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Original Article

The Mitochondria-Targeted Plastoquinone–Derivative SkQ1 Promotes Health and Increases *Drosophila melanogaster* Longevity in Various Environments

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Abstract

Mitochondria play an important role in aging. Strongly reduced function of the mitochondria shortens life span, whereas moderate reduction prolongs life span, with reactive oxygen species production being the major factor contributing to life span changes. Previously, picomolar concentrations of the mitochondria-targeted antioxidant SkQ1 were shown to increase the life span of *Drosophila* by approximately 10%. In this article, we demonstrate that SkQ1 elevates locomotion, which is often considered a marker of health and age. We also show that mating frequency and fecundity may be slightly increased in SkQ1-treated flies. These results indicate that SkQ1 not only prolongs life span but also improves health and vigor. An important property of any potential therapeutic is the stability of its effects in an uncontrolled and changing environment as well as on individuals with various genetic constitutions. In this article, we present data on SkQ1 effects on *Drosophila* longevity in extreme environments (low temperatures and starvation) and on individuals with severe genetic alterations in the mitochondrial systems responsible for production and detoxification of reactive oxygen species. We hypothesize that in vivo SkQ1 is capable of alleviating the probable negative effects of increased mitochondrial reactive oxygen species production on longevity but is not effective when reactive oxygen species production is already reduced by other means.

Keywords: Antioxidant—Life span—Locomotion—Reproduction—*Drosophila*

Mitochondria play an important role in the aging process, mainly by mediating the oxidative status of cells due to deviations in respiratory chain function and modification of redox status, which eventually results in changes in levels of reactive oxygen species (ROS). Genetic (1), epigenetic (2), and structural (3) changes in the mitochondria can affect longevity. Alterations in mitochondrial functions have either negative (4,5) or positive (6–9) effects on the life span of *Drosophila melanogaster*, *Caenorhabditis elegans*, and mice. Overall, at present, the accepted consensus is that life span can be increased by moderately reducing the function of the mitochondria and, consequently, moderately decreasing ROS production by mitochondria, whereas strong reduction shortens life span. Mitochondria are considered therapeutic targets for both aging and age-related diseases (for reviews, see refs. 10–12). Finding mitochondria-targeted drugs with the potential to increase life expectancy and slow down the aging rate is of considerable theoretical and practical interest. However, longevity-promoting drug interventions should be finely tuned, taking into consideration complicated relationships between mitochondrial function, ROS production, and life span, as a radical change in life expectancy may lead to a serious disruption of homeostasis.

Several attempts to extend longevity with antioxidant drugs have been successful (13–15). In our experiments, extremely low concentrations of the mitochondria-targeted antioxidant SkQ1 (10-(6′-plastoquinonyl) decyltriphenylphosphonium) were shown to

increase the life span of male and female *D. melanogaster* by approximately 10% (16). Moreover, SkQ1 treatment given exclusively at an early age increased life span to the same extent as life-long treatment. Taken together, our results indicated that SkQ1 not only predominantly increased the survival of young flies aged less than 10 days but also slightly reduced the rate of aging (16,17). To confirm these results, in this article, we present data demonstrating the effects of SkQ1 on general locomotor activity, which is often used as a marker of vitality and age (for review, see ref. 18).

SkQ1 did not increase the life span of mated *D. melanogaster* females and males, and the effect on the early survival was not observed in mated females, whereas early fertility and the total number of adult progeny were elevated (19). The increase in reproduction ability observed in young mated females instead of the increased survival typical in young virgin females may illustrate the trade-off between life span and reproduction described in many articles (for a review, see ref. 20). Our experiments did not provide any explanation for why this negative correlation was observed in the case of SkQ1 treatment. Frequency of mating was shown to be significantly associated with the extent of the female survival cost of mating (21). An increased reproduction ability of SkQ1-treated flies can also be explained by the increase in the number of eggs laid and by the increased survival of their embryos, larvae, and pupa, which developed in medium with SkQ1. To pursue this line of investigation, we assessed experimentally whether mating frequency, fecundity and viability are affected by SkQ1 treatment.

An important property of any potential geroprotector is the stability of its effects in an uncontrolled and changing environment, under different administration methods, to individuals with genetic constitutions that vary within a broad range. We have already demonstrated that the nature of the SkQ1 effect on *D. melanogaster* longevity was constant for 6 years, regardless of fluctuations in the control life span, differences in preparation and administration of a solution of SkQ1, or the year and season when the experiments were conducted; laboratory lines of different genotypes demonstrated a positive response to SkQ1 (17). In this article, we present data that describe the range of SkQ1 effects on *D. melanogaster* longevity in extreme environments and on individuals with severe genetic alterations in the mitochondrial systems responsible for production and detoxification of ROS.

Materials and Methods

Fly Strains

Transgenic lines with single insertions of the P-element–based vector were obtained from the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu/).

w^{1118}; P{GT1} ND-B17^{BG02008}/CyO has a lethal mutation caused by an insertion in the gene *ND-B17* (http://flybase.org/reports/FBgn0001989.html) that encodes a subunit of NADH dehydrogenase (complex I of the electron transport chain [ETC]).

The Bloomington stock #3605 *w[1118]* is the isogenic control line for *P{GT1}* insertions (http://flypush.imgen.bcm.tmc.edu/pscreen/transposons.html). This line was obtained from Trudy Mackay (North Carolina State University).

w^{1118}; PBac{PB}Sdh^{e00364}/CyO has a semilethal mutation caused by an insertion in the gene *SdhB* (*Succinate dehydrogenase B*) (http://flybase.org/reports/FBgn0014028.html) that encodes a subunit of succinate dehydrogenase (complex II of the ETC).

w^{1118}; PBac{RB}cype^{e03803}/CyO has a lethal mutation caused by an insertion in the gene *cype* (*cyclope*) (http://flybase.org/reports/FBgn0015031.html) that encodes one of the subunits of cytochrome c-oxidase (complex IV of the ETC).

w^{1118}; PBac{WH}CG30096^{f05490} has a viable mutation caused by an insertion in the gene *Sod2* (http://flybase.org/reports/FBgn0010213.html; http://flybase.org/reports/FBti0042460.html; http://flybase.org/reports/FBrf0221061.html) that encodes mitochondrial Mn-superoxide dismutase.

The Bloomington stock #6326 *w[1118]* is the isogenic control line for PBac insertions (http://flystocks.bio.indiana.edu/Reports/6326.html).

The line *w[1118]* #3605 obtained from Trudy Mackay was used in all other experiments.

Flies were kept at 25°C on a medium of semolina, sugar, raisins, yeast and agar with nipagin, propionic acid, and streptomycin. For all experiments, flies were collected from cultures with controlled density: In each vial, 10 fertilized females of approximately the same age (from 5 to 20 days old) were allowed to lay eggs for 4 days.

All lines were checked for the presence of *Wolbachia* via quantitative polymerase chain reaction (Chromo4 Real-Time PCR Detector, Bio-Rad) with primers to the 16S rRNA gene, 5′-CATACCTATTCGAAGGGATAG-3′ and 5′-AGCTTCGAGTGAAACCAATTC-3′ (22). Only lines without Wolbachia were used.

Chemical Solutions

A fresh 20 mM stock solution of SkQ1 was prepared in 96% ethanol for each experiment and stored at −20°C. A working 20 pM solution of SkQ1 was prepared in distilled water ex tempore. This working concentration was previously determined as the most effective (16). The control was 96% ethanol dissolved in distilled water at 10^9 times (to match the concentration of ethanol in the SkQ1 working solution). In each vial, 0.1 mL of SkQ1 or control solution was applied to the surface of the food and dried overnight at 25°C.

Life-Span Assays

Five virgin flies of the same genotype and sex, all collected on the same day from cultures with moderate density, were placed in replicate vials. Flies were transferred weekly to new vials with fresh food and the appropriate solution, without live yeast on the surface. Dead flies were recorded daily. Experiments comparing fly life spans were conducted simultaneously. In each experiment, sample sizes were 50–100 flies/sex/genotype/treatment. Most experiments were repeated two times. Life span was estimated for each fly as the number of days from day of eclosion to day of death. Mean life span and survival curves were primarily used to characterize life span.

Depending on the experiment, flies were kept at 25°C, 18°C, or 8°C either on regular food or on regular food diluted fourfold or eightfold. In the two last cases, cotton wool was placed into the food to avoid its fluidity. In all experiments, flies were kept under 12:12 h light-dark regime.

Locomotion Assays

w^{1118} flies were collected and maintained in the same way as for the life-span assays. To measure locomotion of unmated flies, five newly hatched flies of the same sex were placed in replicate vials with regular food, and on the next day, locomotion was measured, and flies were transferred to fresh vials on medium with either SkQ1 or alcohol solution. Locomotion was measured in unmated females at ages 1, 10, 20, 30, and 40 days (the first experiment), in unmated females

and males at ages 1, 10, and 20 days (the second experiment), and in unmated females and males at ages 1–10 days (the third experiment). To measure locomotion of mated flies (the fourth experiment), three newly hatched males and three newly hatched females were placed together in replicate vials with regular food, and after that, the procedure was the same as for unmated flies. Locomotion was measured in mated males and females at ages 1, 10, and 20 days. Every time, males and females were separated prior to measurements and placed together after measurements.

In the first experiment, females died over time, and measurements were taken on the remaining ones. In other experiments, flies remained alive in most vials throughout the experiment. If necessary, dead flies were replaced with living flies from additional vials, where they were maintained on the same medium as the flies in the experiment. Experiments comparing locomotion were conducted simultaneously and at the same time of the day. In each experiment, sample sizes were 50–150 flies/age/treatment.

To measure locomotion in the second, third, and fourth experiments, vials were placed in a *Drosophila* Population Monitor (TriKinetics), either horizontally or vertically. Fly movement along the walls or in the middle of the vial interrupts infrared beam rings along the length of the vial. Beam intersections were counted electronically, and totals were reported every 5 minutes to a host computer. Two measurements were made every 5 minutes for each vial. Locomotion was reported as the mean number of beam intersections per vial during 5 minutes. In the first experiment, one circular line was drawn on the wall of each vial, and the number of intersections of this line was recorded visually. Locomotion was reported as the mean number of line intersections per vial per female.

Feeding Assays

The methods described in ref. (23) were used, with some modifications. w^{1118} flies were collected and maintained in the same way as for the life-span assays. When flies were 10 days old, they were transferred to vials with fresh food containing 0.25% FD&C Blue No. 1 (Erioglaucine, Applichem) for 6 hours. Thereafter, flies were collected from each vial into separate packets and stored at –70°C.

To measure weight, five frozen flies from each packet were weighed using a torsion balance (WT-500, Labimex). To measure the absorption of the dye, five frozen flies from each packet were milled in 200 µL of distilled water and filtered through a column (CentriSep, Princeton Separations). Optic absorbance of the solution was measured at a wavelength of 629 nm with a spectrophotometer (Genesys 10 UV-Vis, Thermo Scientific). The mean weight and the mean optical density per five flies served as indicators of the intensity of feeding. Sample sizes were 50 flies/sex/treatment.

Viability and Fecundity Assays

To measure mating success, w^{1118} males and females were collected and maintained together, in the same way as for the life-span assays, or separately until the time when the measurements were done. When flies were 10 days old, three males and three females were placed together in empty replicate vials, and copulations were recorded in each vial for 90 minutes. One experiment with males and females kept together and four experiments with males and females kept separately prior to the measurements were performed with sample sizes of 25, 15, 25, 20, and 20 vials (75, 45, 75, 60, and 60 fly pairs, respectively). All vials in each experiment were analyzed at the same time, and the analyses in different experiments were performed at the same time of the day. The last experiment was carried out 2 years later than the others. The mean number of copulations per vial (per three pairs of flies) during 90 minutes was used to quantify the mating activity of the flies.

To measure fecundity, in four of the five experiments described above, three pairs of flies were left in each vial to lay eggs overnight. On the next day, flies were discarded and eggs counted. Sample sizes were 326–1,085 eggs/treatment. Fecundity was quantified as the mean number of eggs laid per vial (per three pairs of flies) during 24 hours.

To measure viability, w^{1118} females aged 1–3 days or 10 days were used. Fertilized females, all collected from cultures with moderate density, were placed in vials, allowed to lay eggs for 12 hours, and removed. Eggs were counted and transferred to replicate vials on the medium with SkQ1 or alcohol solution for development. F_1 pupa and adult flies were counted in each replicate vial. In each experiment, sample sizes were 220–400 eggs/age/treatment. All experiments were repeated twice. Mean egg-to-pupa and egg-to-imago viability per vial were used to characterize progeny viability.

Statistical Analyses

To compare control and SkQ1-treated flies, a Student's *t*-test was used for initial analysis of locomotion, fecundity, viability, and mating success. The nonparametric, distribution-free Mann–Whitney–Wilcoxon test and Kolmogorov–Smirnov test were used to confirm the results and to analyze life span.

Results

SkQ1 Effects on Locomotion

Locomotor activity decreases with normal aging in animals (for a review, see ref. 18). Indeed, in both control and SkQ1-treated females, locomotion reached a maximum at 10–20 days of age and then decreased (Figure 1A). No difference in locomotion was detected in 1-day-old females; however, locomotion of 10-, 20-, and 30-day-old SkQ1-treated females was significantly higher than locomotion of control females. Though this trend continued in females at 40 days of age, it did not reach statistical significance (Figure 1A; Supplementary Table 1). The difference in locomotion was first noticeable at 10 days of age and remained constant at older ages. To determine whether this early effect was reproducible, we remeasured locomotion of 1-, 10-, and 20-day-old females. Again, no difference was detected in 1-day-old females, whereas locomotion of 10- and 20-day-old SkQ1-treated females was significantly higher than locomotion of control females (Figure 1B; Supplementary Table 1). Flies naturally tend to move against gravity. We investigated whether SkQ1 treatment altered fly geotaxis independently of locomotion per se. The results were similar regardless of whether vials were fixed in horizontal or vertical positions, that is, horizontal or vertical motion was primarily measured (Supplementary Table 1). We did not use mechanical stimuli to induce movements, which might explain the lack of difference between the results. Locomotion was also measured in 1-, 10-, and 20-day-old males. No difference was detected in 1- and 10-day-old males, whereas locomotion of 20-day-old SkQ1-treated males was significantly higher than locomotion of control males (Figure 1C; Supplementary Table 1).

We analyzed changes in locomotion of 1- to 10-day-old flies in more detail. Starting from the second day, locomotion of

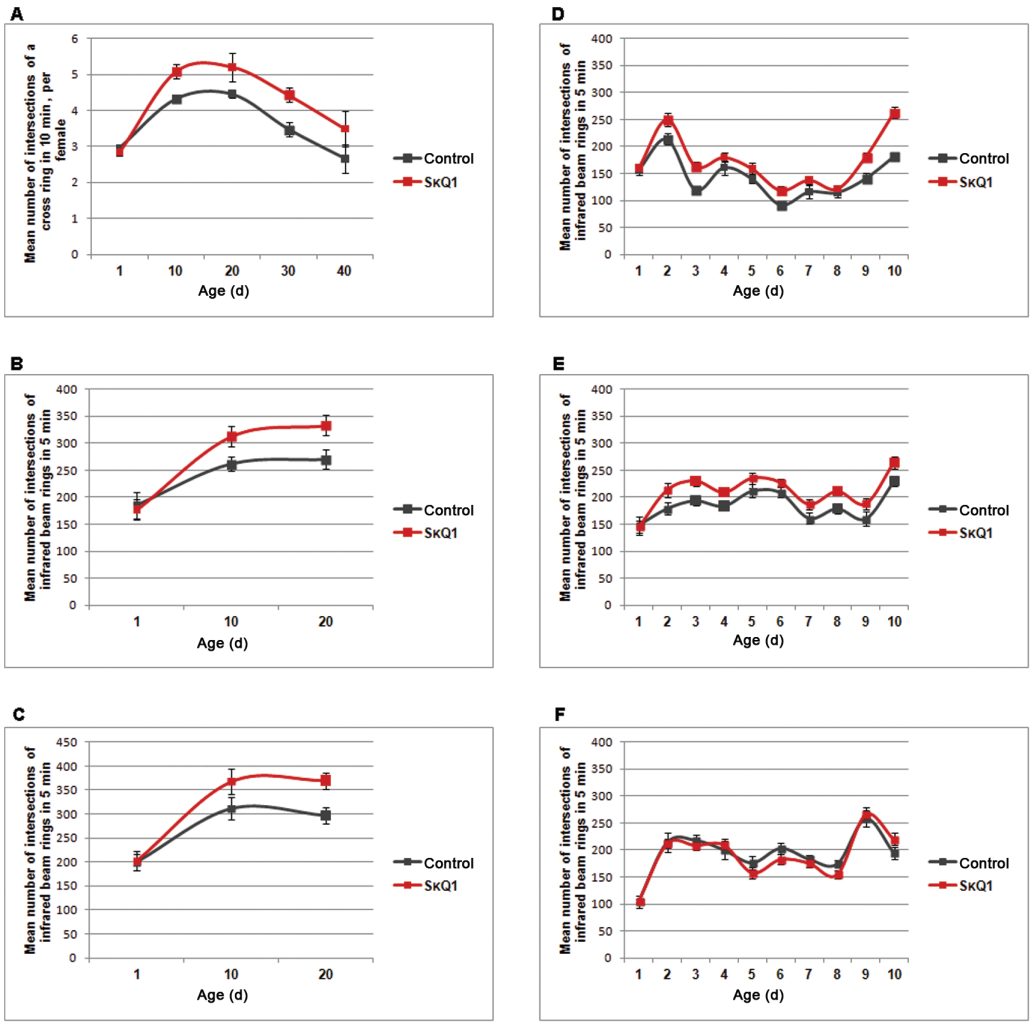

Figure 1. Comparative locomotion of SkQ1-treated and control *w1118* flies. Locomotion of 1- to 40-day-old females, horizontal motion, mornings (**A**). Locomotion of 1- to 20-day-old females (**B**) and males (**C**), horizontal motion, mornings. Locomotion of 1- to 10-day-old females, horizontal motion, mornings (**D**) and evenings (**E**). Locomotion of 1- to 10-day-old males, horizontal motion, mornings (**F**). Large squares denote days with significant difference in locomotion of SkQ1-treated and control flies, see Supplementary Table 1 for statistical details.

SkQ1-treated females became higher compared to controls, though the difference at some days did not reach the level of statistical significance (Figure 1D and E; Supplementary Table 1). In accordance with the previous experiment with males, no significant difference was detected in locomotion of SkQ1-treated and control males at ages 1–10 days (Figure 1F; Supplementary Table 1).

Locomotion was also measured in 1-, 10-, and 20-day-old mated males and females. No significant difference was detected in locomotion of SkQ1-treated and control mated males and females at all ages (Supplementary Table 1).

SkQ1 Effects on Reproduction

Considering that the SkQ1 effect on life span (16) became noticeable at 10 days of age, we asked whether SkQ1 could affect reproduction in 10-day-old flies. When males and females were kept together prior to the measurements, in the same as in the experiments reported in ref. (16), the number of copulations observed during 90 minutes was very low; however, it was somewhat higher for SkQ1-treated flies (Supplementary Table 2). To increase the number of copulations, in the next four experiments, we kept males and females separately prior to the measurements. The number of copulations observed during 90 minutes was also somewhat higher for SkQ1-treated flies; however, the difference was insignificant both in each of the four replicate experiments and when the data were combined (Figure 2A; Supplementary Table 2). Still, the total number of copulations was always higher for SkQ1-treated flies than control flies (Figure 2B).

The difference in the number of eggs laid by 10-day-old SkQ1-treated females versus control females was not statistically significant in the first experiment with males and females kept together prior to the measurements (Supplementary Table 2) and both in each replicate experiment with males and females kept separately prior to the measurements and when the data were combined (Figure 2C; Supplementary Table 2).

No significant difference was found in the egg-to-pupa or egg-to-adult viability of progeny from SkQ1-treated versus control 1- to 3-day-old females (Figure 2D; Supplementary Table 2). There was also no significant difference in the viability of progeny of 10-day-old females (Figure 2D; Supplementary Table 2).

Overall, SkQ1 did not significantly affect the reproductive ability of young flies; however, it is possible that mating activity and

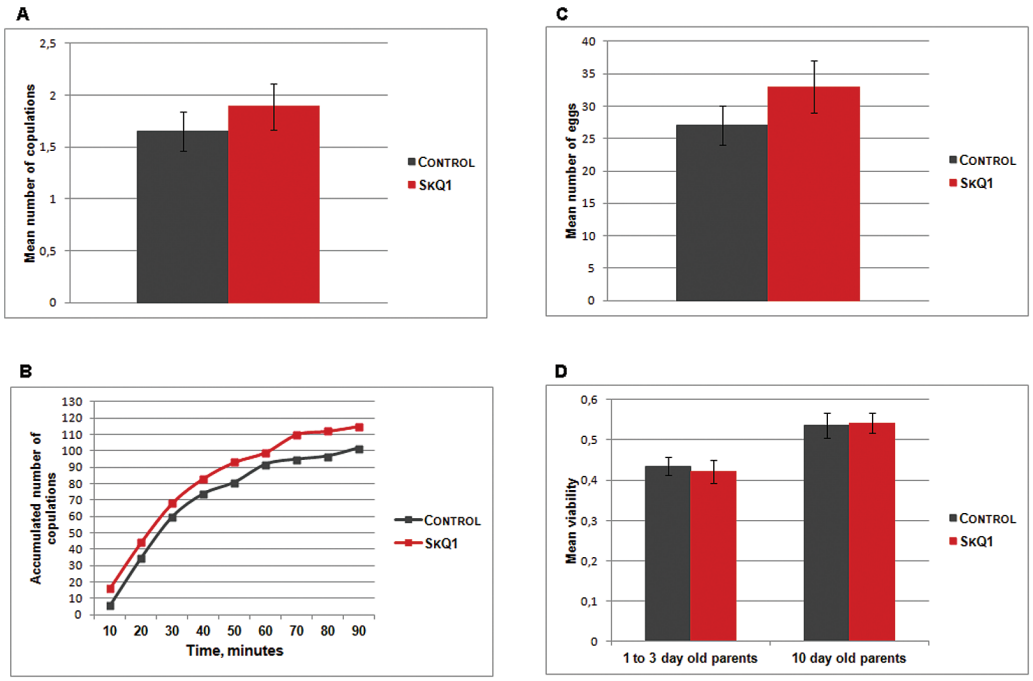

Figure 2. Comparative reproductive ability of SkQ1-treated and control *w1118* flies. Mating activity measured as the mean number of copulations per vial (per three pairs of flies) during 90 minutes (**A**). Accumulated number of copulations (**B**). Males and females were kept separately prior to the measurements. Fecundity measured as the mean number of eggs per vial (per three pairs of flies) laid during 24 hours (**C**). Total (egg-to-imago) viability (**D**). See Supplementary Table 2 for statistical details.

fecundity were slightly improved in SkQ1-treated mated flies compared to controls.

SkQ1 Effects on Life Span in Extreme Environments

SkQ1 treatment did not typically affect the rate of feeding. Neither weight nor accumulation of dye was different between control and SkQ1-treated flies (Supplementary Table 3). Accumulation of weight and dye was significantly correlated in all cases except one (r = .732, p = .0161 and r = .787, p = .0069 for comparisons between the control and SkQ1-treated males; r = .445, p = .1974 and r = .797, p = .0057 for comparisons between the control and SkQ1-treated females).

We assessed the effects of SkQ1 on life span of unmated females and males kept under partial starvation (12.5% and 25% of regular food supply). For each experiment, all formal parameters were calculated (Supplementary Table 4). In both cases, a decreased life span was detected in SkQ1-treated males compared to control males (Figure 3A). The difference in life span was only 1 day; however, this is equal to a 25% and 10% decrease in mean life span at 12.5% and 25% of regular food supply, respectively. However, statistical analysis did not confirm the difference (Supplementary Table 4). In both cases, no difference was found between the life spans of SkQ1-treated and control females (Figure 3B).

We assessed the effect of SkQ1 on the life span of unmated females and males kept at 18°C and 8°C. For each experiment, all formal parameters were calculated (Supplementary Table 4). For each experiment, two independent replicates performed 6 months apart gave consistent results and were combined for further analysis. At 18°C, a significantly increased life span was detected in SkQ1-treated males compared to control males, whereas no difference was found between the life spans of SkQ1-treated and control males at 8°C (Figure 3C; Supplementary Table 4). Of note, at 8°C, males lived much longer than at 18°C, and in both cases, their life span was longer than at 25°C. Similarly, at 18°C, a significantly increased life span was detected in SkQ1-treated females compared to control females, whereas no difference was found between the life spans of SkQ1-treated and control females at 8°C (Figure 3D; Supplementary Table 4). Female life spans were the same at 18°C and 8°C, and in both cases longer than at 25°C.

Effects of SkQ1 on the Life Span of Flies With Genetic Alterations in the Mitochondrial Systems Responsible for Production and Detoxification of ROS

We assessed the effect of SkQ1 on the life span of unmated females and males with mutations in genes encoding components of the ETC in mitochondria. The choice of lines with mutations caused by insertions, which ensure availability of a proper control line, was rather poor compared to other Drosophila genes (http://flybase.org). For this study, we selected one mutation in the *ND-B17* gene, which encodes a subunit of NADH dehydrogenase (complex I of the ETC); one mutation in the *SdhB* gene, which encodes a subunit of succinate dehydrogenase (complex II of the ETC); and one mutation in the *cype* gene, which encodes a subunit of cytochrome c-oxidase (complex IV of the ETC). We also used one mutation in the well-characterized *Drosophila* gene *Sod2*, which encodes the mitochondrial Mn-superoxide dismutase.

Most mutations were lethal or semilethal; therefore, life span was measured in heterozygous flies, hybrids between the control and mutant lines. In the first pilot experiment, life spans of w^{1118} (#3506) and $ND\text{-}B17^{BG02008}/w^{1118}$ (#3506) control and SkO1-treated males and females were measured. In the second and third

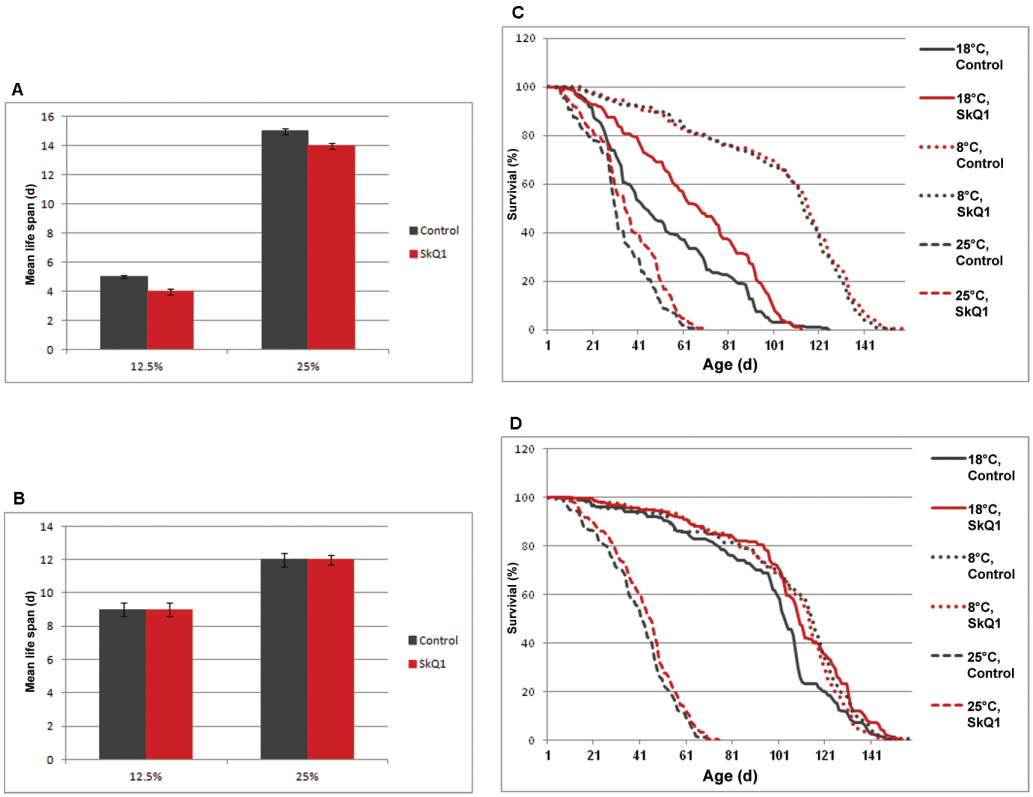

Figure 3. Comparative life span of SkQ1-treated and control *w1118* flies under starvation and at low temperatures. Life span of males (**A**) and females (**B**) on 12.5% and 25% of regular foods supply. Life span of males (**C**) and females (**D**) at 18°C and 8°C, 12:12 light regiment. See Supplementary Table 4 for statistical details. Survival data for 25°C are from ref. (17).

experiments, life spans of w^{1118} (#6326), *ND-B17*^{BG02008}/w^{1118} (#6326), *Sdh*^{e00364}/w^{1118} (#6326), and *cype*^{e03803}/w^{1118} (#6326) control and SkO1-treated males and females were measured. Only one experiment was made for males with the *SdhB* mutation. In the fourth and fifth experiments, life spans of w^{1118} (#6326) and *CG30096*^{f05490}/w^{1118} (#6326) control and SkO1-treated males and females were measured. For each experiment, all formal parameters were calculated (Supplementary Table 4). Replicate experiments yielded consistent results and were combined for further analyses (Supplementary Table 4).

Earlier, SkQ1 effects on the life span of w^{1118} (#3506) males and females have been thoroughly analyzed (17). In this study, we demonstrate that SkQ1 significantly increased life spans of w^{1118} (#6326) males and females (Supplementary Table 4), in a similar way as in w^{1118} (#3506) flies.

Mutations in genes encoding subunits of NADH dehydrogenase, succinate dehydrogenase, and cytochrome c-oxidase increased both male and female life spans compared to the control line; however, SkQ1 did not further increase the life span in males or females with these mutations (Figure 4A–H; Supplementary Table 4). The effects of the mutations were smaller in males than in females but were statistically significant. The effects of *ND-B17*^{BG02008} on male and female life spans was the same both when the proper control line (w^{1118} #3506) and the w^{1118} (#6326) line were used (Figures 4A–D). A mutation in the gene encoding Mn-superoxide dismutase substantially decreased both male and female life spans. In this case, SkQ1 treatment resulted in a significant positive effect and restored life span in flies of both sexes (Figure 4I and J; Supplementary Table 4).

Discussion

Chemical and therapeutic properties of the mitochondria-targeted antioxidant SkQ1 (10-(6′-plastoquinonyl) decyltriphenylphosphonium) have been extensively characterized (for reviews, see refs. 24,25). Studies have demonstrated that, in mammals, SkQ1 retarded and/or alleviated development of a large number of age-related pathologies, including ischemia/reperfusion of the kidney, septic shock resulting from pyelonephritis, stroke and heart muscle infarction, glaucoma, macular degeneration, cataracts, and dry eye syndrome (25). The therapeutic effects of Visomitin eye drops (based on SkQ1) on dry eye syndrome were confirmed in clinical trials (26). We have used the well-established model organism *D. melanogaster* to investigate the consequences of diet supplementation with SkQ1 on life-history traits. To expand upon our previous results (16,17,19), we characterized the effects of SkQ1 on locomotion, mating activity, fecundity, and viability, as these traits are considered markers of vitality and age. We also characterized the effects of SkQ1 on life span in extreme environments (low temperature and low food supply) and on individuals with severe genetic alterations in the mitochondrial systems responsible for production and detoxification of ROS in an effort to define conditions under which SkQ1 is effective and to evaluate the robustness of the drug.

SkQ1 Effects on Locomotion

SkQ1 strongly increased the survival of young unmated females aged less than 10 days, whereas in unmated males this effect was not pronounced (16). SkQ1 also increased the mean life span and slightly slowed down aging in both sexes (17). Unmated females reared on a

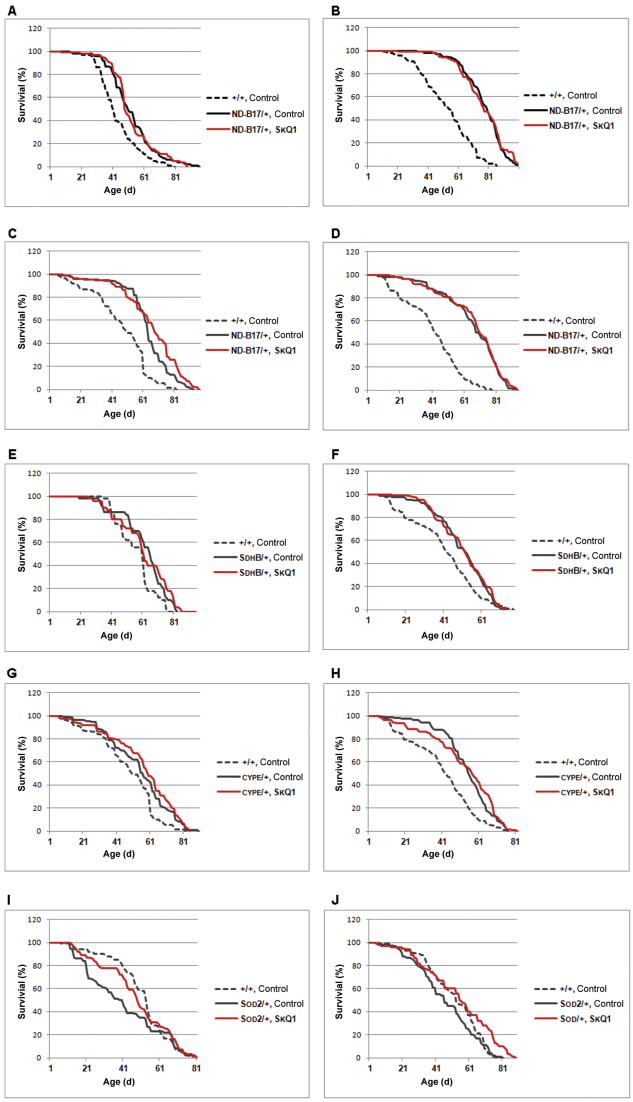

Figure 4. Comparative life span of SkQ1-treated and control flies with genetic alterations in the mitochondrial systems responsible for production and detoxification of ROS. Life span of males (**A**, **C**) and females (**B**, **D**) heterozygous for a mutation in the gene *ND-B17* encoding one of the subunits of NADH dehydrogenase. Life span of males (**E**) and females (**F**) heterozygous for a mutation in the gene *SdhB* (*Succinate dehydrogenase B*) encoding succinate dehydrogenase B. Life span of males (**G**) and females (**H**) heterozygous for a mutation in the gene *cype* (*cyclope*) encoding one of the subunits of cytochrome c-oxidase. Life span of males (**I**) and females (**J**) heterozygous for a mutation in the gene *Sod2* encoding mitochondrial Mn-superoxide dismutase. The *w1118* line #3605 was used as a control in **A** and **B**; the *w1118* line #6326 was used as a control in all other experiments. See Supplementary Table 4 for statistical details.

SkQ1-supplemented diet demonstrated an increase in locomotion as early as the second-day posttreatment, and the difference achieved by the age of 10 days was maintained for the life of the fly. No significant difference in locomotion was observed between 10-day-old SkQ1-treated and control males; however, the difference reached statistical significance later, by the age of 20 days. SkQ1 did not affect both life span (19) and locomotion of mated flies. Thus, SkQ1 effects on locomotion parallel SkQ1 effects on life span and confirm an important property of the drug, namely, its improvement of vitality in unmated females and males at a young age. In mated flies, the improvement affects reproduction at a young age (ref. 19 and see discussion below), without negative effects on life span (19) and locomotion. However, we cannot completely exclude the possibility that, in unmated flies, the drug directly stimulates locomotion independent of effects on life span, as do some mutations (O. Rybina, PhD, E. Pasyukova, PhD, unpublished data, 2016).

Our results indicate moderate sex-specificity of SkQ1 effects on life span and locomotion. In *Drosophila*, sex-specificity of life-span regulation associated with changes in protein homeostasis (27), insulin (28), and steroid signaling (29) has previously been reported. It was demonstrated that changes in expression of sexual differentiation pathway genes in adults can affect adult life span (30). The fundamental evolutionarily conserved systemic regulation of aging by the reproductive system may also account for the sex-specificity of life-span control (31).

Analysis of a correlation between the parameters of a Gompertz function in normal physiological conditions (Strehler–Mildvan correlation, ref. 32) that apparently reflects internal physiological patterns of mortality (33) allowed us to suggest that feeding flies SkQ1 reduced the rate of decrease in fly vitality and, consequently, slowed aging (17). In old flies, we observed a decline in locomotion in both SkQ1-treated and control unmated females. However, the difference in locomotion observed at 10 days of age remained constant at older ages, indicating that SkQ1 did not affect the age-dependent rate of locomotion decline. This result failed to confirm the effect of SkQ1 on aging, likely because this effect is very small. Unfortunately, we were not able to measure locomotion in flies aged more than 40 days because they hardly moved at all. Generally, the difference in age-dependent rate of locomotion decline can be very well manifested in 40-day-old females (34). As it were, a steady increase in locomotion can be regarded as an indicator of health-beneficial effects of SkQ1, which is an important property of any therapeutic.

SkQ1 Effects on Reproduction

Reproduction is believed to shorten life span, and trade-offs between life span and reproduction are widespread (for a review, see ref. 20). Earlier, we described a trade-off between the effects of SkQ1 on the life span of mating flies and their reproduction. SkQ1 did not increase the life span of mated females and males, and the early effect on female survival was absent. At the same time, a significantly higher number of progeny was observed for 10-day-old SkQ1-treated parents compared to controls. The difference became insignificant at 20 days and disappeared completely later in life. However, the early effect of SkQ1 on reproduction was sufficient to provide a significant increase in the total number of progeny generated by SkQ1-treated flies (19). The increase in reproduction stimulated by SkQ1 could be due to elevated mating activity, fecundity, and improved viability of offspring. We assessed experimentally whether these individual traits are affected by SkQ1 treatment. Viability of larvae and imago was not affected by SkQ1, while a reproducible increase in mating activity and fecundity was observed in flies reared on SkQ1-supplemented diet; however, these trends did not reach the level of statistical significance. The accumulation of these small effects during 10 days could be responsible for the increase in overall reproductive ability observed in ref. (19). Indeed, in ref. (19), the statistically significant effect on fecundity was observed in the experiment with 180 control and 180 SkQ1-treated females that laid 9,129 and 9,893 eggs during the first 10 days of their lives, respectively. In this article, fecundity was measured in 315 control and 315 SkQ1-treated females that laid 1,993 and 2,250 eggs during the 10th day

of their lives, respectively. As the tendency of reproductive increase in flies reared on SkQ1-supplemented diet was clear, we decided that further fivefold increase in sample size, which potentially allows to reach significance, is not rational.

Of all the traits, frequency of mating is significantly associated with the extent of the female survival cost of mating (21). SkQ1 affects locomotion, and a reasonable speculation would be that general activity of flies is raised due to SkQ1 treatment. Increased mating frequency could just be a part of this general effect. Gruber and coauthors (35) suggested that our results on mated flies "point to a possible increase in energy production … as a consequence of treatment, an effect that may be explainable by taking into consideration the feedback mechanisms between ROS and energy production in mitochondria." Another possibility is that the effect of SkQ1 on reproduction is more specific and based on an interaction with the metabolism of sex peptides. These male seminal fluid proteins can profoundly change female gene expression and physiology, including egg production and frequency of mating (36).

SkQ1 Effects on Life Span in Extreme Environments

Earlier (17), we showed a steady 10% increase in *Drosophila* life span under SkQ1 treatment in standard laboratory conditions. In this article, we assessed the effectiveness of SkQ1 treatment when changing environmental factors: temperature and diet.

Physiologically, the possible temperature range for fruit flies varies from 5°C to 31°C; pupae and adult insects can tolerate short-term cooling to −5°C, though larval development is only possible at temperatures above 10°C (for references, see ref. 37). Lowering the temperature below 11°C induces a state of diapause (38). The environmentally optimal temperature for flies is considered to range from 17°C to 25°C (39). Hence, lowering the temperature to 18°C places flies on the border of the optimal environment, and we can view these conditions as moderately stressful. Under this moderate stress, SkQ1 increased the life span of males by 27% and of females by 12%, that is, slightly more effectively than in a standard environment. This result supports the idea that the impact of antioxidants is more effective in weak organisms or under suboptimal conditions (40) and is in agreement with the fact that the effect of SkQ1 on life span was more pronounced in mice kept in a vivarium with unfavorable conditions (41). Thus, SkQ1 that is effective in healthy organisms is even more effective when some factors weakened the organism's status. Lowering the temperature to 8°C places flies in a much more stressful condition, as does starvation. In our experiments, in both cases of severe stress, SkQ1 failed to affect life span of either males or females. Thus, SkQ1 was not effective when the environment severely impaired the organism's status.

The ambient temperature affects the life span and the rate of aging of fruit flies (42). Generally, low temperatures are associated with a longer life span in both wild populations and in laboratory conditions (for a review, see ref. 43). This conclusion was fully confirmed in our experiments. Life span was considerably increased at low temperatures: In males, the effect was more pronounced at 8°C, and in females, the effect was similar at both temperatures. It is always of interest to see if a drug is able to increase a life span that is already quite long. Our data show that SkQ1 effectively increased the life span of long-living animals under certain conditions. Overall, the SkQ1 effect did not depend on the mean life span. Under starvation conditions, it was extremely low, whereas at 8°C, it was extremely high, and in both cases, SkQ1 failed to increase survival under severe stress conditions. Whether mechanisms of SkQ1 influence are similar regardless of the nature of the stress remains to be elucidates.

A widely accepted point of view is that a moderate decrease in ROS production is the major factor contributing to the life span increase in animals with reduced mitochondrial function, even though the relationship between alterations in mitochondrial function and intensity of ROS production is not always obvious. Indeed, severe reduction in mitochondrial function and ROS production is regarded as a life-shortening factor (for a review, see ref. 44). In flies raised at 18°C, mitochondrial performance is increased (45). It is possible that at this temperature ROS production is also increased, which explains why the SKQ1 effect on life span is more pronounced at 18°C compared to 25°C. It is also possible that in both stressful conditions (low temperature and diet restriction) the metabolic rate became extremely low and mitochondria function was minimized, which decreased ROS production; in this case, the antioxidant (SkQ1) diet that further decreased the amount of ROS was not advantageous. Several observations make our speculation reasonable. First, it was demonstrated that at 6°C the metabolic rate in *Drosophila* is indeed very much decreased (46). Second, though diet restriction is not necessarily associated with a reduction in metabolic rate or in the rate of the generation of superoxide and hydrogen peroxide from isolated mitochondria (for a review, see ref. 47), starvation is associated with a decrease in carbohydrate and lipid metabolism (48). An alternative point of view regards the increased formation of ROS as a signal that induces endogenous defense mechanisms leading to increased longevity (for a review, see ref. 49). Considering this, antioxidants that reduce the amount of ROS may not be expected to promote health and life span. Although SkQ1 did not increase life span under severe stress conditions, overall, our data on SkQ1 presented here and elsewhere (16,17) are not consistent with this view.

SkQ1 Effects on the Life Span of Flies With Genetic Alterations in the Mitochondrial Systems Responsible for Production and Detoxification of ROS

Mitochondria are an important source of ROS. At various sites within the mitochondrial ETC, which is composed of four multiprotein complexes (complexes I–IV), in particular in complexes I, II, and III, electrons may occasionally leak to oxygen, partially reducing this molecule to a superoxide anion (50,51). The superoxide anion, the precursor of most ROS, is converted to hydrogen peroxide through spontaneous dismutation or through a reaction catalyzed by superoxide dismutases, including mitochondrial Mn-superoxide dismutase. Mitochondria themselves are sensitive to ROS damage. Given the central role that mitochondria and mitochondrial dysfunction play in cancer, neurodegeneration, and the aging process, there is great interest in strategies to protect mitochondria from ROS-mediated damage (35).

Homozygous lethal mutations in genes encoding components of the ETC most likely reduce their function and, consequently, ROS production by the ETC to a level that is incompatible with life. It was experimentally demonstrated that RNAi knockdown of some components of cytochrome c-oxidase has deleterious effects on survival (52). Presumably, suppression of the cytochrome c-oxidase activity was too strong to extend life span. The positive effects of mutations in genes encoding components of the ETC on the life span of heterozygous males and females demonstrated in this article can also be regarded in the context of the hypothesis considering moderately decreased amount of ROS to be the factor contributing to the life-span increase. However, our pilot experiments failed to reveal any difference in the

amount of ROS between control and mutant females. The whole bodies of 10-day-old females were used in these experiments, and a possibility remains that the amount of ROS may be different in particular tissues and cells and even in cell compartments and organelles, and at different ages. These issues remain to be elucidated in our further experiments. We also recognize that, though control and mutant lines with initially the same genetic background were used in our experiments, effects of heterosis cannot be completely excluded, as control and mutant lines were maintained independently for years.

In both males and females heterozygous for lethal mutations in genes controlling enzymes of the ETC, increased life span was not further increased by SkQ1 treatment. This is in agreement with the suggestion that mutations and SkQ1 act via the same pathway: In mutants, the amount of ROS produced by mitochondria was already reduced, and SkQ1 was not effective. Interestingly, the other mutation in *SdhB*, which reduced transcription of the gene, was associated with an increased level of mitochondrial hydrogen peroxide production and the decreased life span of the mutant flies (53). It should be stressed, however, that mutation in the gene encoding one of the subunits of cytochrome c-oxidase (complex IV of the ETC that is not supposed to generate ROS) had the same effect on life span, both in the presence or absence of SkQ1, as mutations in genes encoding enzymes of complexes I and II. This result indicates that either not ROS production, but other characteristics of mitochondria are altered in mutants and cured by SkQ1, or complex IV of the ETC, contrary to what is known now (50,51), is capable to generate ROS, directly or indirectly.

In both males and females heterozygous for a mutation in *Sod2*, life span was decreased compared to controls, hypothetically due to decreased detoxification of superoxide anion in mitochondria. In both males and females, decreased life span was restored by SkQ1 treatment. This result also agrees with our hypothesis. SkQ1 is a small-molecule antioxidant targeted on the mitochondria due to the presence of a lipophilic cation. It can combat ROS produced by mitochondria directly in the mitochondria (25) and compensate for the impaired function of *Sod2*.

Overall, given the well-known properties of SkQ1 (24,25), we speculate that, in vivo, SkQ1 acts as a mitochondria-targeted antioxidant capable of alleviating the negative effects of increased production of mitochondrial ROS on longevity but is not effective when ROS production is already reduced by other means. However, accumulating evidence suggests that mitochondrial role in aging is largely associated with mitochondrial biogenesis and turnover, energy sensing, apoptosis, and calcium dynamics (54), and we cannot exclude that SkQ1 may interfere with these processes.

Supplementary Material

Please visit the article online at http://gerontologist.oxfordjournals.org/ to view supplementary material.

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