Light-harvesting chlorophyll pigments enable mammalian mitochondria to capture photonic energy and produce ATP

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Summary

Sunlight is the most abundant energy source on this planet. However, the ability to convert sunlight into biological energy as adenosine-5'-triphosphate (ATP) is thought to be limited to chloroplasts in photosynthetic organisms. Here we show that mammalian mitochondria can also capture light and synthesize ATP when mixed with a light-capturing metabolite of chlorophyll. The same metabolite fed to the worm Caenorhabditis elegans leads to increase in ATP synthesis upon light exposure, along with an increase in life span. We further demonstrate the same potential to convert light into energy exists in mammals, as chlorophyll metabolites accumulate in rodents when fed a chlorophyll-rich diet. Results suggest chlorophyll type molecules modulate mitochondrial ATP by catalyzing the reduction of coenzyme Q, a slow step in mitochondrial ATP synthesis. We propose that through consumption of plant chlorophyll pigments, animals, too, are able to derive energy directly from sunlight.

Introduction

Determining how organisms obtain energy from the environment is fundamental to our understanding of life. In nearly all organisms, energy is stored and transported as adenosine-5'-triphosphate (ATP). In animals, the vast majority of ATP is synthesized in the mitochondria through respiration, a catabolic process. However, plants have co-evolved endosymbiotically to produce chloroplasts, which synthesize light-absorbing chlorophyll molecules that can capture light to use as energy for ATP synthesis. Many animals consume light-absorbing chlorophyll through diet. Inside the body, chlorophyll is converted into a variety of metabolites (Ferruzzi and...
Blakeslee, 2007; Ma and Dolphin, 1999) that retain the ability to absorb light in the visible spectrum at wavelengths that can penetrate into animal tissues. We sought to elucidate the consequences of light absorption by these potential dietary metabolites. We show that dietary metabolites of chlorophyll can enter circulation, are present in tissues, and can be enriched in the mitochondria. When combined with a light-capturing metabolite of chlorophyll, isolated mammalian mitochondria and other animal derived tissues, have higher concentrations of ATP when exposed to light, compared to animal tissues not mixed with the metabolite. We demonstrate that the same metabolite increases ATP concentrations, and extends the median life span of *C. elegans*, upon light exposure; supporting the hypothesis that photonic energy capture through dietary derived metabolites may be an important means of energy regulation in animals. The presented data are consistent with the hypothesis that metabolites of dietary chlorophyll modulate mitochondrial ATP stores by catalyzing the reduction of Coenzyme Q. These findings have implications to our understanding of aging, normal cell function and life on earth.

**Results**

**Light-driven ATP synthesis in isolated mammalian mitochondria**

To demonstrate that dietary chlorophyll metabolites can modulate ATP levels, we examined the effects of the chlorophyll metabolite pyropheophorbide-a (P-a) on ATP synthesis in isolated mouse liver mitochondria in the presence of red light ($\lambda_{max} = 670$ nm), to which chlorin type molecules such as P-a strongly absorb (Aronoff, 19), and to which biological tissues are relatively transparent. We used P-a because it is an early metabolite of chlorophyll, however, most known metabolites of chlorophyll can be synthesized from P-a by chemistry that normally take place in animal cells. Control samples of mitochondria without P-a, and/or kept in the dark were also assayed. In the presence of P-a, mitochondria exposed to red light produce more ATP than mitochondria without P-a (Fig. 1A) or mitochondria kept in the dark (supplementary material Fig. S1A-D). Mitochondrial membrane potential, Fig. 1B, and oxygen consumption, Fig. 1C, both increased upon increased light exposure in P-a treated mitochondria. Light or P-a alone had no effect on any of the above measures of mitochondrial activity (supplementary material Fig. S1E-G). With too much added P-a, ATP concentrations and the rate of oxygen consumption started to return to levels that of mitochondria not incubated with P-a.
Addition of the electron transport inhibitor, sodium azide, reduced the light/P-a–fueled oxygen consumption by 57% (supplementary material Fig. S1H-I), consistent with oxygen consumption through the electron transport system. Observations were consistent with enhanced ATP production driven by oxidative phosphorylation.

To determine whether P-a associates with mitochondria, we measured P-a fluorescence at 675 nm in the presence of increasing amounts of heart mitochondrial fragments obtained from sheep (Fig. 2A and 2B). After increasing the concentration of mitochondria, P-a fluorescence increased abruptly, by 5-fold, and quickly reached a plateau (Fig. 2B). The abrupt change in fluorescence reflects a change in P-a’s environment, consistent with its change from an aqueous environment to one in which it is associated with a protein. This threshold-sensitive behavior is consistent with zero-order ultrasensitivity, or positively cooperative binding, as described by Goldbeter and Koshland (Goldbeter and Koshland, 1981), and suggests a coordinated interaction between the metabolite and mitochondrial fragments. In contrast, this threshold sensitivity was not observed when increasing amounts of bovine serum albumin was added to a solution of P-a; instead, fluorescence steadily increased (supplementary material Fig. S1J).

Catabolic reduction of CoQ_{10} is a rate limiting step in respiration (Crane, 2001). The majority of CoQ_{10} molecules exist in two alternate states of oxidation: ubiquinone, the oxidized form, and ubiquinol, the reduced form. To show the P-a metabolite could catalyze the photoreduction of mitochondrial CoQ_{10}, we measured the oxidation state of CoQ_{10} in the above sheep heart mitochondrial fragments in response to exposure to red light. We exposed the mitochondria to light for 10 minutes and measured the percent of reduced and oxidized CoQ_{10} by high performance liquid chromatography (HPLC) (Qu et al., 2013). In the isolated mitochondria fragments, nearly all the CoQ_{10} was oxidized in the form of ubiquinone. However, when we incubated the mitochondria with P-a and exposed the suspension to light, 46% of CoQ_{10} was reduced (Table 1, entry 1). In comparison, as a positive control, we energized the mitochondria with glutamate/malate and kept the suspension in the dark, yielding a 75% reduction of CoQ_{10} within 10 minutes (entry 2). In the absence of light, no reduction occurred (entry 3). Upon denaturing the mitochondrial proteins with heat, no reduction occurred (entry 4). Likewise, there was a lack of CoQ_{10} reduction with CoQ_{10}, P-a and light in the absence of mitochondria (entry...
These observations are consistent with the fluorescence data in Fig. 2A and B, showing that mitochondrial proteins sequester and organize P-a. In the absence of added P-a, a 2-14% reduction was observed, depending on the mitochondrial preparation used (entry 6). We attribute this “background reduction” to the actions of endogenous chlorophyll metabolites, which we were able to detect by fluorescence spectroscopy (see: Distribution of dietary chlorophyll, below).

**Light-driven ATP synthesis in rodent tissue homogenates**

To determine whether chlorophyll metabolites and light could influence ATP production in conditions that more closely resemble those found *in vivo*, we treated mouse brain homogenates with P-a and exposed them to 670-nm light. The treated brain homogenates synthesized ATP at a 35% faster rate than a control homogenate that was not incubated with P-a (Fig. 3A). No linear correlation between the increase in ATP concentrations and the amount of added P-a was observed. Increasing concentrations of P-a elicited the same increase in ATP (supplementary material Fig. S2A-B).

To demonstrate that photon absorption by P-a is necessary to enhance ATP production, we exposed the P-a–treated brain homogenates to greenish (500 nm) and red (630, 670 and 690 nm) light, all with the same total energy. As expected, the wavelengths of light that were strongly absorbed by P-a produced the largest increase in ATP. For example, the ATP concentration increased by approximately 16-fold during exposure to 670 nm light; relative to the same sample kept in the dark, it only increased by two-to-five-fold during exposure to 500, 630 and 690-nm light of equal energy (Fig. 3B).

In addition to brain homogenates, P-a also enhanced ATP production in adipose, lens and heart homogenates (supplementary material Fig. S2C-E). Quantification of ATP by both the luciferase assay and high-performance liquid chromatography (HPLC) gave similar results (supplementary material Fig. S2E-F).

**Distribution of light-absorbing dietary chlorophyll**

Chlorophylls and its metabolites, both chlorins, have signature absorption and admission spectra
Namely they absorb strongly ($\varepsilon \approx 50,000 \text{ M}^{-1} \text{ cm}^{-1}$) at approximately 665-670 nm and demonstrate intense fluorescence emissions at approximately 675 nm, which differentiate chlorins from endogenous molecules in mammals (Aronoff, 19). To examine whether dietary chlorophyll and/or its metabolites were present in animal tissue after oral consumption, we fed mice a chlorophyll-rich diet. Brain (Fig. 4A) and fat (Fig. 4C) extracts from these mice exhibited red fluorescence at 675 nm when excited with a 410-nm light. The excitation spectrum of this 675-nm peak (Fig. 4B) was similar to that of known chlorophyll metabolites with an intact chlorin ring: possessing maxima at 408, 504, 535, 562 and 607 nm. This red fluorescence diminished, as measured by the area under the 675 nm peak, when animals were given a chlorophyll-free diet for two weeks. Red fluorescence could also be seen using fluorescence imaging; it was stronger in the bodies and brains of animals fed chlorophyll than in animals given a chlorophyll-poor diet (Fig. 4D). The red fluorescence was enriched in the gut and intestines, consistent with dietary chlorophyll being the source of the fluorescence.

To determine if the red fluorescence was localized to mitochondria, we measured the relative 675-nm fluorescence in whole liver homogenates and mitochondria isolated from these homogenates. As measured by fluorescence intensity, isolated mitochondria contained 2.3-fold as much of the 675-nm fluorescent metabolite/s per milligram of protein as did the whole liver homogenate. This observation suggests that P-a was concentrated in the mitochondria, consistent with data summarized in Fig. 2A and B, and literature reports (MacDonald et al., 1999; Tang et al., 2006)

Fat and plasma extracts from rats fed chlorophyll-rich diets were further analyzed by HPLC to elucidate the source of the red 675-nm fluorescence. Fig. 5A shows a representative chromatogram where we detected compounds in the eluting solvent that displayed 675-nm fluorescence when excited with 410-nm light. Rat fat extracts and plasma extracts both contained similar chlorophyll-derived metabolites and had similar chromatograms (not illustrated). Two groups of compounds eluting at 23-30 min and 40-46 min were detected. Compounds eluting between 23-30 min had similar retention times to those of the chlorophyll metabolites without the phytol tail, possessing at least one carboxylate group such as P-a. The absorption spectra (the
locations of the absorbance maxima and the Soret-to-Q_y-band ratios) of this group of compounds were consistent with demetalated chlorophylls (Rabinowitch, 1944), as shown in Fig. 5B. In addition, this group of peaks displayed spectra indicative with coordination to a metal ion. A representative spectrum of such a metalated metabolite is shown in Fig. 5C, showing a red shifted Soret band, a blue shifted Q_y-band and a Soret-to-Q_y-band ratio of approximately 1.

Compounds between 40-46 min eluted with similar retention times to that of the demetalated chlorophyll-a standard (pheophytin-a). In addition, these compounds portioned in hexanes (polarity index = 0.1) when mixed with hexanes and acetonitrile (polarity index = 5.8). This latter characteristic is consistent with a lack of a carboxylic acid group, or an esterified P-a, such as pheophytin-a. Similar HPLC chromatograms from fat extracts of swine fed chlorophyll rich diets (Mihai et al., 2013) were recorded (supplementary material Fig. S2G), suggesting that uptake and distribution of chlorophyll metabolites were not unique to mice and rats.

We quantified total blood pigments in the rat absorbing at 665 nm. Using an extinction coefficient of 52,000 at 665 nm (Lichtenthaler, 1987), which is typical of chlorophyll a derived pheophytins, we estimated a plasma concentration of 0.05 μM in two rats fed a chlorophyll-rich diet. The 665-nm peak was absent in animals fed a chlorophyll-poor diet. The amount of measured total metabolite is five- and two-times higher than that reported in the rat for the fat soluble vitamins K (Tovar et al., 2006) and D (Halloran and DeLuca, 1979), respectively.

**Light-driven ATP synthesis in C. elegans**

Next, we used *C. elegans* to evaluate the effects of light-stimulated ATP production in a complex organism. As *C. elegans* ages, there is a drop in cellular ATP (Braeckman et al., 1999; Braeckman et al., 2002). We hypothesized that the worm would live longer if it could offset this decline in ATP by harvesting light energy for ATP synthesis. As our model system, we used firefly luciferase-expressing *C. elegans*; upon incubation with luciferin, these worms emit a luminescence that is proportional to their ATP pools (Lagido et al., 2009; Lagido et al., 2008; Lagido et al., 2001). Upon incubation with P-a, worms incorporated the metabolite, as measured by fluorescence spectroscopy (supplementary material Fig. S3A). To determine whether there were changes in ATP stores in response to light, we plated two groups of worms into 96-well plates containing luciferin substrate. We measured worm luminescence to obtain time zero. We
then exposed one group to 660-nm light and kept the other in the dark and periodically measured luminescence in both groups of worms (summarized in Fig. 6A and 6B). To determine whether ATP increased in light-exposed animals, we subtracted the luminescence signal of the worms kept in the dark from that of the worms exposed to light (Fig. 6C). Worms that were given P-a displayed a statistically significant increase in ATP when exposed to light, while control worms showed no increase. The metabolite alone had no effect on ATP levels when the worms were kept in the dark (i.e., luminescence intensity remained constant throughout the experiment). The elevated luminescence signal persisted for one hour after the light was turned off, at which time measurement ceased. However, the luminescence intensity did not further increase during the time the light was off. It is currently unclear whether this persistent signal reflected the kinetics of the luciferase-luciferin reaction, luciferase expression, or actual ATP pools. Thus ATP was quantified by additional methods.

Alternatively, to determine whether light stimulated ATP synthesis, we plated luciferase-expressing worms into a 96-well plate without the luciferin substrate, and exposed them to light. ATP status was determined at time zero, immediately before light exposure, and at 15-minute intervals for a total of 45 minutes by adding the luciferin substrate to a group of worms and measuring luminescence (Fig. 6D and 6E). Using this method, we measured an increase in ATP when 5-day-old and 10-day-old adult worms were fed the metabolite and exposed to light.

We further confirmed the in vivo increase in ATP using two additional ex vivo methods. After light treatment, we lysed the worms, extracted their ATP and quantified ATP in the homogenate using either the firefly luciferase assay or HPLC (supplementary material Fig. S3B-C). Both methods were consistent with the in vivo ATP measurements.

In addition to an increase in ATP, worms treated with P-a exhibited a 13% increase in respiration when exposed to light, as measured by oxygen consumption. On the other hand, light had no effect on the respiration rates in untreated worms (supplementary material Fig. S3D). This observation is consistent with an increase in ATP through oxidative phosphorylation, in accordance with the mitochondrial data. Despite the increase in ATP, the levels of reactive oxygen species (ROS) were equivalent in treated and untreated worms during 5 h of light
exposure, as measured using 2\(^{-}\),7\(^{-}\)-dichlorofluorescin diacetate (supplementary material Fig. S3E). In fact, although the difference was not statistically significant, treated worms exhibited, on average, lower levels of ROS.

**Light harvesting to extend life span**

We next tested whether photonic energy absorption by P-a could prolong life. Life span measurements were taken in liquid cultures according to Gandhi et al. (Gandhi et al., 1980) and Mitchell et al. (Mitchell et al., 1979). Adult worms were incubated with P-a for 24 hours. Beginning at day 5 of adulthood, we exposed the worms to red light in a daily 5:19 light:dark cycle. Control worms were not given P-a or were not exposed to light, but otherwise kept under identical conditions. Counts were made at 2-3 day intervals and deaths were assumed to have occurred at the midpoint of the interval. To obtain the half-life, we plotted the fraction alive at each count verses time and fitted the data to a two-parameter logistic function, known to accurately fit survival of 95% of the population (Vanfleteren et al., 1998). The group treated with P-a and light had a 17% longer median life span than the group that was not treated with P-a, but exposed to light (Fig. 7A and 7B). P-a treatment alone, in the absence of light, had no effect on life span (supplementary material Fig. S4B). Light treatment alone decreased life span by 10% (supplementary material Fig. S4B), in accordance with reports that nematodes survive better in complete darkness (Thomas, 1965). This decrease in median life span brought on by light was reversed when the worms were treated with P-a. The increased median life span with light and P-a was reproducible with different batches of worms (supplementary material Fig. S4B-E).

Increasing the amount of P-a past a certain threshold, however, lead to a gradual decrease in lifespan approaching that of animals not treated with P-a (supplementary material Fig. S4B-C).

We also examined life span longitudinally. We placed 6-day-old adult P-a- and non-P-a-treated worms into a 96-well plate, exposed them to red light for 5 h per day and compared the percent dead and alive after 15 days. Result: 47% of the P-a-treated worms were alive (175 alive; 200 dead) after 15 days, vs. 41% of the control worms (111 alive; 163 dead), consistent with the cross-sectional experiments above.

**Discussion**
Photoreduction of coenzyme q

Here, upon incubation of: 1) isolated mouse mitochondria; 2) mouse brain, heart and lens homogenates; 3) homogenated duck fat, and 4) the in vivo C. elegans, with a representative metabolite of chlorophyll, light exposure was able to increased ATP concentrations. These observations in a variety of animal tissues perhaps demonstrate the generality of this phenomenon. To synthesize ATP, mitochondrial NADH reductase (complex I) and succinate reductase (complex II) extract electrons from NADH and succinate, respectively. These electrons are used to reduce mitochondrial CoQ$_{10}$, resulting in ubiquinol (the reduced form of CoQ$_{10}$). Ubiquinol shuttles the electrons to cytochrome c reductase (complex III), which uses the electrons to reduce cytochrome c, which shuttles the electrons to cytochrome c oxidase (complex IV), which ultimately donates the electrons to molecular oxygen. As a result of this electron flow, protons are pumped from the mitochondrial matrix into the inner membrane space, generating a trans-membrane potential used to drive the enzyme ATP-synthase.

The “pool equation” of Kröger and Klingenberg describes the total rate of electron transfer: $V_{\text{obs}} = V_{\text{ox}} \times V_{\text{red}} / (V_{\text{ox}} + V_{\text{red}})$, where $V_{\text{red}}$ signifies CoQ$_{10}$ reduction and $V_{\text{ox}}$, ubiquinol oxidation (Kroger and Klingenberg, 1973). Based on this equation, the major roles of complexes I and II can be viewed as mechanisms to maintain the mitochondrial ubiquinol pool, and an additional mechanism to reduce ubiquinone should result in increased ATP synthesis. We reasoned the reduction of CoQ$_{10}$ could be a potential step in the respiratory pathway where chlorophyll metabolites could influence ATP levels, as the photoreduction of quinones by chlorophyll-type molecules is known (Chesnokov et al., 2002; Okayama et al., 1967). Indeed, a primary step during photosynthesis is the reduction of the quinone, plastoquinone, by a photochemically excited chlorophyll a (Witt et al., 1963). We hypothesized that if the reduction of mitochondrial ubiquinone could be catalyzed by a photoactivated chlorophyll metabolite, such as P-a, then ATP synthesis would be driven by light in mitochondria with these dietary metabolites. In the proposed mechanism, electrons would be transferred by a metabolite of chlorophyll to CoQ$_{10}$, from a chemical oxidant present in the mitochondrial milieu. Many molecules, such as dienols, SH-compounds, ferrous compounds, NADH, NADPH, and ascorbic acid, could all potentially act as electron donors. Throughout mammalian evolution, red photons from sunlight have been
present deep inside almost every tissue in the body. Photosensitized electron transfer from excited chlorophyll-type molecules is widely hypothesized to be a primitive form of light-to-energy conversion that evolved into photosynthesis (Krasnovsky, 1976). Thus it is tempting to speculate that mammals possess conserved mechanisms to harness photonic energy.

Photoexcitation of chlorophyll and derivatives produces the excited singlet state (*1). Oxidative quenching of this singlet state by ubiquinone is possible. Electron transfer could take place through protein or solution. Escape from the charge transfer complex and protonation would yield ubisemiquinone, which accounts for 2-3% of the total ubiquinone content of mitochondria (De Jong and Albracht, 1994). Ubisemiquinone can be reduced to ubiquinol by repeating the above sequence or by disproportionation to give one molecule of ubiquinol and one molecule of ubiquinone. Back-electron transfer, from the photoreduced metabolite to the oxidized quinone, could be inhibited by disproportionation or by organizing the chlorophyll derivative and ubiquinol through protein binding. In line with the CoQ10 photoreduction hypothesis, we observed mitochondrial CoQ10 was reduced when isolated mitochondria were exposed to light and P-a (Table 1). Also consistent with light/P-a acting upstream of complexes I and II, in isolated mitochondria we observed an increase in ATP in the absence of added electron transport substrates, such as glutamate and malate (Figures 1A, S1A-C). However, further evidence is needed to confirm this mechanistic hypothesis.

**Photons in vivo**

Intense red light between 600 and 700 nm has been reported to modulate biological processes (Hashmi et al., 2010; Passarella et al., 1984; Wong-Riley et al., 2005), and has been investigated as a clinical intervention to treat a variety of conditions (Hashmi et al., 2010). Exposure to red light is thought to stimulate cellular energy metabolism and/or energy production by poorly defined mechanisms (Hashmi et al., 2010). In the presence of P-a, we observed changes in energetics in animal derived tissues initiated with light of intensity and wavelengths (∼670 nm at ∼0.8 ± 0.2 W/m²) that can be found in vivo when outdoors on a clear day. On a clear day the amount of light illuminating your brain would allow you to comfortably read a printed book (Benaron et al., 2002). In humans, the temporal bone of the skull and the scalp attenuate only 50% of light at a wavelength of approximately 670 nm (Eichler et al., 1977; Wan et al., 1981). In
small animals, light can readily reach the entire brain under normal illumination (Berry and Harman, 1956; Brunt et al., 1964; Massopust and Daigle, 1961; Menaker et al., 1970; Vanbrunt et al., 1964). Sun or room light over the range of 600 to 700 nm can penetrate a 1.5-inch-thick abdominal wall with only three-to-five orders of magnitude attenuation (Bearden et al., 2001; Wan et al., 1981). Photons between 630 and 800 nm can penetrate 25 cm through tissue and muscle of the calf (Chance et al., 1988). Adipose tissue is bathed in wavelengths of light that would excite chlorophyll metabolites (Bachem and Reed, 1931; Barun et al., 2007; Zourabian et al., 2000). Thus, identification of pathways, which might have developed to take advantage of this photonic energy, may have far-reaching implications.

**Dietary chlorophyll in animals**

A potential pathway for photonic energy capture is absorption by dietary derived plant pigments. Little is known about the pharmacokinetics and pharmacodynamics of dietary chlorophyll or its chlorin-type metabolites in human tissues. Here, we observed the accumulation of chlorin type molecules in mice, rats, and swine administered a diet rich in plant chlorophylls (Figures 4, 5 and S2G). Data suggests that sequestration of chlorophyll-derived molecules, which are capable of absorbing ambient photonic energy, from diets might be a general phenomenon.

To date, the reported chlorophyll metabolites isolated from animals have been demetalated (Egner et al., 2000; Fernandes et al., 2007; Scheie and Flaoyen, 2003). The acidic environment of the stomach is thought to bring about loss of chlorophyll’s magnesium (Ferruzzi and Blakeslee, 2007; Ma and Dolphin, 1999). Our absorbance data of extracted pigments from rat fat is consistent with the presence of chlorophyll metabolites bonded to a metal (Figure 5). If true, the presence of a metal derivative in fat tissue suggests that the pigment was actively remetalated to take part in coordination chemistry. The identification of several metabolites in the fat and plasma of rats and swine fed a chlorophyll-rich diet is consistent with what is found in plants: several metabolites are present and play active roles in plant maintenance. The structures of the metabolites remain to be elucidated. Chlorin-type molecules are similar in structure and photophysical properties and thus can carry out similar photochemistry (Gradyushko et al., 1970). Our data demonstrates that dietary metabolites of chlorophyll can be distributed throughout the body where photon absorption may lead to an increase in ATP as demonstrated for the chlorin P-a. Indeed, P-a could have been transformed into other metabolites, as most
known metabolites of chlorophyll can be formed from P-a by chemistry that normally takes place in animal cells.

There was not a linear relation between the increase in ATP and the amount of added P-a (Figure S2A and B). ATP stimulation by light in the presence of P-a better fit a binary on / off, rather than a graded response to P-a. Increasing concentrations of P-a elicited the same increase in ATP, after light exposure. However, with too much added P-a, ATP levels began to fall. This on / off response was also consistent with the observed cooperative binding mode of P-a with mitochondria fragments, suggesting that the threshold response may be regulated by mitochondrial binding of P-a. If chlorophyll metabolites are found to be involved in energy homeostasis, a better understanding of their pharmacodynamics and pharmacokinetics will be needed.

**ATP stores and life span**

Light of 670 nm wavelength that penetrates the human body, yields approximately 43 kcal/mol (1.18 x 10^{-22} kcal per photon). Given estimated concentrations of chlorophyll derivatives in the body (Egner et al., 2000; Fernandes et al., 2007; Scheie and Flaoyen, 2003) and the photon flux at 670 nm (Bachem and Reed, 1931; Barun et al., 2007; Bearden et al., 2001; Benaron et al., 2002; Chance et al., 1988; Eichler et al., 1977; Menaker et al., 1970; Vanbrunt et al., 1964; Wan et al., 1981; Zourabian et al., 2000), each chlorophyll metabolite would be expected to absorb only a few photons per second. As such, one might anticipate negligible amounts of additional energy. Organization of chlorophyll metabolites into supramolecular structures, like chlorophyll antenna systems in photosynthetic organisms, would increase the effective cross section of photon absorption and, thus, photon catch. Indeed, our observed positively cooperative binding with mitochondrial fragments is evidence for such organization. Even so, to approach the rate of ATP synthesis powered by NADH or FADH_{2}, sufficient P-a pigment would have to be added to turn animals green. Nevertheless, in model systems, we measure an increase in ATP upon light absorption and changes in fundamental biology (extention in life span). Regardless of the mechanism by which ATP is increased or the measured amount of the increase, the larger question is: how much of an increase in ATP is enough to make a biological difference?
In animals, treatment with P-a and light both increased ATP and median life span suggesting that light in the presence of these light absorbing dietary metabolites can significantly fundamental biological processes. We previously observed that chlorophyll metabolites enabled photonic energy capture to enhance vision using a mouse model (Isayama et al., 2006; Washington et al., 2004; Washington et al., 2007). Because ATP can regulate a broad range of biological processes, we suspect that ATP modulation also played a role in vision enhancement. The increase in life span may seem contradictory, given that there are observations suggesting limiting metabolism and ATP synthesis increases this worm’s life span. It has been proposed that the life span of this worm is determined by the metabolic status during development (Dillin et al., 2002) and that there is a coupling of a slow early metabolism and longevity (Lee et al., 2003). Observations have led to the hypothesis that increased life span may be achieved by decreasing total energy expenditure across the worm’s entire life span (Van Raamsdonk et al., 2010). However, most studies decrease ATP synthesis from hatching through genetic engineering. By contrast, here, we were able to increase ATP during adulthood at a time when ATP stores reportedly begin to decline. For example, by day four of adulthood, the level of ATP and oxygen consumption can drop by as much as 50% compared to day zero (Braeckman et al., 1999; Braeckman et al., 2002). This difference in timing may account for why we observe an increase in life span in response to an increase in ATP. We note that besides caloric restriction, there are only a few interventions that are known (Petrascheck et al., 2007) to increase life span when given to an adult animal.

Alternative mechanisms of life-span extension cannot be ruled out. For example, an increase in reactive oxygen species (ROS) is thought to increase life span in C. elegans (Heidler et al., 2010; Schulz et al., 2007). Upon photon absorption, metabolites of chlorophyll can transfer energy to oxygen, resulting in the generation of singlet oxygen, a ROS. Thus lifespan extension seen here might be a result of an increase in ROS due to the generation of singlet oxygen. However, our published data with blood plasma (Qu et al., 2013) and data here from C. elegans do not show an increase in ROS. As ubiquinol is a potent lipid antioxidant (Frei et al., 1990) any ROS increase might be offset by an increase in ubiquinol generated from the photo-reduction of coenzyme Q. Indeed, by producing ubiquinol, P-a might have alternatively increased life span by protecting against long-term oxidative damage, also a mechanism that has been shown to increase C. elegans life span.
Conclusion

Both increased sun exposure (Dhar and Lambert, 2013; John et al., 2004; Kent et al., 2013a; Kent et al., 2013b; Levandovski et al., 2013) and the consumption of green vegetables (Block et al., 1992; Ferruzzia and Blakesleeb, 2007; van't Veer et al., 2000) are correlated with better overall health outcomes in a variety of diseases of aging. These benefits are commonly attributed to an increase in vitamin D from sunlight exposure and consumption of antioxidants from green vegetables. Our work suggests these explanations are incomplete. Sunlight is the most abundant energy source on this planet. Throughout mammalian evolution, the internal organs of most animals, including humans, have been bathed in photonic energy from the sun. Do animals have metabolic pathways that enable them to take greater advantage of this abundant energy source? The demonstration that: 1) light-sensitive chlorophyll-type molecules are sequestered into animal tissues; 2) in the presence of the chlorophyll metabolite P-a, there is an increase in ATP in isolated animal mitochondria, tissue homogenates and in the C. elegans, upon exposure to light of wavelengths absorbed by P-a; and 3) in the presence of P-a, light alters fundamental biology resulting in an up to 17% extension of life span in the C. elegans suggests that, similarly to plants and photosynthetic organisms, animals also possess metabolic pathways to derive energy directly from sunlight. Additional studies should confirm these conclusions.

MATERIALS AND METHODS

General

Two light sources were used for all experiments, either a 300 W halogen lamp equipped with a variable transformer and band pass interference filters (500, 632, 670, 690 nm with full width-half maximum [FWHM] of 10 nm) or a 1.70 W, 660 nm, LED light bulb. Luminous power density was set to 0.8 ± 0.2 W/m² as measured by a LI-250A light meter (LI-COR Biosciences, Lincoln, NE). The intensity of red light used was 30–60 times less than the level of red light that we measured on a clear March afternoon in New York City and is less than the level of what several organs are exposed to in vivo. Pyropheophorbide-a (P-a, 95% purity) was obtained from...
Frontier Scientific, Logan, UT. For all experiments, prior to exposing samples to light, we minimized light exposure by preparing samples/experiments with laboratory lights turned off, using a minimum amount of indirect sunlight that shown through laboratory windows (>0.001 W/m²).

Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University. Mice (ICR, Charles River, Wilmington, MA) weighing 22–28 g were used. Rats (Fisher 344, Harlan Teklad, Indianapolis, IN), weighing 300 g were used. Swine, fed a chlorophyll rich diet have been described (Mihai et al., 2013).

**Continuous ATP monitoring in isolated mouse liver mitochondria**

Animals were fed a chlorophyll-poor, purified rodent diet supplied by Harlan (Indianapolis, IN) for a minimum of 2 weeks. We isolated mouse liver mitochondria by differential centrifugation according to existing procedures (Frezza et al., 2007) and used only preparations with a minimum respiratory control ratio above 4.0 [state III/II, using glutamate (5 mM final) and malate (2.5 mM final) as measured by an oxygen electrode from Qubit Systems Inc., Kingston, ON, Canada]. Mitochondria at a final concentration of ≈ 1 mg protein/mL as determined by the Coomassie Plus (Bradford) protein assay (Thermo Fisher Scientific, Rockford, IL) in buffer A (see below) were incubated with P-a for 30 min at 0 °C. ADP was added (0.5 mM final concentration) and then 250 µL aliquots of this suspension were placed in 9 wells of a 96-well plate for light exposure at room temperature. At various times, 20 µL aliquots were withdrawn, added to 150 µL lysis buffer (see below), and ATP levels were determined with a commercial kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Controls were treated in the same way, except they: a) were incubated at 0 °C without P-a (shown), b) not exposed to light and c) were incubated without P-a and not exposed to light.

Buffer A: 0.250 M mannitol, 0.02 M HEPES, 0.01 M KCl, 0.003 M KH₂PO₄, 0.0015 M MgAc₂·H₂O, 0.001 M EGTA, 1 mg/mL fatty acid–poor bovine serum albumin (BSA), pH 7.4. Lysis buffer: 10 mM Tris, pH 7.5; 100 mM NaCl; 1 mM EDTA and 1% Triton X-100.

**Membrane potential**
Mitochondrial membrane potential was monitored in buffer A as described by Feldkamp et al. (Feldkamp et al., 2005). Measurements were made in a 3 mL cuvette placed inside a fluorescence spectrometer (Fluorormax-4, HORIBA Jobin Yvon, Horiba Scientific, Kyoto, Japan) with a final reaction volume of 1 mL. For light exposure, we used a fiber optic light guide to capture and direct light from a 660 nm LED light bulb into the spectrometer. The end of the fiber optic cable was positioned 1 cm above the reaction mixture. Prior to these experiments, light power was measured 1 cm from the end of the fiber optic cable.

Oxygen consumption
Mitochondrial oxygen consumption was measured using an oxygen electrode cuvette (OX1LP-1 mL, Qubit Systems Inc. Kingston, ON, Canada) according to the manufacturer’s instructions. Reactions were run with mitochondria at a concentration of ≈ 1 mg protein/mL in buffer A. For light exposure, a 660-nm LED was directed at the plastic (Poly-(methyl methacrylate)) chamber. For inhibition of respiration, sodium azide was added at a final concentration of 0.005 M from a stock solution in water. Sodium azide inhibits cytochrome oxidase (Complex IV); oxygen consumption during state 3 respiration is progressively inhibited by increasing concentrations of azide (Bogucka and Wojtczak, 1966).

Zero-order ultrasensitivity
Sheep heart mitochondria was prepared according to literature (Smith, 1967) on two separate occasions from 2 and 1 sheep heart/s using “Procedure 1”. We used mitochondrial fragments to allow P-a direct access to the respiratory chain, to minimize potential complications due to variable rates of P-a import. Mitochondrial isolation started within one hour after sacrifice and the hearts were transported to the laboratory in an bath of 0.25 M sucrose, 0.1 M tris(hydroxymethyl)aminomethane (Tris) at pH 7.5, which was surrounded by ice. Mitochondria was isolated and stored in 250 μL aliquots at a concentration of approximately 60 mg of protein per mL in 300 mM trehalose, 10mM HEPES–KOH pH 7.7, 10 mM KCl, 1 mM EGTA, 1 mM EDTA and 0.1% BSA at -80°C (Yamaguchi et al., 2007) until use. The thawed mitochondria exhibited RCR of approximately 1, indicating mitochondrial fragmentation.
Coenzyme Q redox status

We used sheep heart mitochondria because it contains relatively large amounts of CoQ₁₀, which expedited analysis. For evaluation of CoQ₁₀ redox ratios, frozen mitochondria was thawed at 37°C and diluted with 500 μL buffer A to create a mitochondrial stock solution, which was kept on ice until use. For reactions, 50 μL of this stock suspension was added to 500 μL of buffer A, containing 0.5 μg / mL antimycin A from a 25 μg / mL stock solution in ethanol. Antimycin A binds to the Q₁ site of cytochrome c reductase (complex III), thereby inhibiting the upstream oxidation of any produced ubiquinol. Pa was added (25 μM final concentration) from a 1.3 mg / mL stock solution in DMSO. The suspension was added to a test tube, mixtures purged with argon and the reactions initiated by placing the tube between two LED light bulbs (previously described). We irradiated the samples for 10 minutes at room temperature. For negative controls, we repeated the above sequence changing the following parameters: 1) in the absence of light; 2) in the absence of added P-a; 3) with heat denatured mitochondria; and 4) in the absence of added mitochondria but with added Coenzyme Q. For a positive control we added 10 μL of a stock solution of 0.25 M glutamate / 0.125 M malate in Tris buffer at pH 7. For mitochondrial denaturing, 200 μL of mitochondria stock was purged with argon and placed in a bath at 70°C for 5 minutes. For control reactions without added mitochondria, a Coenzyme Q stock solution in Buffer A was prepared by adding ALL-QTM (DSM Nutritional products, Switzerland), a water soluble coenzyme Q solution containing 10% coenzyme Q, modified food starch, sucrose and medium chain triglycerides, to buffer A. For these reactions 50 μL of the water-soluble Coenzyme Q stock or the denatured suspension was used as above in place of the mitochondrial stock solution. All reactions were adjusted to give the same amount of Coenzyme Q in the reaction mixture as measured by HPLC.

To quantify relative ubiquinone and ubiquinol concentrations, a 50 μL aliquot was taken from the reaction mixture and was added to 200 μL of 0.4M perchloric acid and 100 μL isopropyl ether containing 1 mg of butylated hydroxytoluene / ml as an antioxidant. The solution was vortexed for 1 minute, centrifuged for 2 min at 15,000 r.p.m and the organic phase analyzed by HPLC. HPLC conditions have been reported (Qu et al., 2009; Qu et al., 2011). Briefly, we used an isocratic elutent consisting of 1% sodium acetate 3% glacial acetic acid, 5% butanol in methanol at 0.6 mL per minute. The HPLC column was 50 x 2.1 mm, C-18, 2.6 u, 100Å
A PDA detector set at 290 nm for ubiquinol and 275 nm for ubiquinone was used. We determined relative ubiquinol and ubiquinone concentrations by their online absorption spectra using extinction coefficients of 14,200 M$^{-1}$ cm$^{-1}$ at 275 nm in ethanol for ubiquinone and 4,640 M$^{-1}$ cm$^{-1}$ at 290 nm in ethanol for ubiquinol (Lester et al., 1959).

**ATP synthesis in mouse brain homogenates**

To produce brain homogenates, the frontal lobe was homogenized using 2 strokes of a Potter S homogenizer (Sartorius AG, Goettingen, Germany) at 4 °C (20 mg of brain to 1 mL buffer A). The homogenate (80 μL) was added to buffer A (920 μL) and treated as described above for liver samples. Reactions were run in triplicate and data obtained between 5 and 50 min after lysis. ATP production showed a linear increase during this time, which was fitted to a line, the slope of which is reported as the relative ATP synthesis rate.

**ATP synthesis in mouse lens and heat homogenates**

Lenses were homogenized (KONTES® DUALL® tissue grinder with glass pestle) in ATP assay buffer (0.15 mM sucrose, 0.5mM EDTA, 5mM magnesium chloride, 7.5 mM sodium phosphate, 2mM HEPES) at 50 μL buffer per lens. We added 1 μL of P-a stock (1 mM) and 1 μL of ADP stock (10 mM) were added into 100 μL lens homogenate. The mixture was exposed to red light (671 nm at 0.8 W/m²) or kept in dark for 20 minutes. ATP concentrations were determined using a luciferase based ATP quantification kit according to the manufactures instructions (Life Technologies, Grand Island, NY).

Heart tissue (20 mg) was homogenized as above in 1 ml ATP assay buffer. 10 μL of P-a (1 mM) and 10 μL of ADP (10 mM) and 940 μL of ATP assay buffer were added into 40 μL tissue homogenate. The mixture was exposed to red light and ATP was determined as described above using a luciferase based ATP kit.

**ATP concentrations in duck adipose**
We removed visceral fat from a duck (*Anas platyrhynchos domestica*) less than 30 minutes after suffice by decapitation and homogenized the fat at 4 °C (without buffer) in a loose-fitting Potter-Elvehjem homogenizer. We then added P-a (70 µL of a 3.3 mg/mL stock solution) and ADP (800 µL of a 10 mg/mL stock solution). The homogenate was divided into two groups: one group was kept in the dark, while the other was exposed to red light (671 nm at 0.8 W/m²); both dishes were kept at 37 °C. 200-µL aliquots were taken from each dish and ATP was measured using the luciferase assay or by HPLC, as described in the literature (Ally and Park, 1992).

**Effect of light wavelength**

The entire brain was homogenized with a Dounce homogenizer (20 mg of brain to 1 mL buffer C) at 4 °C. We took a 40-µL aliquot of the homogenate and added it to 940 µL buffer C. We added 10 µL P-a (from a 1 mM stock in DMSO) and placed the sample on ice for 1 hour. We then added 10 µL ADP (from a 10 mM stock). Five 100-µL portions of the suspension were added to each well of a 96-well plate and exposed to light for 40 min. Then, 20-µL aliquots of the mixture were lysed with 200-µL lysis buffer for 1 h on ice, and ATP levels were determined as above using a luciferase based ATP kit.

Buffer C: 0.15 mM sucrose, 0.5 mM EDTA, 5 mM MgCl₂, 7.5 mM Na₂HPO₄, 2 mM HEPES.

**Red fluorescence in tissue extracts**

The chlorophyll-rich diet (Harlan Teklad, Indianapolis, IN) contained 15% by weight spirulina (a food supplement produced from cyanobacteria (Ciferri, 1983)), which translates to approximately 0.15% by weight chlorophyll-a. The control diet was a purified diet devoid of dietary chlorophylls (Harlan Teklad). The swine chlorophyll rich diet has been described (Mihai et al., 2013).

For fluorescence spectroscopy, five animals each were given these respective diets *ad libitum* for two weeks. Whole brain or 2-7 grams of abdominal fat was homogenized with a hand-held homogenizer (Omni Micro Homogenizer (µH), Omni International, Kennesaw, GA), HPLC grade acetone (40 mL) was added and the sample was vortexed for 1 minute. Insoluble material was precipitated by centrifugation and the acetone evaporated with a rotary evaporator. The samples were re-suspended in 3 mL of chloroform and measured directly.
For HPLC and UV-spectroscopy, we extracted 2.5 grams of fat, as described above, from rats or swine administered a chlorophyll rich diet to give a clear oil. We then added 10 mL of absolute ethanol, cooled the sample to -20 °C for 30 min, pelleted the insoluble material by centrifugation, separated and evaporated the ethanol with a rotary evaporator and re-suspended the sample in 500 μL of absolute ethanol. For plasma, we added 4 mL of plasma to 1 mL of saturated NaCl and 10 mL ethyl acetate, vortexed the sample for 1 minute and separated the layers by centrifugation. We removed the ethyl acetate layer, evaporated the ethyl acetate and re-suspended the resulting film in 300 μL of absolute ethanol. The samples were then used for HPLC and UV-spectroscopy. HPLC was done on a Waters (Milford, MA) HPLC system with 600 pump, 2475 fluorescent detector, a 2998 photodiode array (PDA) detector and a C18, 2.6 μ, 100Å, 150 x 2.10 mm column (Phenomenex, Torrance, CA). Excitation was set to 410 nm and emission set to 675 nm. Absorbance between 275 and 700 was recorded. We used a mobile phase of acetonitrile containing 10% isopropyl alcohol and 0.1% formic acid (solvent A) and water containing 0.1% formic acid (solvent B). Compounds were eluted at a flow rate of 0.3 mL per minute with a 50:50 mixture of A:B for 5 min, which was changed linearly to 100:0, A:B over 15 min. At 35 minutes, the flow was increased to 0.5 mL per minute.

**In vivo imaging**

Animals were imaged with a Maestro™ In-Vivo Imaging System (CRi, Hopkinton, MA), as described by Bouchard et al. (Bouchard et al., 2007); the animals were skinned to reduce interference from skin autofluorescence.

**General C. elegans maintenance**

Worms were a gift from Dr. Cristina Lagido from the Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, UK (Lagido et al., 2009; Lagido et al., 2001). Nematode husbandry has been described (Wood, 1988). Briefly, animals were maintained on Nematode Growth Medium (NGM) agar (Nunc) using *E. coli* strain OP50 as a food source. To obtain synchronous populations, we expanded a mixed population on egg yolk plates (Krause, 1995). Worm eggs were isolated from the population by treatment with 1% NaOCl/0.5 M-NaOH solution (Emmons et al., 1979) and
transferred to a liquid culture with *E. coli* strain OP50, carbenicillin (50 μg/mL) and amphotericin B (0.1 μg/mL) (Complete medium).

Real time ATP monitoring in *C. elegans*

We administered the P-a chlorophyll metabolite by adding it to the culture medium for a minimum of 24 h. To confirm P-a uptake, we washed away the culture medium containing P-a, suspended the worms in fresh medium and determined the fluorescence spectra in the worms. Treated worms had signature chlorophyll-derived fluorescence, while control worms that were not given P-a exhibited no such fluorescence, confirming metabolite uptake.

Method A - Worms were grown in liquid culture at a density of 10,000 worms/mL. Twenty-four hours before the experiment, the culture was split into control and treatment groups and varying amounts of a P-a stock solution in DMSO were added to the treated groups. Control worms were given DMSO vehicle. Worms were washed with M9 buffer (IPM Scientific, Eldersburg, MD) to remove food and unabsorbed P-a and resuspended at 3,000 worms/mL. 50 μL of worm suspension from each of these groups were plated into a well of a 96-well plate. Each experimental group was plated into a minimum of 12 wells. To assay ATP stores by luminescence, 100 μL of luminescence buffer containing D-luciferin was added to each well, according to the literature (Lagido et al., 2009; Lagido et al., 2001) and luminescence was recorded in a plate reader. The luminescence buffer was a citric phosphate buffer at pH 6.5, 1% DMSO, 0.05% Triton X-100 and D-luciferin (100 μM). After initial ATP measurements, half of the worms from each experimental group were exposed to LED light centered at 660 nm at 1 ± 2 W/m²; the other half was kept in the dark by covering the plate with aluminum foil. ATP (luminescence signal) was recorded periodically. The amount of ATP synthesized was reported as the difference within an experimental group between the luminescence signal of worms kept in the dark and the worms exposed to light. All experimental procedures outside of red light exposure were performed under dim light. The experiment was repeated three times with different populations of worms.

Method B – Worms were plated as above, with each experimental group divided into 12 wells of a 96-well plate. Four identical 96-well plates were made, each containing worms treated with
varying concentrations of P-a and control worms. At time zero, 100 μL of luminescence buffer was added to a plate and in vivo ATP was assayed via luminescence. The remaining three plates were exposed to light and ATP assays were performed every 15 min for 45 min by the addition of 100 μL of luminescence buffer and the recording of luminescence.

**In vitro ATP monitoring in C. elegans**

One-day-old adult worms in liquid culture were incubated with P-a for 24 h, washed with M9 buffer and re-suspended in M9 buffer at 50,000 worms/mL. The control group was incubated in DMSO vehicle without P-a. We placed 100 μL of each worm suspension into 18 centrifuge tubes. At time zero, six tubes from each group were placed in liquid nitrogen and the remaining tubes exposed to red light. Then, at 15 and 30 min, six tubes from each group were placed into liquid nitrogen. To measure ATP, we removed the centrifuge tubes from the liquid nitrogen and placed them in boiling water for 15 min to lyse the worms (Artal-Sanz and Tavernarakis, 2009). The resulting solution was cleared by centrifugation for 5 min at 15,000 rpm and ATP in the lysate was measured using the luciferase assay according to the manufacturer’s instructions or by HPCL according to established protocols (Ally and Park, 1992).

**C. elegans oxygen consumption**

Oxygen consumption was measured using a Clark-type oxygen electrode (Qubit Systems Inc.), as described (Anderson and Dusenbery, 1977; Zarse et al., 2007). One-day-old adults in liquid culture at a density of approximately 10,000 worms/mL were incubated with P-a (25 μM) for 24 h in complete medium. Animals were washed three times with M9 buffer to remove bacteria and excess P-a and re-suspended in M9 buffer at 10,000 worms/mL. One-mL aliquots of this suspension were transferred into the respiration chamber and respiration was measured at 25 °C for 10 min while being exposed to an LED light centered at 660 nm at 1 ± 2 W/m². The control group was treated in the same way but not incubated with P-a.

**C. elegans ROS formation**

ROS formation was quantified as described by Schulz et al. (Schulz et al., 2007). Three-day-old worms were synchronized in liquid culture at a density of 500 worms/mL in complete medium, then divided into control and treatments groups. The treated group was incubated for 24 hours
with 12 μM P-a and the control group in DMSO vehicle. Bacterial food and P-a were removed by three repeated washes with M9 and the worms resuspended to 500 worms/mL M9 buffer. We added 50 μL of the suspension from each group to the wells of a 96-well plate with opaque walls and transparent bottom. A 100 μM 2’,7’-dichlorofluorescin diacetate (Sigma-Aldrich, St. Louis, MO) solution in M9 buffer was prepared from a 100 mM 2’,7’-dichlorofluorescin diacetate stock solution in DMSO. 50 μL of this solution were pipetted into the suspensions, resulting in a final concentration of 50 μM. Additional controls included worms without 2’,7’-dichlorofluorescin diacetate and wells containing 2’,7’-dichlorofluorescin diacetate without animals; these were prepared in parallel. Five replicates were measured for each experimental and control group. Immediately after addition of 2’,7’-dichlorofluorescin diacetate, the fluorescence was measured in a SpectraMax M5 microplate reader (Molecular Devices, LLC, Sunnyvale, CA) at excitation/emission wavelengths of 502 and 523 nm. The plates were then exposed to red LED light and fluorescence was re-measured at 2.5 and 5 hours under conditions equivalent to those used previously.

Life span

Population Studies - Life span measurements were done according to Gandhi, et al. (Gandhi et al., 1980) and Mitchell et al. (Mitchell et al., 1979) with some modifications. Eggs were harvested and grown in darkness in a liquid culture at room temperature. To prevent progeny, 5-fluoro-2’-deoxyuridine (FUDR) (Sigma-Aldrich, 120 μM final) was added at 35 hours after egg isolation, during the fourth larval molt. At day four of adulthood, the culture was split into control and experimental groups. The experimental group was treated with 12 μM P-a from a stock solution in DMSO. The control group was given the DMSO vehicle alone. The treated and control cultures were then split into triplicates or duplicates. The final density of worms in all reaction flasks was 500 worms/mL; each flask contained 10 mL for a total of 5000 worms. The following day (day five of adulthood), worms were exposed to LED light centered at 660 nm at 1 ± 2 W/m² for 5 h. Light exposure was repeated every day until the end of the experiment. For counting, aliquots were withdrawn and placed in a 96-well plate to give approximately 10 worms per well; the worms were scored dead or alive on the basis of their movement, determined with the aid of a light microscope. A total of 60–100 worms (representing 1–2% of the total population) were withdrawn and counted at each time point for each flask. Counts were made at
2-3 day intervals and deaths were assumed to have occurred at the midpoint of the interval. Any larvae that hatched from eggs made before the FUDR was added remained small in the presence of FUDR and were not counted. We used the L4 molt as time zero for life span analysis. To obtain the half-life, we plotted the fraction alive at each count verses time and fitted the data to a two-parameter logistic function using the software GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). The two-parameter model is known to fit survival of 95% of the population fairly accurately (Vanfleteren et al., 1998). Because changes in environment, such as temperature and worm density, the amount of food, may influence life span, control measurements were conducted at the same time under identical conditions. The concentration of P-a dropped (approximately 75%) throughout the life span studies and it was not adjusted (supplementary material Fig. S4F).

*Life Span in 96 Well Microtiter Plates* - We measured life span as described in the literature (Solis and Petrascheck, 2011), except that P-a was added at day 4 and light treatment commenced at day 5. Scoring (fraction alive) was done once on day 15.

**Acknowledgments:**

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**References:**


Fig. 1. Chlorophyll metabolite P-a allows isolated mouse liver mitochondria to capture light to make ATP.

(A) ATP synthesis in mouse liver mitochondria incubated with P-a (treated) and exposed to light compared to controls (no P-a). Light exposure started at time zero and ADP was added at 30 seconds. Aliquots were obtained at times shown and relative ATP levels measured by firefly luciferase assay. Means and standard deviations are shown for each time point. The experiment was run in triplicate with the same batch of mitochondria. *: P value < 0.05

(B) Mitochondrial membrane potential (Δψm) with light exposure as measured by safranin fluorescence. Lower fluorescence equals higher membrane potential. Mitochondria, with or without P-a, were exposed to light for 2 min or kept in the dark. Safranin was added at time zero and Safranin fluorescence was continuously measured while samples remained under light. The experiment was run in triplicate with the same batch of mitochondria. Curves shown are the average traces for triplicate runs.

(C) Representative oxygraph trace (black line) for mitochondria treated with 4 μM P-a. Light was turned on or off at times indicated by the arrow. Steeper slope denotes faster oxygen consumption. Dotted lines show slopes when the light was turned off. When the light was turned on the slope of the black line increased by twofold. That is, oxygen consumption increased when the light was turned on. When the light was turned off, oxygen consumption returned to baseline levels (i.e. the two gray lines have the same slope).

Fig. 2. Cooperative binding of P-a to mitochondrial Fragments

(A) Fluorescence spectra of P-a before and after addition of sheep heart mitochondrial fragments. Upon addition of mitochondrial fragments, the fluorescence intensity of P-a increased and shifted to a longer wavelength, and the shape of the curve (ratio of the shoulder to main peak) changed.

(B) Ultrasensitive steady state response of the P-a-mitochondrial interaction. We plotted fluorescence intensity for a 1 μM P-a solution while increasing the concentration of mitochondrial fragments. A Hill coefficient of 36, with a 95% confidence interval from 7 to 65, was obtained by fitting the data to the Hill equation (y = ax^b / (c^b + x^b) + Offset). Fit (R^2): 0.96.

Table 1. Photoreduction of CoQ_{10} is an early event in light-stimulated ATP synthesis.

Percent of reduced CoQ_{10} (ubiquinol) in sheep heart mitochondria under the listed conditions. Mito: mitochondria. All reactions were for 10 min under an anaerobic atmosphere, employing the same amount of mitochondria. Antimycin A was added to prevent ubiquinol reduction. Longer reaction times did not increase the percentage of reduced CoQ_{10} (data not shown). Ubiquinone and ubiquinol were quantified by HPLC. Entries 1-5 are results for two experiments each. Entry 6 is a range of values observed for two different mitochondrial preparations.

Fig. 3. Chlorophyll metabolite P-a allows mouse brain tissue homogenates to capture light to make ATP.
(A) ATP synthesis in mouse brain homogenate with light exposure. Homogenates were incubated with ADP ± P-a and exposed to light starting at time zero. Aliquots were withdrawn at times shown. Relative ATP in the aliquots was measured by firefly luciferase assay. The experiment was run in triplicate with the same batch of homogenized brains. Relative ATP synthesis rates, as mean with standard error and 95% confidence intervals (CI) were: Treated = 171.7 ± 8.1 (CI: 154.6–188.7); Control = 111.3 ± 9.1 (CI: 92.5–130.0). Means and standard errors are shown for each time point. For the control, the standard errors are smaller than the line markings and thus cannot be seen. * : P value < 0.05

(B) Overlay of the absorption spectrum of P-a (dotted line) and the wavelengths tested for ATP production in samples treated with P-a and exposed to light for 20 min. Peak ATP production correlated with peak P-a absorption. Experiments were done in triplicate. Means and standard errors are shown. However, standard errors are smaller than the markings and thus cannot be seen.

Fig. 4. Dietary chlorophyll results in chlorophyll-metabolite-like fluorescence in tissues.
(A) Representative fluorescence spectra of brain extracts following excitation at 410 nm. Relative peak areas for a total of 6 control animals fed a chlorophyll-poor diet and 6 treated animals fed a chlorophyll-rich diet are displayed as means and standard deviations were:
Treated: 15.4 ± 6.7 (n = 6); Control: 4.2 ± 2.6 (n = 6); p = < 0.01
(B) Representative excitation spectrum (emission at 675 nm) of a brain extract from mice fed a chlorophyll-rich diet.
(C) Representative fluorescence spectra of abdominal fat extracts from mice fed chlorophyll-poor and rich diets.
(D) A 675 ± 10-nm fluorescence image of skinned mice raised on chlorophyll-rich and poor diets. Mean gray value in the boxed areas with standard deviation and minimum and maximum gray value shown in brackets were: Treated Brain, 118 [97–138]; Control Brain, 82 [60–100]; Treated Back Fat Pad, 116 [97–132] and Control Back Fat Pad, 35 [25–46].

Fig. 5. Light-absorbing metabolites of chlorophyll are present in adipose.
(A) HPLC chromatogram of an adipose extract. 2.5 grams of abdominal adipose from a rat fed a chlorophyll-rich diet was extracted with acetone and the acetone concentrate subjected to HPLC. In the chromatogram, only compounds that displayed 675-nm fluorescence, characteristic of chlorophyll and its metabolites possessing a chlorin ring, are shown. Five major peaks are observed along with several minor peaks. For peaks with letters, the corresponding absorption spectra are shown below.
(B-D) Absorption spectra of labeled peaks in panel A. Numbers above peaks are peak maxima in nm. Numbers in the center are the ratios of the Soret band, around 400 nm, to the Q_y band at around 655 nm. All spectra are consistent with those of metabolites of chlorophyll. Spectra C has been assigned to a metalated porphyrin.

Fig. 6. P-a treatment enables worms to capture light to generate ATP.
Black lines show worms incubated with P-a at the indicated concentrations; gray lines show worms not incubated with P-a.
(A) *In vivo*, real-time ATP levels in 1-day-old were tracked during exposure to light. We incubated luciferase-expressing worms with luciferin and exposed them to light at time zero. Luminescence was measured at the times shown. Data represent triplicate experiments of 12 separate sets of worms plated in 12 wells of a 96-well plate. Means and standard deviations are shown for each of the three separate runs.

(B) *In vivo*, real-time ATP levels in worms kept in the dark. The same experiment as in panel A in the same 96-well plate, but the worms were kept in the dark.

(C) Percent ATP increase for worms in panel A relative to worms represented by panel B.

(D) Cross sectional, *in vivo*, real-time ATP monitoring. Groups of worms were incubated with or without P-a; light exposure began at time zero and *in vivo* ATP was determined at the times shown in each group of worms by worm luminescence after the addition of luciferin. Each time point represents a different group of worms exposed to light for the times shown. Each experiment was performed in triplicate sets of 12; averages and standard deviations are shown. *P* values of Student’s t-tests are also shown, representing the significance compared with the controls at the same light exposure.

(E) The same experiment described in panel D, but using 10-day-old worms.

**Fig. 7. P-a and light increase *C. elegans* median life span.**

(A) Median life spans of worms treated with P-a and exposed to light vs. those exposed to light but not treated with P-a. Numbers in parentheses are 95% confidence intervals (CI).

(B) Life span plots for the table in panel A. *P*-value is from the f-test. Experiments were run in triplicate. We used the L4 molt as time zero for life span analysis. Worms were grown in liquid culture at 500 worms/mL. For counting, aliquots were withdrawn and placed in a 96-well plate to give approximately 10 worms per well; the worms were scored dead or alive on the basis of their movement, determined with the aid of a light microscope. A total of 60–100 worms, representing 1–2% of the total population, were withdrawn and counted at each time point for each flask.
Fig. 1
Fig. 2
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<th>Reaction Conditions</th>
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Table 1
Fig. 3
A

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B

Fraction alive

- Treated
- Control

P = 0.003

Fig. 7