Improving mitochondrial function significantly reduces metabolic, visual, motor and cognitive decline in aged Drosophila melanogaster

Tobias W. Weinrich[1], Ariathney Coyne[1], Thomas E Salt[1,2], Christopher Hogg[1] and Glen Jeffery[1]

[1]University College London, Institute of Ophthalmology, London UK.

[2]Neurexpert Ltd. London, UK.

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Correspondence to:

Glen Jeffery

Institute of Ophthalmology

University College London

11-43 Bath St, London EC1V 9EL, UK

Email: g.jeffery@ucl.ac.uk

Abstract

Mitochondria play a major role in ageing. Over time mutations accumulate in mitochondrial DNA (mtDNA) leading to reduced ATP production and increased production of damaging reactive oxygen species (ROS). If cells fail to cope, they die. Reduced ATP will result in declining cellular membrane potentials leading to reduced CNS function. However, aged mitochondrial function is improved by long wavelength light (670nm) absorbed by cytochrome c oxidase in mitochondrial respiration. In *Drosophila*, lifelong 670nm exposure extends lifespan and improves aged mobility. Here we ask if improved mitochondrial metabolism can reduce functional senescence in metabolism, sensory, locomotor and cognitive abilities in old flies exposed to 670nm daily for 1 week. Exposure significantly increased cytochrome c oxidase activity, whole body energy storage, and ATP and mtDNA content and reduced ROS. Retinal function and memory were also significantly improved to levels found in 2 week old flies. Mobility improved by 60%. The mode of action is likely related to improved energy homeostasis increasing ATP availability for ionic ATPases critical for maintenance of neuronal membrane potentials. 670nm light exposure may be a simple route to resolving problems of ageing.

1. Introduction

Aging is driven by numerous factors, but mitochondrial decline plays a key role (Bratic and Larsson, 2013; López-Otín et al., 2013). Mitochondria provide much of the energy needed for cellular function in the form of ATP. However, mitochondrial function declines with age as mitochondrial DNA (mtDNA) is subject to progressive mutation. This is associated with reduced mitochondrial membrane potentials and declining ATP production, and also with increased production of potentially damaging reactive oxygen species (ROS) (Hiona et al., 2010). Hence ageing is associated with reduced energy for cellular function and progressive inflammation. This forms the basis for the mitochondrial theory of aging, which although not without qualification, is still important in our understanding of the ageing process (Harman, 1981; López-Otín et al., 2013).

Age related decline in mitochondrial function can be partly corrected in rodents by exposure to specific longer wavelengths of light that are absorbed by cytochrome c oxidase (COX) in the mitochondrial respiratory chain (Gibson and Greenwood, 1965; Karu, 1999, 2014; Mason et al., 2014). Changes in the redox state of COX can be monitored in real time optically in the rodent retina *in vivo* where it increases rapidly 5 mins after exposure to 670nm light (Kaynezhad et al., 2016). Subsequently, mitochondrial membrane potentials improve (Kokkinopoulos et al., 2013), there is an increase in ATP (Calaza et al., 2015; Gkotsi et al., 2014), and a reduction in both ROS and inflammation (Begum et al., 2013; Kokkinopoulos et al., 2013). There is also improved retinal function (Sivapathasuntharam et al., 2017). In *Drosophila* similar results are obtained in respect of ATP and inflammation, however, here there is also an increase in mean life span and improved aged mobility after 670nm exposure (Begum

et al., 2015). In bumble bees, rodents and primates where mitochondrial function has been undermined chemically, 670nm light improves mobility and corrects behavioral deficits (Darlot et al., 2015; Powner et al., 2016; Reinhart et al., 2016).

Here we explore the metabolic and functional impact of 670nm light given daily over a week to ageing *Drosophila* to determine if 670nm exposure can significantly improve age related decline. If there are improvements in aged mitochondrial function, this may be reflected in improved metabolism and animal function. Hence, we examine changes in metabolism along with motor and retinal function and performance in a memory task.

2. Materials and Methods

2.1. Fly stocks and husbandry. Male *Drosophila melanogaster* Dahomey were maintained under standard lab conditions and diet (12/12 lighting at 25C and 70% humidity). Newly hatched flies were collected and kept at standard density of 30 male flies in vials (25 x 95 mm) containing 10 mL food medium changed three times weekly. Unless stated otherwise flies were killed by instant freezing with dry ice.

2.2. 670 nm treatment. 670 nm light exposure was similar to our previous fly and bee studies using the same light devices (C.H. Electronics UK) (Begum et al., 2015; Powner et al., 2016). For the majority of experiments, five week old flies were exposed to 670 nm for 20 min per day at 40 mW cm^{-2} for 7 days. Flies of this age were used throughout to avoid a survival bias arising from use of older animals where the population may be skewed towards improved survival characteristics and as such be unrepresentative. Each experimental group had a matched untreated control and was also compared to 2 week untreated flies. In the 7 day treatment experiment, an additional group was added for the mitochondrial parameters where ATP, metabolic

rate, mtDNA and ROS were measured. Here 5 week old flies received the same 670 nm exposure as the experimental group, but this was in blacked out vials so that the light exposure did not reach the animals. In none of the experiments was exposure to 670nm associated with measurable warming. Optical attenuation by the clear vials was <1% with no shift in wavelength.

To study the initial impact of 670 nm, 6 week old flies were given a single 90 min exposure and assessed directly for: metabolic rate, whole body ATP content, ROS and locomotor function. 90 mins exposure was chosen on the basis of preliminary experiments designed to target the first measurable changes in animal physiology in response to this light. Here experiments included only light treated and untreated controls.

2.3. Measuring fly metabolic rate. Metabolic rate was assessed by measuring CO_2 production in lab-made respirometers following a protocol previously described (Yatsenko et al., 2014). In each of the groups there were 5 replicates containing 5 flies. Metabolic rate was measured over 120 min (90 min in the case of single exposure)**.**

2.4. ATP measurement. Whole body fly ATP was measured using a commercial ATP kit (Life Technologies, Paisley UK) and samples were processed as previously described (Costa et al., 2013). Five replicates containing 5 flies were used for each group. Unless stated otherwise, all general chemicals and reagents were from Sigma Aldrich (Dorset UK).

2.5. Immunostaining for cytochrome c oxidase. Flies were decapitated in sterile PBS and heads fixed for 2h in 4% paraformaldehyde in PBS. These were placed in 40% sucrose in PBS for cryoprotection overnight. They were then embedded in optimal

cutting compound and cut frozen at 10µm. Mounted sections were stained for cytochrome c oxidase subunit III and beta-actin. They were blocked with 5% normal donkey serum in 0.5% Triton X-100 in PBS. Then exposed to primary antibody (1/800, goat anti cytochrome c oxidase subunit III, Santa Cruz Biotechnology Inc, CA, USA, and 1/500 rabbit anti beta-actin, Abcam ab8227, Cambridge, UK) for 24h at 4C. Slides were washed and stained with conjugated secondary antibody (Alexa donkey anti goat 568 nm and Alexa donkey anti rabbit 488 nm (Invitrogen, UK), 1/1000, diluted in PBS containing 2% NDS, Triton X-100 0.5%). Sections were coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA.). Measurement of COX III and beta-actin expression in the fly retina were analysed and processed using Fiji is Just Image J v. 1.49e (Schneider et al., 2012). The freehand tool was used to draw a line around the fly retina, excluding the cornea and other structures, and pixel intensity measured in the delimited area. The COXIII signal was normalised to beta-actin. 15 fly heads per group.

2.6. Measuring mtDNA. Mitochondrial and nuclear-cytosolic fractions where isolated by differential centrifugation and mtDNA and nuclear DNA quantified as previously described (Villa-Cuesta et al., 2014). Briefly, 10 flies were gently homogenised in 1 mL isolation buffer (225mM mannitol, 75mM sucrose, 10mM MOPS, 1mM EGTA and 0.5% BSA, pH 7.2). The extracts were then centrifuged at different speeds to obtain cytosolic and mitochondrial enriched fractions. For DNA quantification, RNA from the mitochondrial and cytosolic fraction was removed by treatment with RNase A (Thermo Scientific, UK) and the DNA content determined spectrophotometrically (Nanodrop ND-1000). The DNA concentration in the mitochondrial fraction was normalised to the

cytosolic fraction to obtain the mtDNA/nDNA ratio. Five replicates containing 10 flies were used for each of the groups of flies.

2.7. Measuring reactive oxygen species. To assess oxidative stress, ROS levels were measured using dichloro-dihydrofluorescein diacetate (DCFH-DA) as previously described (Haddadi et al., 2014). Flies were decapitated in sterile PBS and heads homogenized in 200 µL ice-cold Tris-HCl buffer (0.4M; pH 7.4) followed by a 10 min centrifugation at 2000g at 4C. From each sample, 100 µL was added to microplate wells containing 15 µL of 5 µM DCFH-DA, and total volume adjusted to 200 µL with homogenizing buffer. After 1 h incubation, the conversion of DCFH-DA to DCF was measured at 489 nm excitation and 525 nm emission. Each replicate contains 10 heads (60-90 total heads per group).

2.8. Protein measurement. Total protein measurement was performed using a commercially available kit, BCA Protein Assay by Thermo Scientific, (Paisley UK). Bovine serum albumin was used as a standard and the amount of protein was measured following the manufacturer's protocol.

2.9. Glucose, glycogen and triglyceride levels. Glucose, glycogen and triglyceride levels were measured using established methods (Tennessen et al., 2014). For the glycogen and glucose assay, the hexokinase method was used. Briefly, for each sample 5 flies were homogenized in 100 µL cold (PBS) and heated to 70C for 10 mins. Homogenates were diluted 1:3 in PBS and centrifuged for 3 min at 4C. The supernatant was collected, and glucose levels determined with a Glucose (hexokinase) assay kit (Sigma). The hexokinase reaction was performed for 15 min and absorbance at 340 nm was measured. For measuring glycogen an additional step was undertaken where

samples were supplemented with 1 µg/mL amyloglucosidase. Amyloglucosidase was used to hydrolyse glycogen into glucose molecules. Absolute glycogen and glucose levels were determined by a standard glucose curve. Samples and standard were analysed in duplicate. Free glucose and glycogen levels were normalised to protein in the corresponding homogenate. Protein concentration was determined in duplicate. Samples were assayed in 5 replicates.

To assess triglyceride levels, 5 flies per group were homogenised in 100 µl PBS 0.05% Tween 20 (PBST) and heat treated as above. Two aliquots of 20 µl for each sample were treated with PBST (free glycerol) and triglyceride reagent and incubated for 45 min at 37C. The triglyceride reagent was used to breakdown triglycerides into fatty acids and glycerol. To measure glycerol, free glycerol reagent was added and incubated for 5 min at 37C and absorbance determined at 540 nm. Samples and standards were analysed in duplicate. Free absolute glycerol and triglycerides levels were normalised to protein in corresponding homogenates. Protein concentration was determined in duplicate using the Pierce BCA Protein Assay Reagent Kit. Samples were assayed in replicates of 5.

2.10. Responses to stress. Three methods were used to measures the response of flies to physiological stress: starvation resistance, heat sensitivity and chill coma recovery time. For starvation resistance flies were placed in vials (20 flies per vial, 80 flies per group) containing 10 mL of 1% agar, which provided no nutrients. Mortality rate was determined by counting dead animals over 6 days. Vials were changed every 24 h. Second, flies were exposed to heat stress (Costa et al., 2013). They were placed in empty vials (10-15 flies per vial, 105 flies per group), which were placed in a 37C water

bath for 1h. They were then transferred into fresh food containing vials. Live flies were recorded after 2, 4 and 24 h later. Third, flies were assessed for chill coma. They were placed in empty 5 mL vial (10 flies per vial, 60 flies per group) and submerged for 2 h in a 0C water bath following a protocol previously published (Andersen et al., 2015). Subsequently, they were left at 20C and the spontaneous chill coma recovery time to a standing position was recorded.

2.11. Physiological and behavioural responses. Electroretinogram recordings were made to assess retinal function. *Drosophila* were immobilised with brief CO_2 exposure and restrained on a microscope slide under a nylon mesh with one eye exposed. The fly was placed in an electrically shielded dark recording chamber. A stainless steel indifferent (earth) electrode was inserted into the thorax and a glass micropipette inserted into the retina. Visual stimulation was trains of white light flashes (50% duty cycle) at 0.5, 2, 5, 10, 20, 30, 50, 100 Hz at 0.3 W/m^2 (background 0.2mW/m^2). Retinal responses were recorded via an Axoprobe 1A amplifier, digitized (5 kHz) by a CED1401 interface and stored on a PC. Experimental groups were: two weeks N=8, 6 weeks N=7 and N=11 for 6 week 670nm exposed.

Locomotor function was assessed using negative geotaxis (Ali et al., 2011). Briefly, flies were placed in clear cylinders with a mark 8 cm from the bottom placed on the side. Flies were tapped to the bottom and number that climbed above the mark in 10 s was counted (10 times). 80-100 flies per group.

To assess cognitive ability an aversive phototaxis suppression assay was used modified from Ali et al. (2011). Two adjoining chambers made from clear tubes were connected by a door. One chamber was brightly illuminated from the far end while the other was

dark. The light chamber was lined with filter paper along much of its length soaked in quinine, which flies find aversive. Flies were trained to avoid the light chamber when the door was opened due to the presence of quinine and remain on the dark side. Successfully trained flies were reassessed after an interval of 6 hours on the same task to determine if they remembered the association of light with quinine. Fly numbers per group were N=15 at 2 weeks, N=17 at 6 weeks exposed to 670nm and N=19 at 6 weeks with no light.

2.12. Statistics. Data were analysed with GraphPad Prism v.6 (SanDiego CA, USA) and statistical analysis was undertaken using two-tailed Mann Whitney U test unless otherwise stated. Data presented are mean + SEM. The significance was asserted as * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3. Results

Levels of cytochrome c oxidase subunit III (COX III) were assessed to determine if 670nm light interacted directly with mitochondria and if this was associated with increased ATP levels and other key metrics. Changes in ATP alone may not be totally reliable in assessing mitochondrial function as it can also be produced independently by glycolysis. There was a significant decline of approximately 20% in retinal COXIII expression between 2 and 6 weeks of age in the fly eye. However, following 670nm exposure in old flies COX III expression increased significantly in relation to both 2 week flies and 6 week untreated animals. The increase against 2 week animals was approximately 15% and against old untreated controls was almost 30%. Hence,

exposure to 670nm in the fly is associated with a positive shift in mitochondrial respiration consistent with previous studies on flies (Begum et al., 2015), bees (Powner et al., 2016) and mice (Gkotsi et al., 2014) (Fig. 1).

3.1. Responses to initial single 670nm exposures. While the impact of 670nm light exposure has been widely documented, particularly in experimental pathology (Fitzgerald et al., 2013), little is known about the response to single or multiple exposures. Hence, as a baseline for this study, the impact of a single exposure of 670nm for 90min on aged flies was examined. Six week animals were given a single 670nm exposure that resulted in a significant increase in the production CO_2, demonstrating that there was a metabolic response to light exposure. However, over this limited period there was no measurable increase in ATP or change in ROS levels. We also measured locomotor function and found no difference between experimental and control groups. Hence there was a change in whole fly metabolic rate but this did not translate into other metrics in this relatively short time window (Fig.2).

3.2. Responses to 7 day 670nm exposures. In aged flies there was a significant decline of approximately 30% in respiration as measured by CO_2 production when compared with young flies. This decline was corrected in 6 week old flies exposed to 670nm, returning levels of respiration to those found at 2 weeks of age (Fig 3A). This pattern between the groups was repeated when ATP levels were measured. A significant age related decline of around 50% was found in ATP levels at 6 weeks, but this decline was corrected by 670nm light exposure (Fig 3B). MtDNA content was also measured in these groups and normalised against nuclear DNA. MtDNA declined significantly by approximately 75% between 2 and 6 weeks of age, but increased

significantly following a week of 670nm exposure. While mtDNA content more than doubled in 670nm exposed flies compared to 6 week untreated flies, it did not return to levels found at 2 weeks of age, although the difference between 2 week old and 6 week 670nm treated flies was not statistically significant (Fig.3C). There was a significant age related increase in ROS in the flies between 2 and 6 weeks of age of around 30%. This increase was reduced significantly by 670nm light exposure to levels comparable with that found in young flies (Fig.3D). There were no significant differences in these parameters between 6 week old flies and age matched flies treated in the same manner as the experimental group but in blacked out vials. Hence, daily exposure to 670nm over 1 week increases metabolic rate, ATP concentration and mtDNA content, and also reduced ROS. While it is known that 670nm improves mitochondrial function as measured by their membrane potentials and ATP production in mice (Gkotsi et al., 2014; Kokkinopoulos et al., 2013), results from mtDNA measurements imply that it may also influence autophagy and/or mitochondrial dynamics by increasing mitochondrial number.

3.3. Changes in metabolites. We assessed changes in whole body metabolism in response to 670nm light exposure in flies exposed daily for a week in the same groups as above, except the group of flies in blacked out vials. It is known that with normal fly ageing there is a reduction in triglycerides, glycogen and glucose (Biteau et al., 2010), indicating that ageing leads to an increase in catabolic activity. If 670nm exposure modifies mitochondrial function, then metabolic homeostasis may adapt to this and there may be a move away from catabolic activity. There is a significant age related decline in triglycerides, glycogen and glucose, which in each case is between 20-40%.

However, there was a significant increase in the concentration of each of these molecules in response to 670nm light exposure (Fig4A-D). In the case of triglycerides and glucose the improvement found after 670nm light exposure brought their levels back into the range found in 2 week old flies. It is only in the case of glycogen that the improvement did not reach this level. Hence, in each case light exposure resulted in a significant increase in these metabolites. One interpretation of these data is that improved mitochondrial efficiency may result in a reduced drain on these energy stores.

3.4. Heat and cold stress. Improved metabolites may provide protection responses to heat and cold stress. Survival in a heat stress test declined significantly with age by approximately 50% in 6 week old flies, but survival was not improved by exposure to 670nm light (Fig.5A). When flies were placed into a chill coma for an hour there was a delay in the recovery response with age. But 6 week old 670nm treated flies recovered significantly faster than old untreated animals during the early phase of recovery period (Fig.5B). In Fig.5C data between 0-8 mins is re-represented normalised to the young untreated animals. Here it is clear that 670nm exposed older flies recovered more rapidly than untreated old flies. Approximately 50% of the 670nm treated flies have recovered in the first 4 mins while this level does not occur until around 6-7 mins in old untreated animals (Fig.5B&C).

3.5. Starvation. Resistance to starvation was also measured by counting surviving fly numbers when placed in vials containing 1% agar only. In the young fly population 50% of the animals died by 100h and all were dead by 150h. In aged flies 50% of the population were dead at by approximately 40h and all were dead by approximately 100h. This difference was significant. The survival profile of the 670nm treated flies was

almost identical to that of the older untreated animals (Fig.6A). Hence, while metabolism improves with 670nm light this is not associated with improved survival in heat stress or starvation, but it is with chill coma. It is possible that increased energy stores that result from 670nm exposure improve resilience for challenges like chill coma that lasted 2h but do not with starvation where the period of challenge was over a much longer period. In support of this notion, when the metabolites that were improved by a week of 670nm exposure were examined during the early phases of starvation at 24h and 48h, it was clear that their improvement was not robust. By 24h of starvation the improved metabolite levels had declined to that of found in aged matched controls and this was similar at 48h (Fig 6B, C and D).

3.6. Physiological responses and memory. Having established that 670nm light exposure improves fly metabolism we progressed to assess its impact on key behavioural and physiological metrics. Retinal function was measured via the amplitude of ERG responses to flash stimuli between 0.5Hz–100Hz. In young flies the amplitude of responses was maximal at 2Hz stimulation and then declined with the progressive increase in the frequency of the stimuli.

There was a significant difference in amplitudes of the retinal responses between young and old flies with responses in the younger group being approximately 25% greater than those in the older group. Furthermore, there was a greater decline in responses with increasing stimulation frequency in old flies than in young flies. Responses in old flies treated with 670 nm light were almost identical to those in young animals (Fig 7A&B). Hence, there was a significant improvement in retinal function with 670nm exposure

similar to that found in old mice treated with 670 nm light (Sivapathasuntharam et al., 2017).

The impact of 670nm light exposure was also examined on locomotor function in a manner similar to that previously undertaken when 670nm was given throughout life (Begum et al., 2015). Locomotor function declined significantly by >50% with age between 2 and 6 weeks. Exposure to 670nm light for 1 week in 6 week old flies significantly improved this aged related decline, but did not return this to levels found in 2 week old flies (Fig 7C).

The ability of flies to remember an association of light with an aversive stimulus was examined. Flies that had learned to avoid light because of the presence of quinine were held for 6 h outside the testing apparatus and then retested. There were significant differences in the performance of this test with age, with a 75% reduction in performance in older flies that were unable to remember the association when retested. As their failure to remember resulted in them moving towards the light, differences were not related to issues of reduced mobility. This age related reduction in performance was significantly improved in the older flies exposed to 670nm light. These animals performed at a very similar level to 2 week old flies (Fig 7D).

4. Discussion

Our results show that a single exposure to 670nm light initially increases fly metabolic rate as measured by CO_2 production, but does not increase measurable ATP or impact on locomotion. However, when 670nm light exposure is extended to daily exposures

over a week in aged flies a marked and wide range of improvements are found including elevated ATP and mtDNA content, and reduced ROS levels. Also, associated improvements are found in metabolite storage, retinal function, locomotion and memory. In spite of these, no positive impact was found when animals that had been exposed to 670nm light were challenged by starvation or heat stress. It is probable that some of the positive aspects identified in aged flies following light exposure were the result of improvements in ATP production, as neuronal membrane pumps are major consumers of ATP and are known to show an age related decline (De Lores Arnaiz and Ordieres, 2014). Greater energy availability to cellular pumps may underpin some of the improvements, particularly in respect of functional metrics.

Another factor that may play a role in the general improvement in physiological function is the reduced levels of ROS following light exposure. ROS play a significant role in ageing (López-Otín et al., 2013). However, this requires some qualification as increased ROS can prolong lifespan in *C. elegans* (Van Raamsdonk and Hekimi, 2009) and manipulations that elevate ROS do not always increase ageing (Van Remmen et al., 2003). Lopez-Otin et al. (2013) in reviewing the role of ROS point out that there is evidence that ROS expression can be protective. Hence, it is likely that the level of ROS expression may be a key factor, but this may vary with age and tissue type. However, that we find such a large increase in ROS with age and a significant reduction with light exposure implies that in this situation elevated ROS is probably detrimental.

ROS play a role in memory impairment in *Drosophila*, with the formation of giant mitochondria that are dysfunctional and produce ROS in the mushroom bodies (Haddadi et al., 2014; Seo et al., 2010), which are the main site of olfactory and

gustatory memory formation (Kirkhart and Scott, 2015). The finding that the enhanced antioxidant enzyme activity in *Drosophila* that is associated with reduced ROS is also linked to a slower age related decline in memory function highlights the potentially detrimental role of ROS here (Haddadi et al., 2014). Such findings mirror our results showing reduced ROS after 670nm light exposure that is also associated with improved aged memory.

Cytochrome c oxidase in mitochondrial respiration absorbs specific long wavelengths of light (Karu, 1999; Karu et al., 2005). By monitoring the optical reflections from the retina *in vivo*, it is possible to measure relative changes originating from the redox state of COX that increases rapidly within 5 mins after a brief exposure to 670nm and continues to do so for around 1h (Kaynezhad et al., 2016). This is the likely first point of interaction between this light and improved mitochondrial respiration. 670nm light increased fly respiration when animals were initially exposed, but within this window a measurable increase in ATP did not occur. The point at which measureable increases in ATP can be found remains to be defined, however this was clearly present after 1 week in our flies, consistent with data from mice retinae and brain showing ATP elevation at a similar time (Gkotsi et al., 2014).

Reduced mitochondrial function and declining ATP are key features of ageing (López-Otín et al., 2013). Many *in vitro* and *in vivo* studies have shown that specific long wavelengths of light have the ability to impact positively on aged and damaged mitochondrial function (Fitzgerald et al., 2013). These studies include work on insects. Using *Drosophila* and bees we have shown that extended 670nm exposure improves aged ATP and mobility and also extends mean lifespan, consistent with the

mitochondrial theory of ageing (Begum et al., 2015; Harman, 1981; Powner et al., 2016).

Our results in *Drosophila* are consistent with another fly study where a longer wavelength (808nm) that is also absorbed by COX has been used to improve complex IV in mitochondrial respiration and rescue function in the Parkinson pink1 *Drosophila* fly model (Vos et al., 2013). In rodents and primates, 670nm light has significantly improved symptoms in Parkinson's disease models where disease has been induced by MPTP, a toxin that undermines complex I in the mitochondrial respiration chain (Darlot et al., 2015; Johnstone et al., 2014). Similarly, we have used 670nm in bumble bees to protect them from neonicotinoid pesticides that also undermine mitochondrial function (Powner et al., 2016). Hence, specific wavelengths across the deep red to infrared range improve and protect mitochondrial function in both mammals and insects.

Longer wavelengths absorbed by mitochondria that improve respiration are assumed to play a role in improving outcomes in experimental pathology (Fitzgerald et al., 2013), but there may be multiple mechanisms that are initiated by long wavelength light exposure that go beyond elevated ATP production. Our data show that during a week of light exposure, mtDNA content more than doubles compared to age matched controls. Mitochondria undergo fusion and fission and use this to respond to changes in energy demand. The balance of these processes changes significantly with aging and disease (Bereiter-Hahn, 2014; Szklarczyk et al., 2014). Mitochondria also experience autophagy where they can be removed when dysfunctional. We can not determine whether the increase in mtDNA is due to shifts in autophagy or changes in the balance of fission and fusion. However, autophagy may be partly regulated by declining mitochondrial

membrane potentials (Narendra et al., 2012), which we have shown are increased by 670nm light exposure in mice (Kokkinopoulos et al., 2013). Hence, it is possible that increased mtDNA may arise because fewer mitochondria need to be removed by this process, while those that remain may have improved respiration. Our understanding of the relationship between longer wavelength light and mitochondrial function is seriously lacking in many respects, however, to date we have found no strong evidence in the mouse retina that 670nm light markedly changes the balance of fission and fusion. This is in spite of the significant changes that occur in these dynamics in the outer retina with age (Hoh Kam and Jeffery, 2015).

We have shown that there are increases in triglycerides, glycogen and glucose in aged flies exposed to 670nm. Also, that there is an association with this and improved responses to chill coma recovery, but not to starvation and heat stress. In respect of chill coma and starvation this implies that 670nm light has the ability to improve responses to short term stress found in chill coma but not that which extends over a longer period in starvation. This is consistent with our demonstration that although there are improvements in metabolism with 670nm exposure they are rapidly undermined when challenged by starvation. It remains unclear whether longer periods of light exposure could protect against this.

The improvements we show in retinal function with 670nm light are similar to those in both aged mice and bees whose mitochondria have been undermined with insecticide (Powner et al., 2016; Sivapathasuntharam et al., 2017). The very high energy demand of the retina exposes this region to progressive inflammation and cell loss (Cunea et al., 2014; Cunea and Jeffery, 2007; Curcio, 2001; Xu et al., 2009). It is likely that here

neuronal membrane potentials suffer markedly with ageing (De Lores Arnaiz and Ordieres, 2014) and that this is the reason that ERG amplitudes decline with age before there is retinal cell loss. The ability of longer wavelength light to improve neuronal membrane potentials may have widespread impact on ageing and also be the mechanism behind the recovery in aged fly memory and mobility. The improvements in retinal function and memory were proportionally much greater than found for mobility. This may reflect that the former are CNS functions, while mobility depends on coordinated interaction between the CNS and the periphery. Hence, factors such as aged muscle loss may limit overall improvements in mobility.

The use of deep red light to improve mitochondrial function in human ageing and disease may have significant potential, particularly given its potential cost effectiveness. Efficacy in the retina has already been established in diabetes (Gonzalez-Lima and Rojas, 2011; Tang et al., 2013). It may have a significant role to play where mitochondrial function is challenged such as in ischemia (Keszler et al., 2014; Lohr et al., 2009). Further, as mitochondria can play a significant role in signaling cell death as well as reducing inflammation it has been suggested that they may have a function in reducing the burden of dementia (Sommer et al., 2012).

5. Figure captions

Figure 1. Effects of 670 nm exposure on the expression of Cytochrome C oxidase subunit III in the *Drosophila* retina.

Transverse eye sections of 2 weeks, 6 weeks and 670 nm exposed *Drosophila* were stained for cytochrome c oxidase subunit III (COXIII). (**A**) Representative images for each group. (**B**) Quantification of COXIII intensity represented graphically, normalised to beta-actin, a housekeeping protein not expected to change with 670 nm exposure. 6 week old flies had a significantly lower normalised COXIII levels than 2 week old flies. 670 nm treatment for 1 week increased relative signal intensity compared to age matched controls, and compared against the 2 week old flies. Results are shown as mean + SEM. Scale bar= 20 μm. Abbreviations R: retina, C: cornea.*$p<0.05$, ***$p < 0.001$.

Figure 2. Effect of single 670 nm exposure on *Drosophila*.

(**A**) CO_2 production was measured in control 6 week old flies and 6 week old flies treated with 670 nm for 90 min. Measurements were simultaneously to light exposure. During the exposure metabolic rate increased significantly compared with the controls. (**B**) ATP levels were measured in 6 week old flies and 6 week flies treated with 670 nm for 90 min. There were no differences between groups. (**C**) ROS levels were measured (absolute fluorescence). No changes in ROS levels were found between the groups (**D**) Locomotor function. After 90 min 670 nm exposure no differences were found between groups. Results are mean +SEM. *$p < 0.05$.

Figure 3. Effects of 670 nm exposure on mitochondrial parameters in *Drosophila.*

(**A**) Metabolic rate was assessed in lab-made respirometers. Results are shown as the rate of CO_2 production per hour per fly. At 6 weeks there is a significant reduction in metabolic rate compared with 2 week old flies. 670 nm exposure improved metabolic rate back to the level found at 2 weeks. (**B**) Whole fly ATP levels were measured. At 6 weeks there is a significant reduction in ATP content compared with 2 week flies. 670 nm exposure significantly improved ATP levels. (**C**) Mitochondrial DNA content. Results are the ratio of mtDNA to nuclear DNA. At 6 weeks there is a significant reduction in mtDNA compared with young flies. 670 nm exposure significantly increased mtDNA content to similar levels found in 2 week flies. (**D**) ROS levels were measured. Results are shown as fluorescent units. At 6 weeks there is a significant increase in ROS, which is significantly reduced with 670 nm treatment. There were no statistically significant differences in the assessed parameters between the 6 week old flies and flies treated with 670 nm but in blacked out vials (Control). Results are mean +SEM. mtDNA: mitochondrial DNA, nDNA: nuclear DNA. *$p < 0.05$, **$p < 0.01$.

Figure 4. Effects of 670 nm exposure on protein, lipids, carbohydrates in *Drosophila.*

(**A**) Total protein levels were measured in the 3 different groups with no differences were found. (**B**) Triglycerides were measured and represented as a ratio to protein. At 6 weeks there is a significant reduction triglycerides, but with 670 nm light exposure levels

increased to those found in young animals. (**C**) Glycogen and glucose levels were measured and also represented as a ratio. At 6 weeks there is a significant reduction in glycogen and glucose. 670 nm light exposure improved glycogen and glucose levels significantly. Increased glycogen levels did not reach levels found at 2 weeks. Results are shown as mean + SEM. *p < 0.05, **p < 0.01.

Figure 5. Effects of 670 nm on increased stress levels in *Drosophila.*

Effects of 670 nm on heat sensitivity (**A**) and chill coma recovery (**B** and **C**). (**A**) Percentage of flies surviving after 24 h of heat stress. At 6 weeks there is a significant reduction in survival in comparison to 2 week flies. 670 nm exposure did not change survival rates. (**B**) Chill coma recovery time (CCRT) that required for flies to stand at room temperature after 2h cold stress (0C). Cumulative incidence function of CCRT for 2 weeks, 6 weeks and 670 nm treated flies. Difference are apparent from 1-7 min. After 7 min, the curves are identical. Mean CCRT, 2 weeks=5.7 min, 6 weeks=6.3 min and 670 nm=5.3 min. The 670 group recovered faster compared to the non-treated flies (**C**) CCRT percentage difference in the first 8 min normalised to 2 week animals. 670 nm treated flies recover faster. Results are mean + SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 6. Effects of 670 nm on resistance to starvation in *Drosophila.*

Starvation resistance was assessed. (**A**) At 6 weeks there is a significant reduction in survival when flies are starvation challenged compared to 2 week old flies ($p<0.001$, log-rank test), where the mean lifespan drops from 100h to 50h. 670 nm did not increase survival (2 weeks vs 670 nm, $p<0.001$, log-rank test). Triglycerides (TAG), glycogen and glucose levels were measured in starving flies in each of the 3 groups at 24 and 48h (**B, C** and **D**). The improved levels of each found after 670nm exposure were rapidly undermined by starvation and in each there were no differences between aged animal and those exposed to 670nm. However, in each case both of these group were significantly reduced compared to young flies. Results are mean + SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 7. Consequences of 670 nm exposure on physiological and behavioural responses in *Drosophila*

(**A**) and (**B**) Flicker electroretinograms were recorded to assess retinal function. (**A**) Amplitudes of the ERG response normalised against the lowest frequency. This clearly shows the overall decline in amplitudes between 2 and 6 weeks. This also reveals the significant improvement in the ERG in 6 week old flies exposed to 670 nm of 1 week. One-tailed ANOVA, ($p=0.0166$). Mean + SEM. (**B**) Example ERG waveforms from 2 weeks, 6 weeks and 6 weeks exposed to 670 nm flies to flicker stimulus at progressive frequencies. With age, the amplitude of response declines, but this is improved by 670 nm exposure. (**C**) Climbing ability was tested. At 6 weeks there is a significant reduction in the percentage of flies that climb above the 8 cm mark. 670 nm significantly improve

the capacity of flies climb; however this improvement does not achieve that found in two weeks. (**D**) Memory was tested in an aversive photo tactic setting. At 6 weeks there is a significant reduction in the percentage of flies not being able to remember an association between light and an aversive stimulus compared with 2 week old flies. 670 nm increased the capacity of flies to remember the association.

Results are mean + SEM, except retinal function, mean + SD. *p < 0.05, **p < 0.01, ***p < 0.001.

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7. Competing financial interests

The authors declare no competing financial interests.

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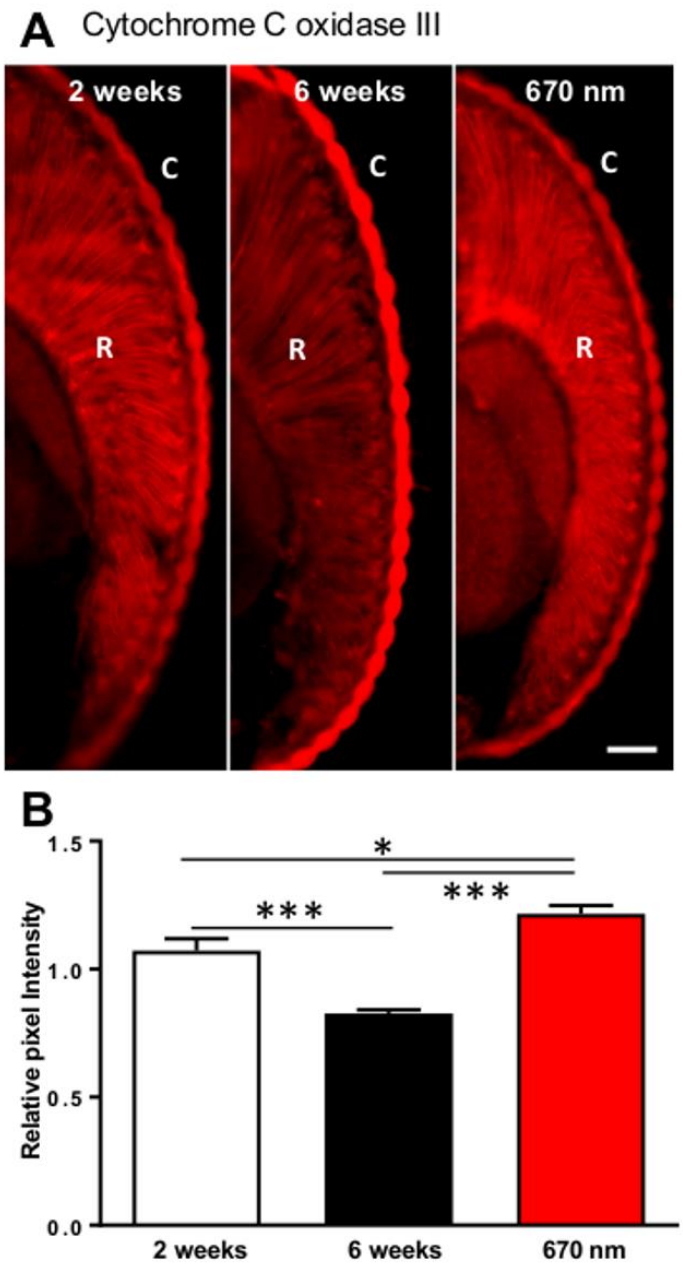
A
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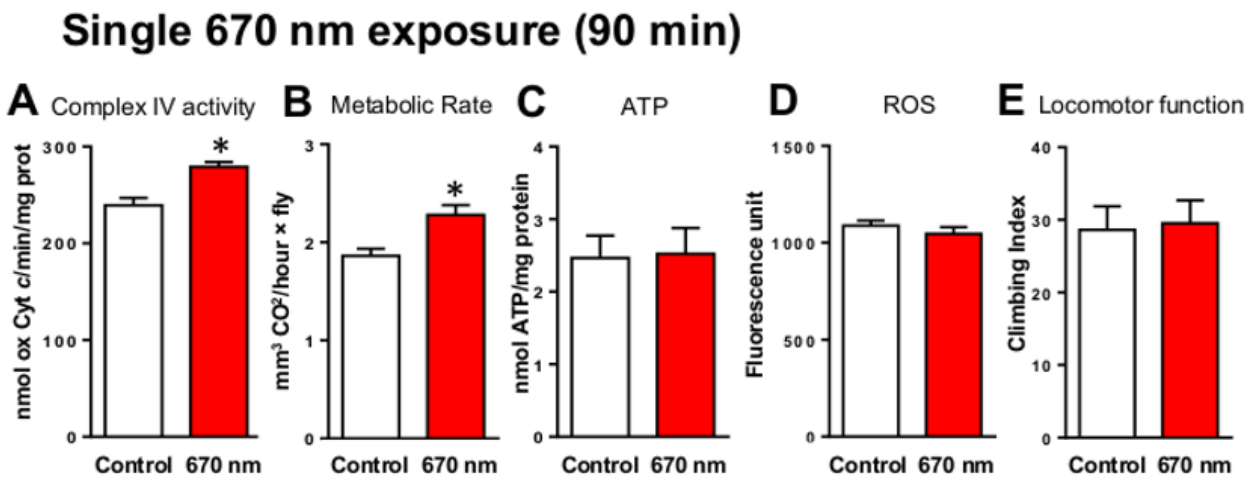
Single 670 nm exposure (90 min)
A Complex IV activity
nmol ox Cyt c/min/mg prot
300
200
100
0
*
Control
670 nm
B Metabolic Rate
mm³ CO²/hour × fly
3
2
1
0
*
Control
670 nm
C ATP
nmol ATP/mg protein
4
3
2
1
0
Control
670 nm
D ROS
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40
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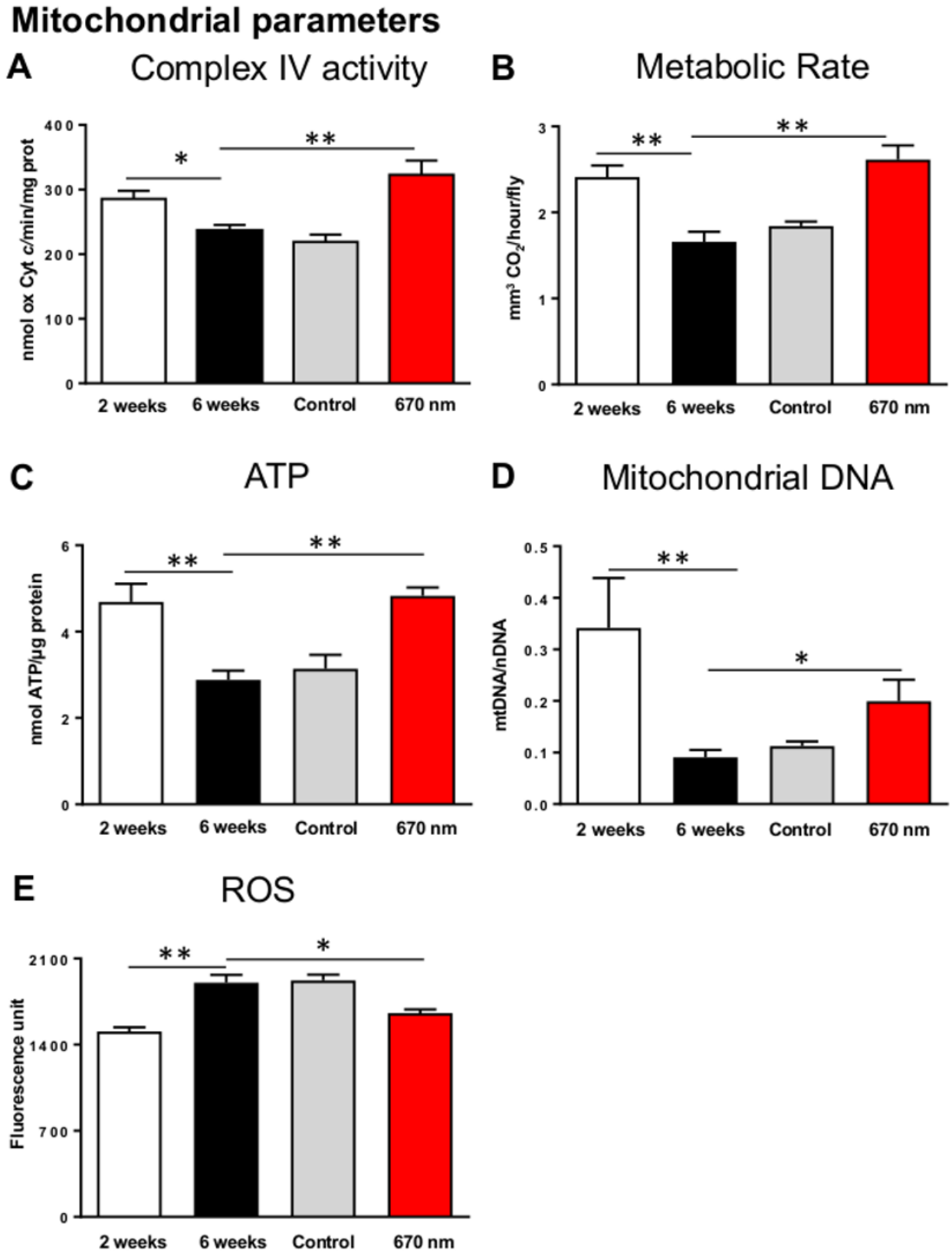
Mitochondrial parameters
A
Complex IV activity
nmol ox Cyt c/min/mg prot
400
300
200
100
0
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**
2 weeks
6 weeks
Control
670 nm
B
Metabolic Rate
mm³ CO₂/hour/fly
3
2
1
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**
2 weeks
6 weeks
Control
670 nm
C
ATP
nmol ATP/µg protein
6
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2 weeks
6 weeks
Control
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0.5
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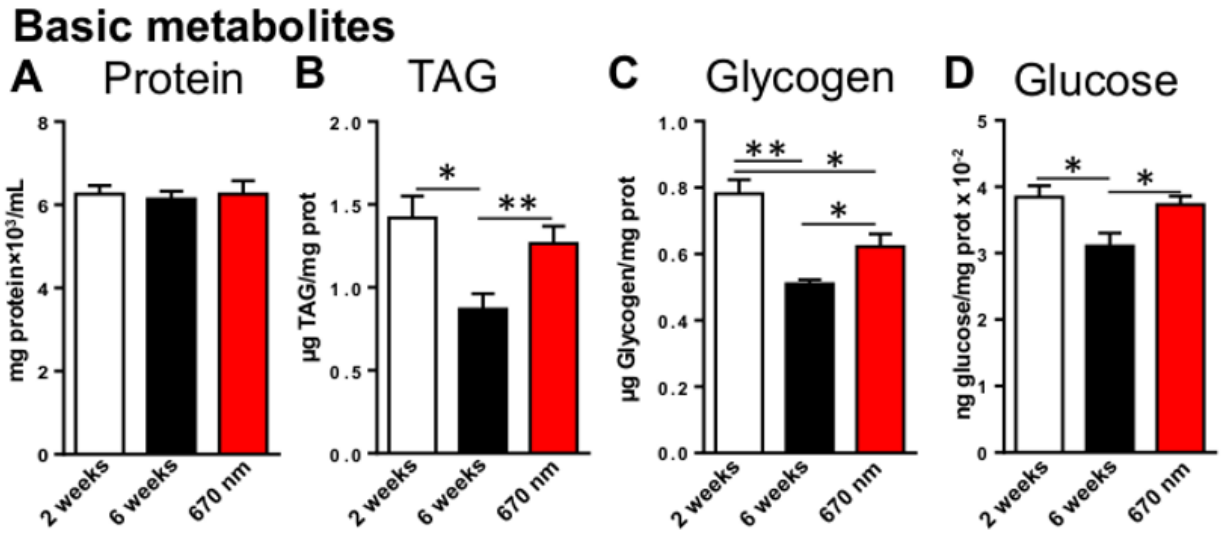
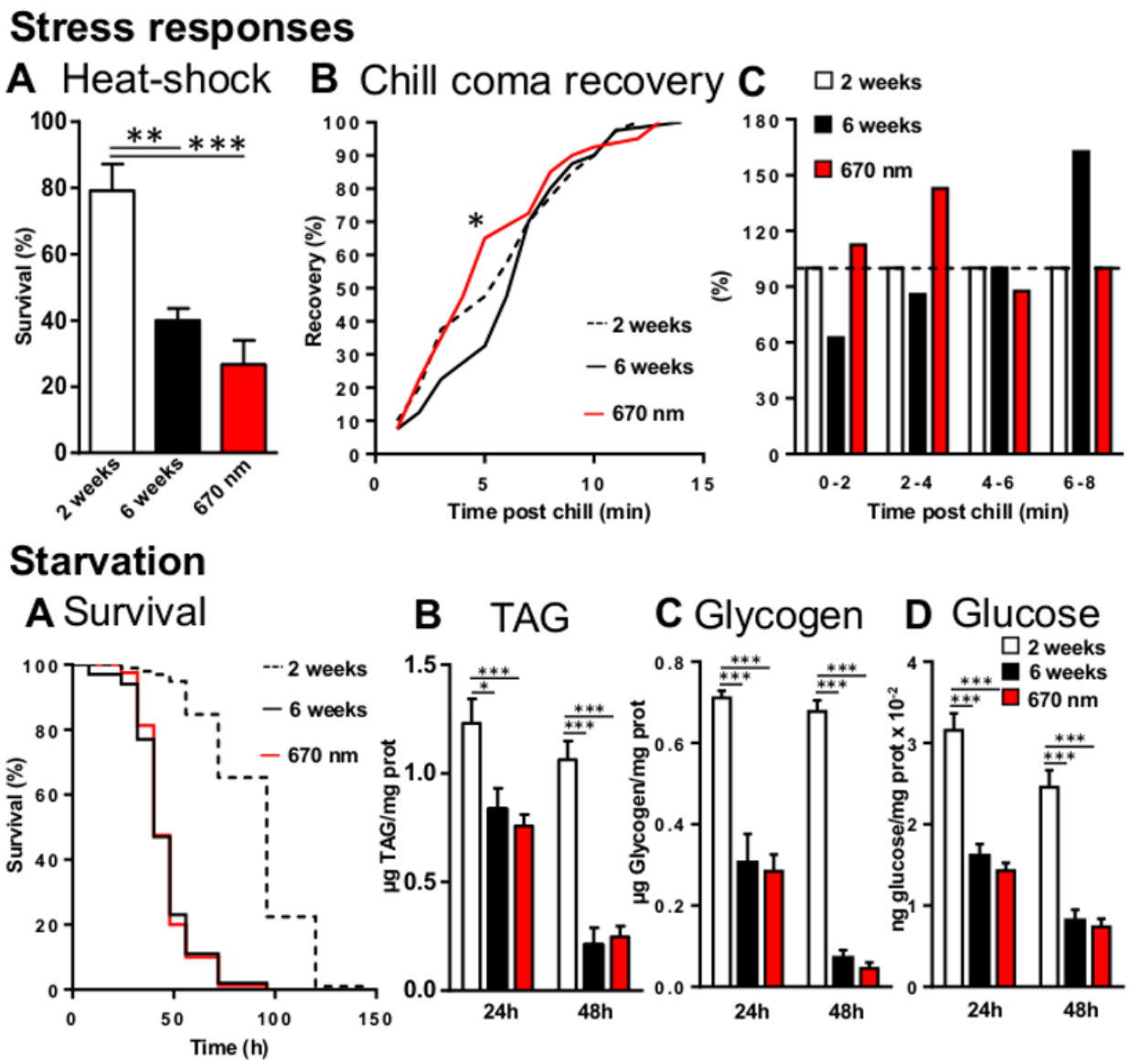

Basic metabolites
A Protein
B TAG
C Glycogen
D Glucose
mg protein×10³/mL
µg TAG/mg prot
µg Glycogen/mg prot
ng glucose/mg prot x 10⁻²
2 weeks
6 weeks
670 nm


Stress responses
A Heat-shock
B Chill coma recovery
C
2 weeks
6 weeks
670 nm
Survival (%)
Recovery (%)
Time post chill (min)
(%)
0-2
2-4
4-6
6-8
Starvation
A Survival
B TAG
C Glycogen
D Glucose
Time (h)
µg TAG/mg prot
µg Glycogen/mg prot
ng glucose/mg prot x 10-2
24h
48h

Physiology and behavioural responses
Visual Function