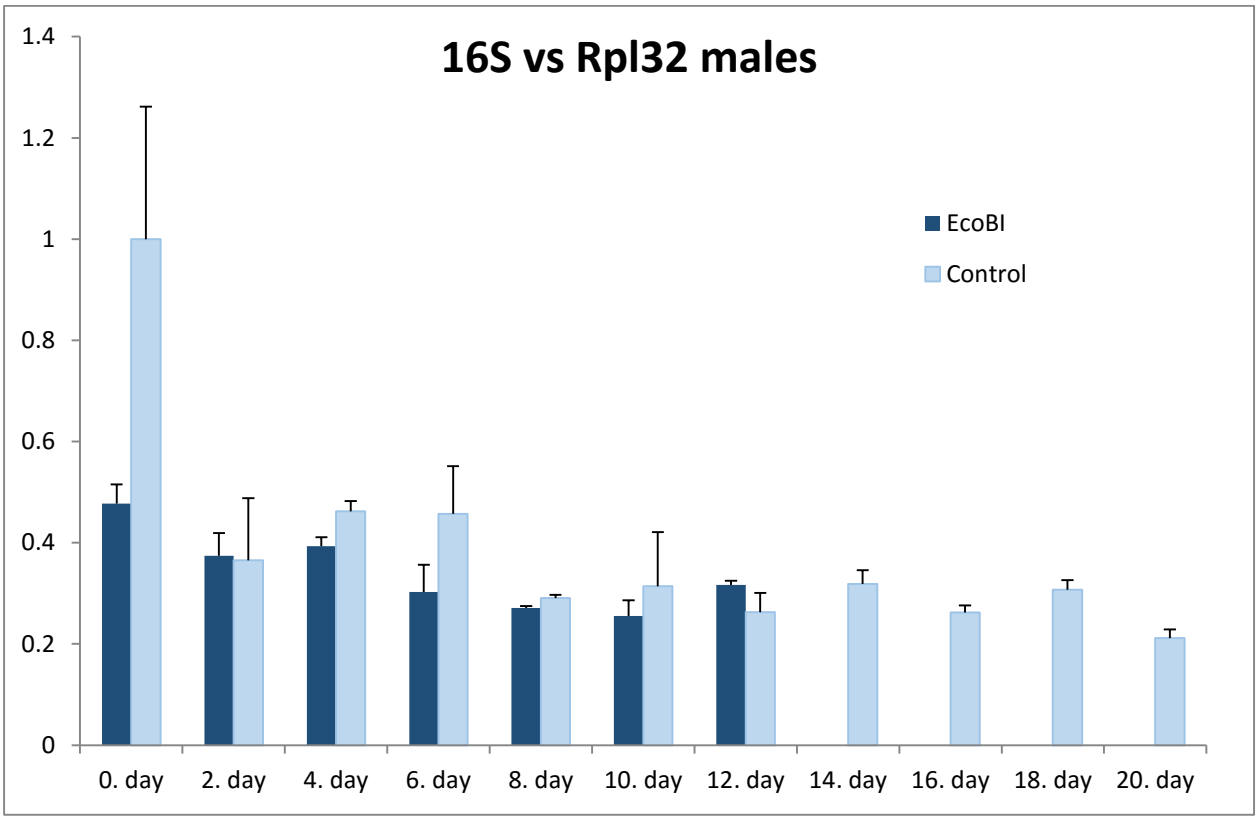

Fig. 7 cDNA QPCR 16S male expression

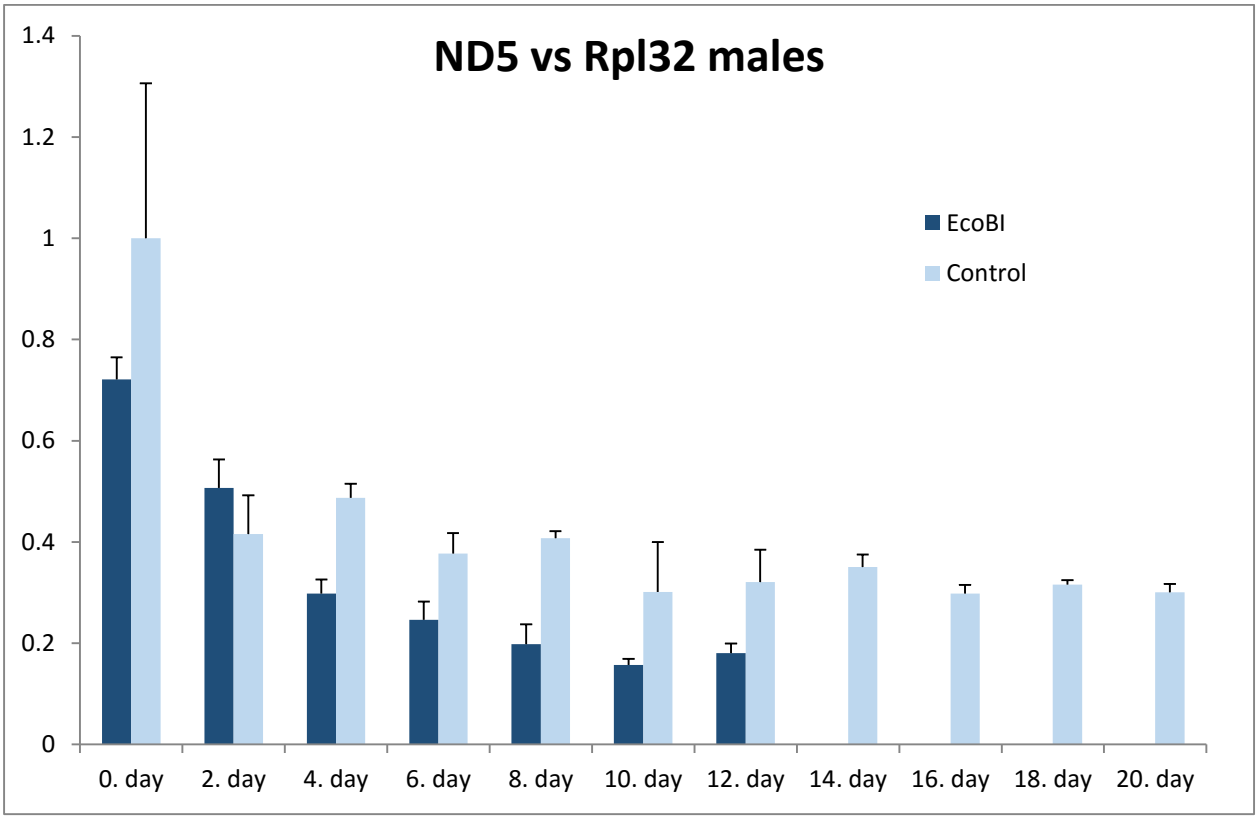

Fig.8 cDNA QPCR ND5 male expression

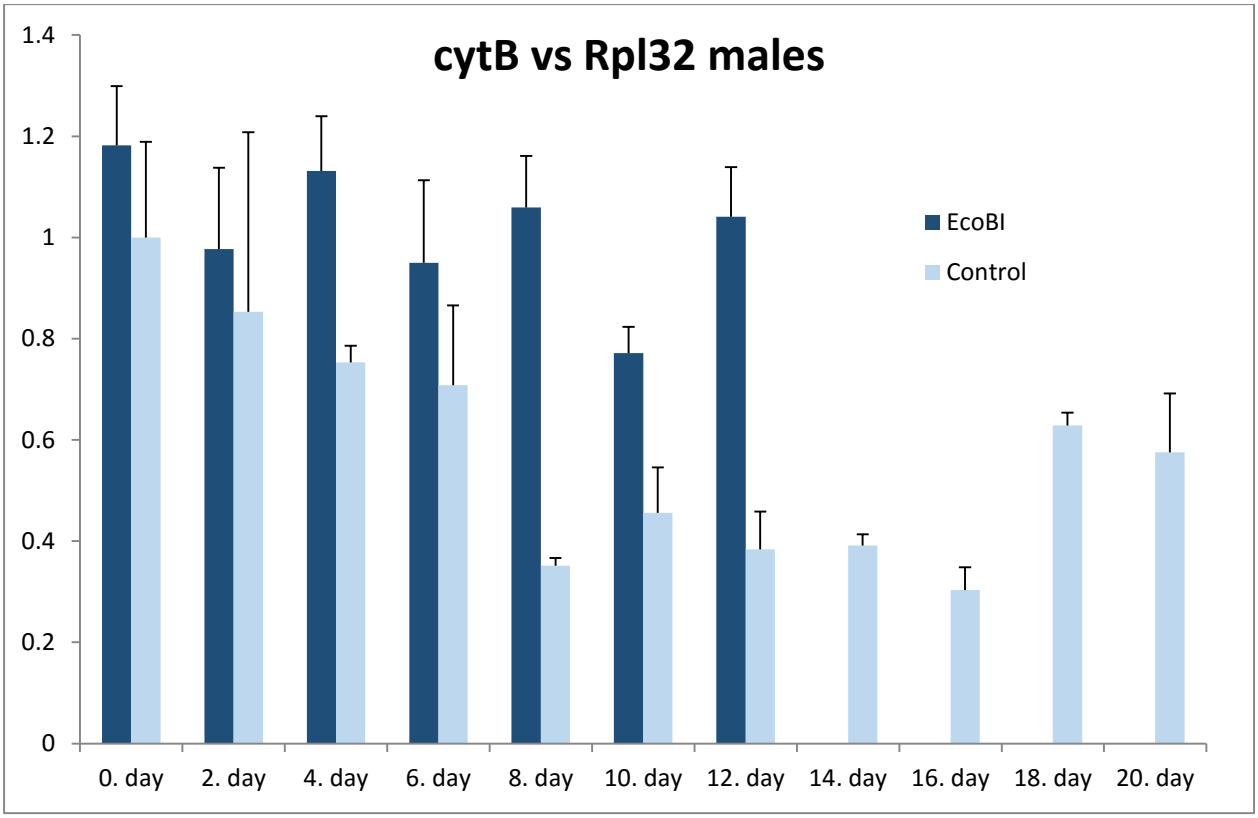

Fig.9 cDNA QPCR cytB male expression

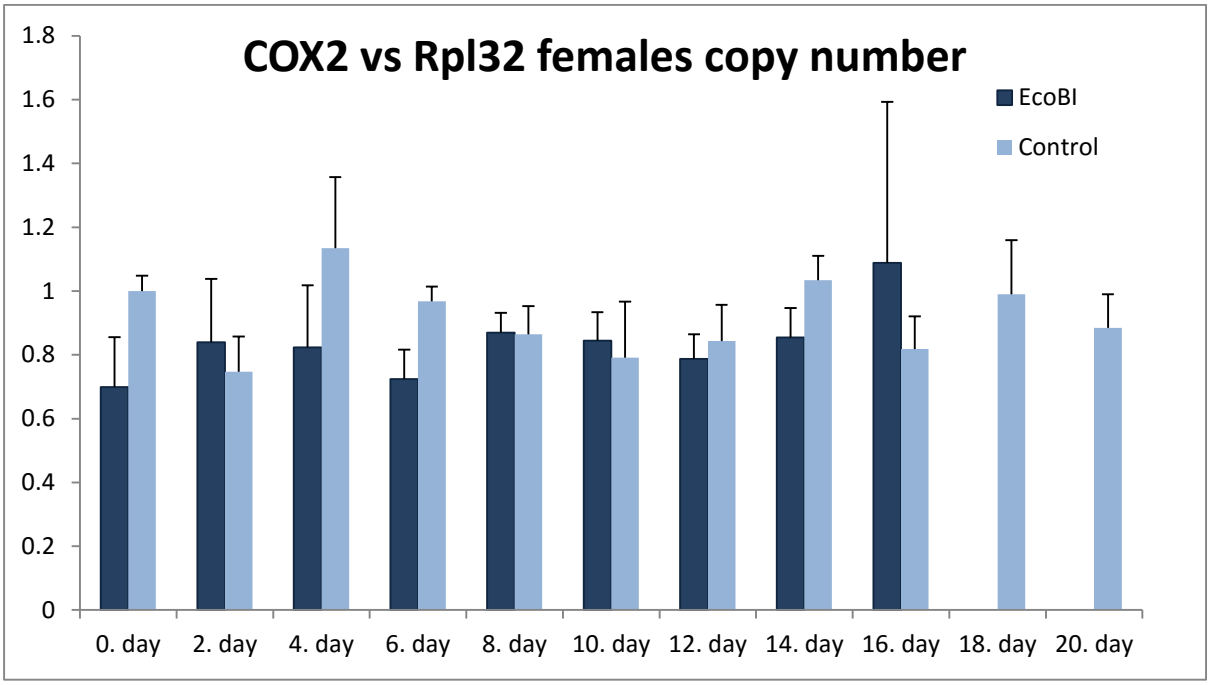

Fig. 10 DNA QPCR COX2 female copy number

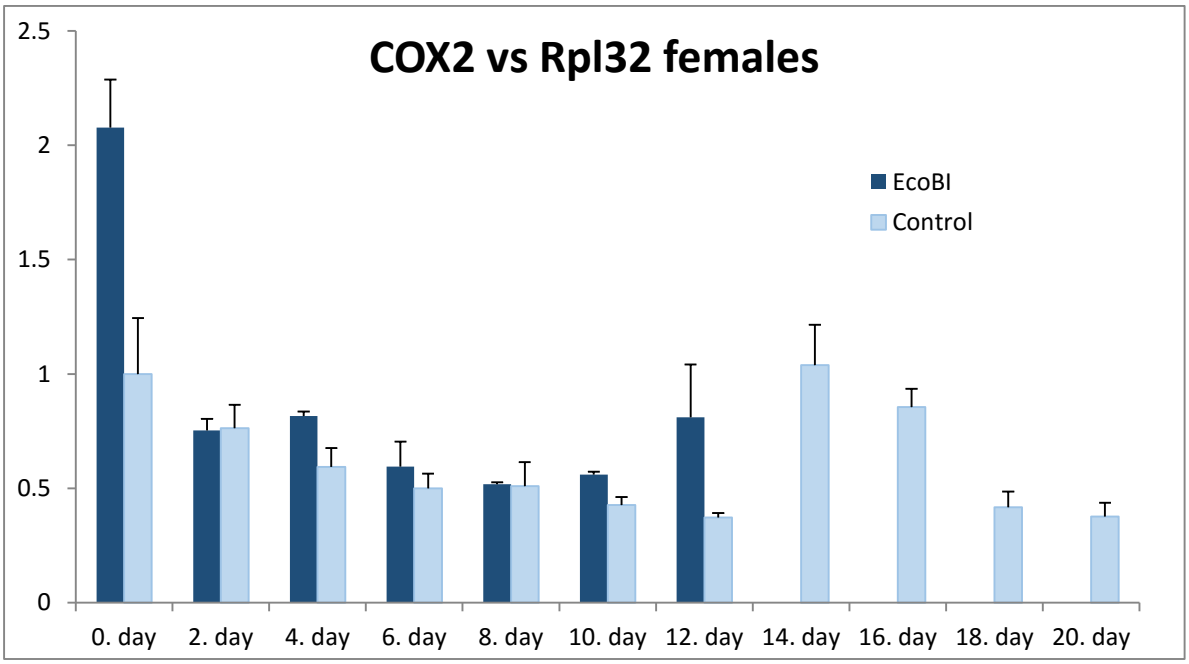

Fig. 11 cDNA QPCR COX2 female expression

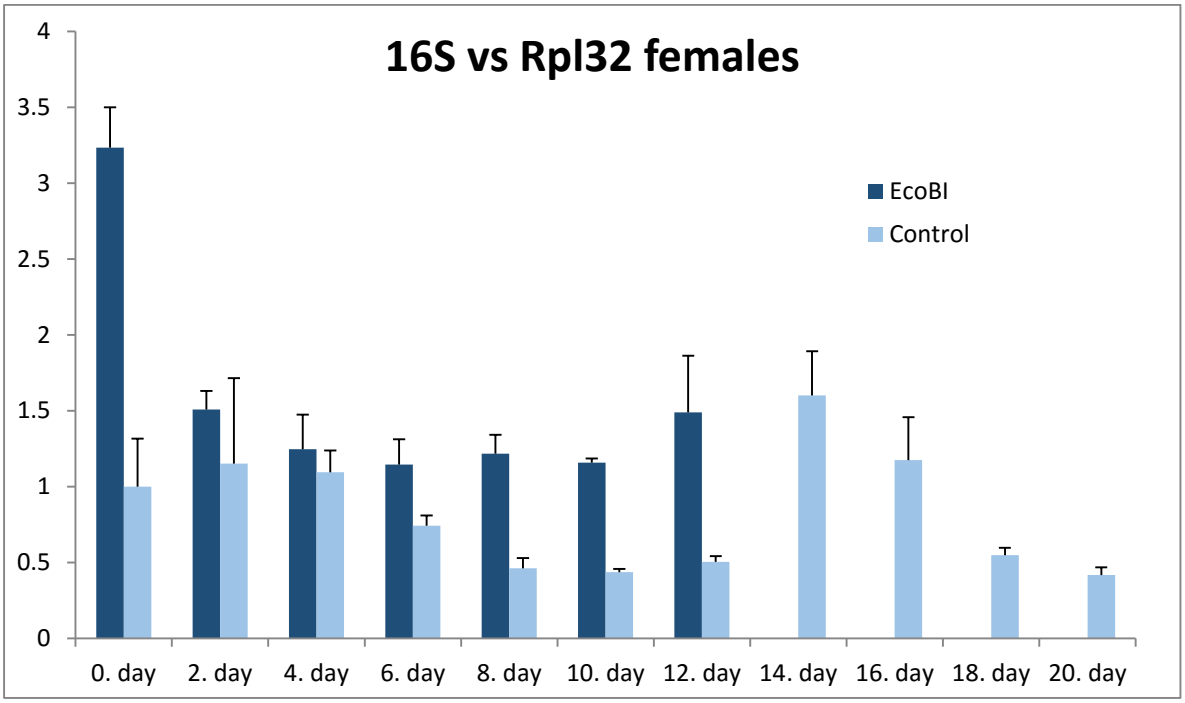

Fig. 12 cDNA QPCR 16S female expression

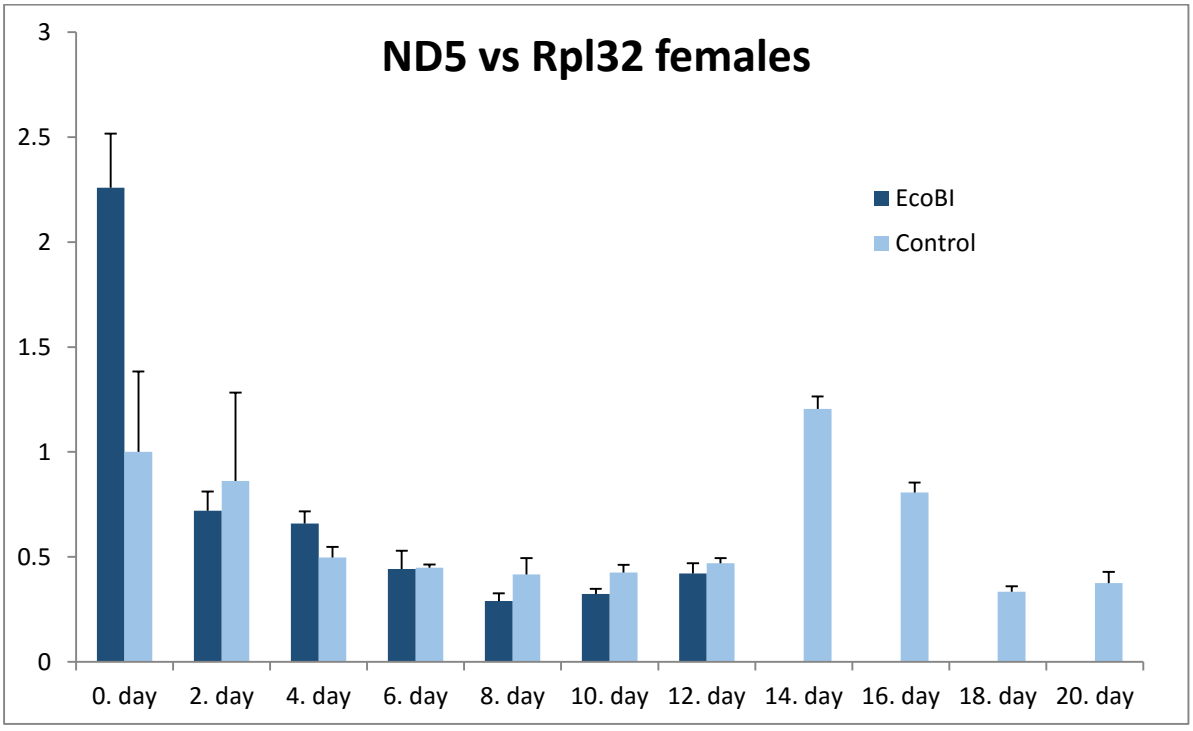

Fig. 13 cDNA QPCR ND5 female expression

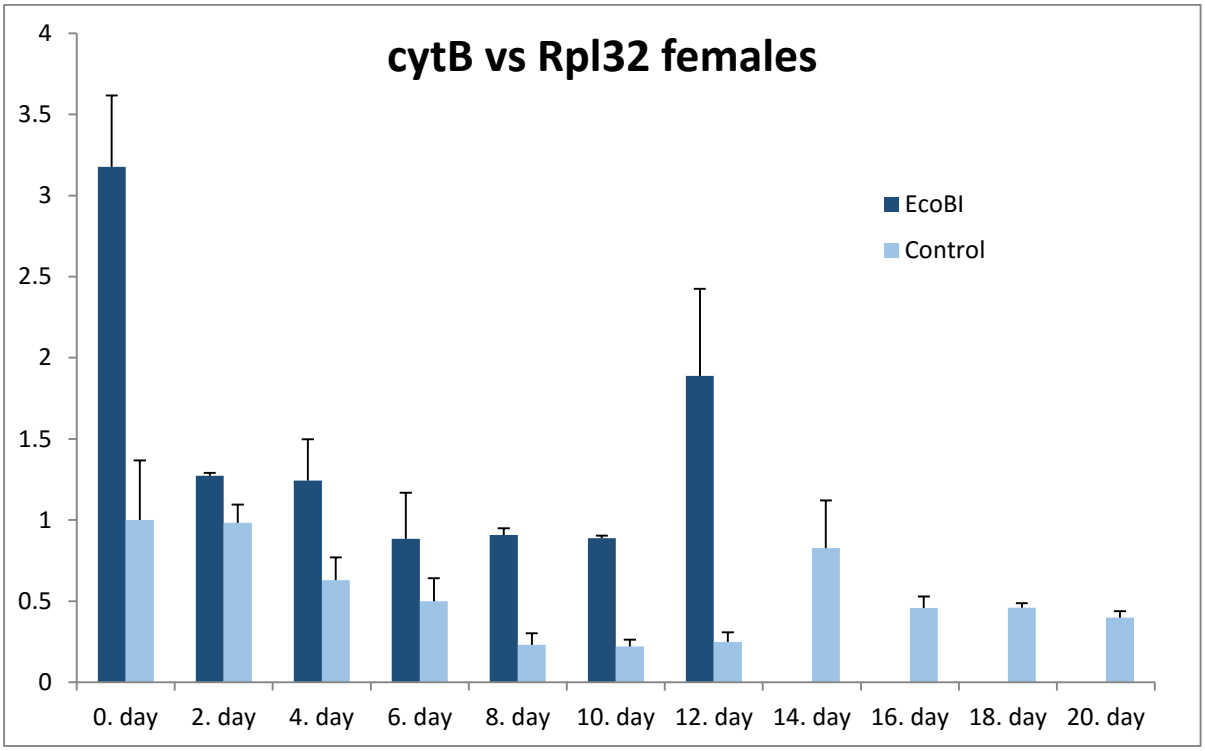

Fig.14 cDNA QPCR cytB female expression

Results

1. **Mitochondrial protein expression (western)**
2.

In the female EcoBI flies the NDUFS3/ATP5a ratio initially rises in days 0-4 but then decreases below starting levels (Fig.1). In NDUFS3/GAPDH ratio the EcoBI females also seem to rise in days 0-4 and reach a plateau at days 4-6 before starting to fall from day 10 onward (Fig.2). No such decrease in NDUFS3 protein expression in comparison to ATP5a and GAPDH levels in the control flies.

In the EcoBI males the protein expression in the NDUFS3/ATP5a stays steady in the 0-6 days but then decreases to 30% on day 14 of the 0-day expression. (Fig.3). In the NDUFS3/GAPDH the EcoBI male fly expression levels rise in days 0-6 but also start ascending levels until the flies die at day 14 (Fig.4).

We conclude that the Complex I subunit expression decreases in muscles in both the males and females flies in response to the EcoBI expression, suggesting drop in complex I amount. However, since these are results from just one biological replication, caution must be exercised when interpreting these results.

3. Mitochondrial copy number(qPCR)

Male mtDNA copy number

The mtDNA copy number in the EcoBI males flies decreases until day 4 and then steadily increases while the control flies copy number stays the same (Fig.5). This could be due to increased DNA replication caused by fragmentation of full-length mtDNA and because of the short amplicon the qPCR identifies both the fragmented and intact mtDNA.

Female mtDNA copy number

The mtDNA copy number in the female COX2 qPCR stay the same as the control with a peak at the last day which could be due to adaptation, since most of the flies had died and this causes a selection in the last days (Fig. 10).

4. Mitochondrial gene expression (RT-qPCR)

Male mitochondrial gene expression

The 16S expression levels stay the same compared to the control (Fig. 7). Expressions levels in the EcoBI decrease in both COX2 and ND5 below control strain levels after day 4 and 10 respectively (Fig.6 and 8). The cytB expression levels are generally higher than the control levels throughout the lifespan of the males flies in the control flies there is a fall in the expression levels after day 0 (Fig.9).

Females mitochondrial gene expression

The COX2 expression in the females flies also stays the same until the last day (Fig.11). The 16S has higher expression in the females than in the control (Fig.12). The ND5 expression does not differ from the control in the EcoBI female flies (Fig.13). The cytB expression are higher than the control in the females EcoBI flies (Fig. 14).

Conclusions & discussion

Protein analysis suggests that complex I decreases in response to EcoBI expression, this finding is supported by observed decrease in expression of ND5 subunit in males. Expression of complex IV mitochondrial subunit COX2 displayed similar reduction. Unchanged 16S levels probably reflect the higher stability of mitoribosomal RNA, which is not as sensitive to fluctuations in transcription as protein-coding mRNA-s. EcoBI degrades mtDNA (data not shown), but it also translocates it. This activity could possibly remove initiating/elongating transcription complexes from DNA and thus affect mitochondrial transcription. The substantial increase in cytB could reflect that the effect of EcoBI on transcription is more complex and specific than just plain overall inhibition. Although the coding sequence on both mt genome strands are being transcribed in their entirety, there are two major sites for transcription termination, implicating currently unknown mechanisms of regulation between initiation and termination. cytB gene is located between these two sites, suggesting that EcoBI activity might actually in some cases favor transcription by inhibiting termination. In any case, it is clear that EcoBI action interferes with normal expression of mitochondrial genes in muscles. These aforementioned effects on transcription were not observed in females. However, it is important to keep in mind that these full body lysates were used in analysis and mitochondria from muscles make up only a part of the total mitochondrial pool, especially in females where large portion of mitochondria are in embryos.

The last day time points might not be reliable in the westerns and qPCRs since most of the flies gathered for testing were dead by the late time points (10-14 days) and this causes a selection of flies. Although these results are preliminary, they confirm the functionality of EcoBI in mitochondria and demonstrate its role in directly interfering with integrity and expression of mt genome.

References

1. Alberts, Molecular Biology of the Cell 5th edition. Chapter 14 Mitochondrion (2008)
2. R.K. Murray, Harpers Illustrated Biochemistry (Lange Medical Book) 29th edition. Chapter 13 and 17. (2012)
3. D.Harman, Aging: a theory based on free radical and radiation chemistry. J Gerontol 11, 298 (Jul 1956)
4. D.Harman, The biologic clock: the mitochondria? J Am Geriatr Soc 20, 145 (Apr 1972)
5. Jiang X & Wang X, Cytochrome C-mediated apoptosis. Annu Rev Biochem 73:87-106 (2004)
6. J.W.Kim, C.V. Dang. Cancer's molecular sweet tooth and the Warburg effect. Cancer Res 66, 8927 (Sep 15, 2006)
7. C.Wang, R.J.Youle, The role of mitochondria in apoptosis. Annu Rev Genet 43, 95(2009)
8. S.DiMauro, E.A.Schon, Mitochondria DNA mutations in human disease. Am J Med Genet 106, 18 (spring, 2001)
9. Chinnery, P F. Turnbull, D M. Mitochondrial DNA mutations in human disease. Molecular Medicine Today. 6(11):425-32, (2000 Nov.)
10. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. Science. 2005;307:384–387.
11. . Mercer, John R.; Cheng, Kian-Kai; Figg, Nichola; Gorenne, Isabelle; Mahmoudi, Melli; Griffin, Julian; Vidal-Puig, Antonio; Logan, Angela; Murphy, Michael P.; Bennett, Martin. DNA Damage Links Mitochondrial Dysfunction to Atherosclerosis and the Metabolic Syndrome. Circulation Research. 107(8):1021-1031, (October 2010)
12. Myhill et al. Chronic fatigue syndrome and mitochondrial dysfunction Int J Clin Exp Med. 2009; 2(1): 1–16.
13. Byung-Ok *et al*. A MELAS syndrome family harboring two mutations in mitochondrial genome. Exp Mol Med. Jun 30, 2008; 40(3): 354–360.
14. Trifunovic, A. *et al*. Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 429, 417–423 (2004).
15. N.Bonnefoy, T.D. Fox, Directed alternation of Saccharomyces cerevisae mitochondrial DNA by biolistic transformation and homologous recombination. Methods Mol Biol 372, 153 (2007)
16. . P.F.Chinnery et al., Peptide nucleic acid delivery to human mitochondria. Gene There 6, 1919 (Dec 1999)
17. A.Muratovska et al., Targeting peptide nucleic acid (PNA) oligomers to mitochondria within cells by conjugation to lipophilic cations: implications for mitochondrial DNA replication, expressions and disease. Nucleic Acids Res 29, 1852 (May 1 2001)
18. J.M Collombet, V.C. Wheeler, F.Vogel, C.Coutelle, Introduction of plasmid DNA into isolated mitochondria by electroporation. A novel approach toward gene correction for mitochondrial disorders. J. Biol chem. 272, 5342 (Feb 21, 1997).
19. Y.G.Yoon, M.D.Koob, Transformation of isolated mammalian mitochondria by bacterial conjugation. Nucleic Acids Res 33, e139 (2005)
20. M.Koulintchenko, R.J.Temperley, P.A.Mason, A.Dietrich, R.N. Lightowlers, Natural competence of mammalian mitochondria allows the molecular investigation of mitochondrial gene expression. Hum Mol Genet 15, 143 (Jan 1, 2006)
21. Minczuk M. Development of a single-chain, quasi-dimeric zinc-finger nuclease for the selective degradation of mutated human mitochondrial DNA. Nucleic Acids Res. 2008 Jul;36(12):3926-38.
22. Bacman SR Specific elimination of mutant mitochondrial genomes in patient-derived cells by mitoTALENs. Nat Med. 2013 Sep;19(9):1111-3
23. S. Srivastava, C. T. Moraes. Double-strand breaks of mouse muscle mtDNA promote large deletions similar to multiple mtDNA deletions in humans. Hum Mol Genet 14, 893 (Apr 1, 2005)

24. S. R. Bacman, S. L. Williams, C. T. Moraes, Intra- and inter-molecular recombination of mitochondrial DNA after in vivo induction of multiple double-strand breaks. Nuclei Acids Res 37, 4218 (Jul, 2009)
25. K. J. Krishnan, A. K. Reeve, D. C. Samuels et al., "What causes mitochondrial DNA deletions in human cells?" Nature Genetics, vol. 40, no. 3, pp. 275–279, 2008
26. Laurent Seroude, Ted Brummel, Pankaj Kapahi and Seymour Benzer, Spatio-temporal analysis of gene expression during aging in Drosophila melanogaster. Aging Cell (2002) 1, pp47-56
27. Reinecke F, Smeitink JAM, van der Westhuizen FH. 2009. OXPHOS gene expression and control in mitochondrial disorders. Biochimica et Biophysica Acta 1792:1113–1121.