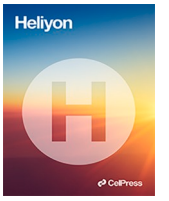
Heliyon 10 (2024) e37854

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Endogenous coenzyme Q content and exogenous bioavailability in *D. melanogaster*

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ARTICLE INFO

Keywords:
Coenzyme Q
Drosophila melanogaster
Aging
Sex differences

ABSTRACT

Development and aging significantly impact the cellular levels of Coenzyme Q (CoQ), which is associated with both pathological and physiological conditions. Aim of this study was to describe the CoQ status throughout the lifetime of *Drosophila melanogaster*, a well-established model in aging studies. CoQ_9 and CoQ distribution was analysed across different body segments and various life stages in both male and female flies. The results indicate that CoQ_9 is the predominant isoform in every phase of flies' life cycle, with the highest concentrations observed in the thorax. We noted distinct trends in CoQ distribution during aging, which varied according to sex and body segments (head, thorax, and abdomen). Supplementation with two concentrations of CoQ_9 and CoQ_{10} (15 μM and 75 μM) for 2 weeks induced a segment- and sex-specific CoQ uptake. Although 75 μM CoQ_{10} was more effective in modulating the CoQ status, lifelong treatment with this concentration did not affect the longevity of the flies.

1. Introduction

Coenzyme Q (CoQ), also known as ubiquinone, due to its ubiquitous presence across biological systems, represents a class of molecules characterized by a benzoquinone ring and a lipid side chain. This side chain varies in length, typically comprising between 6 and 10 isoprene units.

This structure confers to CoQ a lipophilic feature that allows its inclusion in cellular membranes. In mitochondria, CoQ acts as an electron shuttle among respiratory complexes. Recent study showed that CoQ in the respiratory chain is also necessary for the supramolecular organization of the respiratory supercomplexes at the mitochondrial level and is therefore essential for the production of ATP [1]. Therefore, CoQ carries out an essential role in the energetic metabolism: it acts as electron carrier in the mitochondrial respiratory chain transporting the electrons between complex I (NADH-ubiquinone oxidoreductase) or Complex II (succinate-ubiquinone oxidoreductase) and Complex III (succinate-cytochrome c oxidoreductase) [2]. CoQ can accomplish this function owing to the presence of two redox-active sites within the benzoquinone ring, which are the sites of the conversion among its oxidized state (CoQ or ubiquinone), its semi-reduced state (CoQH· or semiquinone), and its fully reduced state ($CoQH_2$ or ubiquinol). Moreover, in its reduced

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https://doi.org/10.1016/j.heliyon.2024.e37854
Received 29 March 2024; Received in revised form 6 August 2024; Accepted 11 September 2024
Available online 12 September 2024

form, CoQ acts as a potent lipophilic antioxidant, directly scavenging free radicals and additionally regenerating other antioxidants by reduction, notably converting α-tocopheroxyl radical back to α-tocopherol [3]. Its antioxidant function efficiently protects lipids from oxidative damage, but also DNA and proteins [4,5]. CoQ plays a role also in other important functions, such as apoptosis [6], cell signaling and gene expression modulation [7,8]. Finally, coenzyme CoQ participates to other secondary pathways being an electron acceptor of different dehydrogenases such as dihydroorotate dehydrogenase (pyrimidine biosynthesis), proline dehydrogenase 1; 2 (proline catabolism), sulfide: quinone oxidoreductase (sulfide detoxification), electron-transferring flavoprotein dehydrogenase (fatty acid oxidation) etc [9]. CoQ is also an essential cofactor for cellular ubiquinone-oxidoreductase ferroptosis suppressor protein 1 (FSP1). In fact, FSP1 activity, by increasing ubiquinol percentage in plasma membranes, was shown to enhance resistance to Ferroptosis [10]. The length of the isoprene chain differs between species; consequently, different organism may present a different major CoQ isoform. For example, *Saccharomyces cerevisiae* contains CoQ with six isoprene units (CoQ_6), *Escherichia coli* CoQ_8, rodents and *Caenorhabditis elegans* CoQ_9, humans and *S. pombe* CoQ_{10}. Nevertheless, some species have more than one CoQ form such as rodents that possess CoQ_9 and CoQ_{10} [11] and *Drosophila melanogaster* that contains CoQ8 (~5 %), CoQ_9 (~82 %) and CoQ_{10} (~13 %) [12,13].

Despite its ubiquitous distribution CoQ content may vary in different organs and tissues. As a general rule, tissues with high energy requirements or metabolic activity, such as heart, kidney, liver and muscle, contain relatively higher concentrations of CoQ [14]. This is also due to the fact that, at cellular level, the distribution of CoQ shows a large portion (40–50 %) localized in the inner mitochondrial membrane, with smaller amounts in the other organelles, reflecting its important role in mitochondrial function [15].

In addition, CoQ content varies in response to different pathophysiological conditions [16]. In humans, the cellular CoQ content varies throughout their lifetime, increasing in the first 20 years of life in all organs, and subsequently enduring a gradual and tissue-specific decrease during aging. A similar trend was observed in rodents [17], on the contrary in nematode *C. elegans* CoQ_9 concentration significantly increases in advanced age [18].

CoQ_{10} deficiency has been described both as a primary and secondary defect and has been involved in the pathogenesis of different conditions. Primary CoQ_{10} deficiencies are rare and severe genetic diseases in which the CoQ deficiency can afflict many organs and tissues, these diseases are associated with the mutations of different ubiquinone biosynthetic genes such as PDSS1, PDSS2, COQ2, COQ4, COQ5, COQ6, COQ7, COQ8A, COQ8B, and COQ9. Secondary CoQ deficiencies are acquired deficit in CoQ biosynthesis that, independently from the genetic background, depend on disorders in mitochondrial and extra-mitochondrial processes [19,20]. Secondary CoQ deficiency is often related to increased oxidation, often observed in pathophysiological conditions associated with increased oxidative stress such as metabolic diseases (diabetes, familial hypercholesterolemia), inflammation, pharmacotherapies and age-associated degenerative diseases (cardiovascular disease, liver disease, neurodegeneration, sarcopenia) [21,22]. In all of these conditions, CoQ supplementation was found to partially improve symptoms [23,24].

However, it is difficult to establish optimal CoQ supplementation strategies due to the remarkable variability in clinical trials associated to experimental designs using different CoQ formulations, doses and length of treatments, as well as patient dependent variability associated with different age and different basal CoQ level [25].

In addition, it is estimated that only 2–3% of the orally administered CoQ is absorbed by the body. CoQ uptake, similarly to its distribution, is tissue-specific; concentrations are higher in plasma and liver, whereas in heart, kidney, brain and skeletal muscle levels are low or potentially undetectable [26–30]. Notably these tissues are the richest in mitochondria highlighting that exogenous CoQ supplementation has specific limitations in reaching mitochondria where they are metabolically active.

Drosophila melanogaster is extensively employed as a model organism for studying the etiology, progression, and treatment of human diseases thanks to its characteristics such as easy genetic manipulation and short life cycle. Drosophila has also proven to be a reliable model for studying human mitochondrial diseases associated with primary coenzyme Q (CoQ) deficiency, particularly when any of the biosynthetic *COQ* genes are silenced [31].

However, to the best of our knowledge, comprehensive description of CoQ content and distribution in *D. melanogaster* with respect to sex and developmental stage remains incomplete. Moreover, as the bioavailability of exogenous CoQ has not been fully characterized, understanding these aspects is essential for employing Drosophila as a model organism to investigate the role of this coenzyme in specific pathophysiologic conditions.

Differently from humans, that present only CoQ_{10} characterized by ten isoprenoid units, Drosophila presents three CoQ forms of which CoQ_9 is the most prevalent form. The simultaneous presence of different CoQ isoforms presents advantages in terms of bioavailability studies enabling the opportunity to discriminate between endogenous and exogenous CoQ uptake that would be more difficult in organisms characterized by a single isoform such as humans.

Given these premises, the objective of this study was to characterize the CoQ content, redox status, and distribution across different body segments (head, thorax, and abdomen) of *D. melanogaster* throughout its development and at various ages in both males and females. Moreover, the effect of CoQ supplementation on the endogenous CoQ content in the three body segments was analysed. This knowledge is fundamental to better characterized CoQ functions but also to identify general approaches to increase CoQ level in pathological conditions caused by CoQ deficiency or in age-related CoQ declines.

2. Methods

2.1. Drosophila culture and supplementation

The *Drosophila melanogaster* strain used in this study was Canton-S (a kind gift of Prof. Daniela Grifoni - University of l'Aquila, Italy). Flies were raised on Formula 4–24® media (Carolina Biological, Burlington, NC, USA) at constant temperature (25 °C) and humidity (60 %) with a 12/12 h light–dark cycle as previously reported [32]. Formula 4–24® media is composed by oat flour, soy flour, wheat

flour, other starches, dibasic calcium phosphate, calcium carbonate, citric acid, niacinamide, riboflavin, sodium chloride, sodium iron pyrophosphate, sucrose, thiamine, mononitrate, brewer's yeast, emulsifier preservatives, mold inhibitor, food staining [33]. The lyophilized diet was hydrated with water and yeast pellets (*Saccharomyces cerevisiae*) were added to the tubes. *Drosophila melanogaster* intended for the characterization of the CoQ_9 and CoQ_{10} contents (520 females and 550 males) and for the longevity curve description (n. 150 for each sex), were sorted by sex within two days after eclosion and moved into tubes with fresh food every 2–3 days until the desired sample age has been reached or lifelong. The tubes containing the laid eggs were maintained until the development of the different larval stages which were collected in four replicates (n. for each replicate: 15 larval L3, 15 pre-pupa, 15 pupa) for the CoQ_9 and CoQ_{10} content analysis by HPLC.

Flies (nearly 80 for each treatment and sex) reared to evaluate the efficacy of different CoQ supplementation strategies were maintained on medium culture (replaced with fresh food every 2–3 days) up to two weeks of life, then they were supplemented for 15 days with different concentrations of oxidized CoQ_9 and CoQ_{10} administered with a micellar delivery system, using a mixture of 3:5 Glycerol:Cremophor® RH 40 (Sigma-Aldrich - Saint Louis, MO, USA). In particular, 1.2 mM CoQ_9 and CoQ_{10} (kind gift from Kaneka, Japan) stocks were prepared and kept at −20 °C until use. CoQ_9 and CoQ_{10} were diluted in the water used to soak 1.0 g of diet at two different concentrations: 15 μM and 75 μM. The water used to prepare the control food (Crem groups) contained the same concentration of Glycerol-Cremophor® RH 40 mix (0.027 %). The concentration of 15 μM CoQ_9 and CoQ_{10} was selected because it corresponds to the supplementation of 50 μg/g of food with various CoQ isoforms used by Grant et al. in Drosophila [34]. The concentration of 75 μM was used to ensure that any lack of effect could not be attributed to an insufficient dosage.

Finally, further flies (n. 210 flies for each treatment and sex) were reared to assess the effects on lifespan (longevity assay) of CoQ supplementation.

2.2. Body segments dissection

With the aid of a stereomicroscope, two dissection tweezers, and after 2 h of fasting followed by anesthesia with FlyNap (Carolina Biological), different flies' body samples were analysed. In particular, pools of 30 heads, 20 thoraxes and 20 abdomens were collected in three replicates for each sex. After flickering, males were collected at 0, 2, 3, 4, 6 weeks, while females at 0, 2, 4, 6, and 8 weeks. These samples served to characterize, by HPLC analysis, the CoQ_9 and CoQ_{10} contents in relation to the different life stages/sex. Further three replicates of flies' body samples were collected from each treated group/sex to evaluate, by HPLC analysis, the efficacy of different CoQ supplementation strategies in modulating endogenous CoQ_9 and CoQ_{10} content.

2.3. CApillary FEeder (CAFE) assay

To evaluate the potential effect of the CoQ supplementation on food intake, we used the capillary feeder method (CAFE) as reported in Ref. [35] with minor modifications. Flies were housed in shortened culture vials measuring 7 cm in length. At the bottom of each tube, cotton balls, soaked with 500 ⌈L of water, were placed to maintain humidity and the tubes were subsequently sealed with parafilm to minimize evaporation and maintain a high level of humidity within the vials. Four microcapillary tubes were inserted in the foam plug of each vial and filled with CoQ_9 or CoQ_{10} (at 15 ⌈M or 75 ⌈M concentrations) diluted in 2.5 % sucrose. The control groups (Crem) received only Glycerol-Cremophor® RH 40 mix (0.027 %) diluted in 2.5 % sucrose. On the day of the experiment, two weeks old flies never supplemented were weighted and starved for 3 h, and then separated into CAFE vials, with eight flies for vial (40 flies/sex/condition; n = 80 flies). To account for evaporation of the liquid food, three vials were set up with feeding capillaries but without flies. Fly consumption was evaluated after 3 h measuring the amount of liquid consumed from the microcapillary tube (in mm) as described by *Fiocca* et al. [36] and data were reported as μL/mg of fly.

2.4. Analysis of body weights

Body weights of male and female flies supplemented with one of the two treatments (CoQ_9 or CoQ_{10}) at different concentration (15 ⌈M or 75 ⌈M), or with the Glycerol-Cremophor® RH 40 mix (Crem) as control, were recorded on 0 and 4 weeks. Briefly, 20 flies in each group (five replicates) were anesthetized by FlyNap (Carolina Biological) and then weighed on a balance. The mean body weights of the flies in each group were calculated.

2.5. Longevity assay

The synchronization of male and female flies after eclosion was carried out as reported by *Linford* et al. [37], flies were allowed to mate freely for two days before separating them according to sex [38] under FlyNap (Carolina Biological) anesthesia. Flies were moved to tubes with fresh food every 2–3 days, and the number of living flies was recorded. This operation was repeated until all the flies died. Kaplan–Meier survival curves were generated for lifespan assessment. Survival curves were prepared by Kaplan-Meier survival analysis and analysed using the OASIS2 software [39].

2.6. Coenzyme Q quantification

The two main isoform of Coenzyme Q, CoQ_9 and CoQ_{10}, in both oxidized and reduced forms, were quantified in all larval stages (L3, pre-pupa and pupa) and in previously dissected heads, thoraxes and abdomens of adult flies. Each sample, previously weighed, was

subjected to a single extraction by solubilization in 1 mL of 1-propanol followed by sonication. Specifically, four sonication cycles on L3, pre-pupa and pupa and two cycles on all body segments were performed. Each cycle lasted 3 min and was alternated with 1 min of cooling on ice. The number and duration of sonication cycles were previously optimized to ensure total extraction of ubiquinones from the samples and minimize their oxidation. The samples were then centrifuged at 4 °C for 2 min at 20,900 g and 40 μL of supernatant were injected in HPLC. CoQ_9 and CoQ_{10} level and their oxidative status were analysed by using a Shiseido Nanospace HPLC (Shiseido Co. Ltd, Tokyo, Japan) equipped with an electrochemical detector (ECD) model 3005, pumps one and two model 3201, a flow-channel degasser model 3009, a switch valve model 3012, a column oven model 3014 and a refrigerated automatic autosampler model 3023. The system used 3 chromatographic columns: a Capcell Pack C18 MG concentrating column (2 mm I.D. x 35 mm, 5 μm), a Capcell Pack C18 AQ analytical column (2 mm I.D. x 150 mm, 3 μm), an OSAKA SODA CQ-R reducing column (2.0 mm I.D x 20 mm) [40].

Two mobile phases were used in the chromatography, The first mobile phase was used to flux the sample through the concentrating column and its composition was 50 mM sodium perchlorate in methanol/distilled water (95/5 v/v). The phase was pumped at a flow rate of 200 μL/min. After 2 min, using a column-switching system, coenzymes were eluted from the concentrating column to analytical one by a second mobile phase which composition was 50 mM sodium perchlorate in methanol/isopropanol (70/30 v/v) that was fluxed at a flow rate of 80 μL/min. After separation, molecules were eluted to a reducing column capable of fully reducing the peaks of

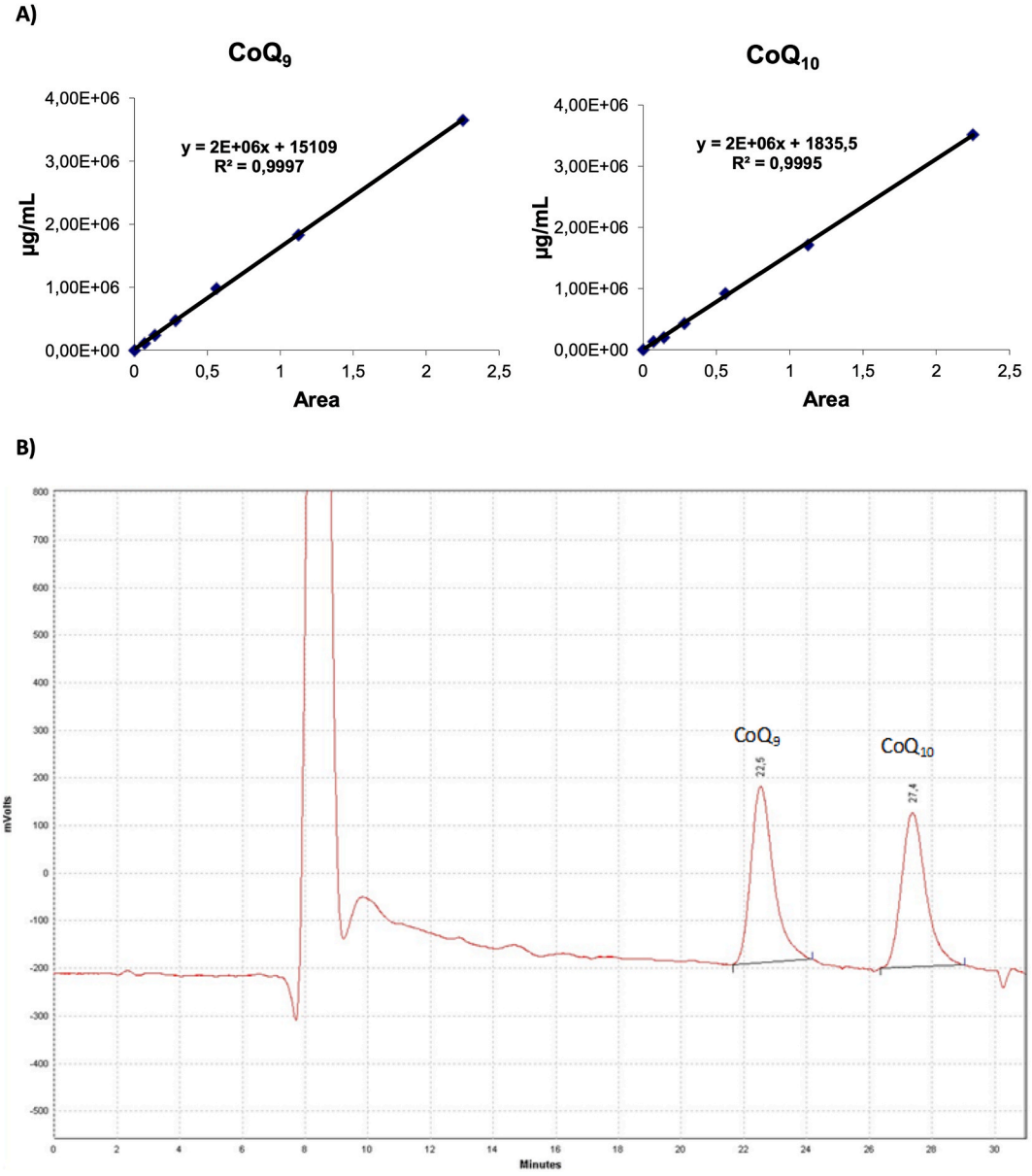

Fig. 1. A) Quinones Standard calibration curve, B) HPLC-ECD trace of CoQ_9 and CoQ_{10}, the standards were only in the oxidized form.

ubiquinone, making possible the discrimination of both oxidized forms of CoQ_9 and CoQ_{10} simultaneously. The column oven was set at 40 °C and the oxidation potential for ECD was 650 mV.

Ubiquinones were identified and quantified by using an ultrapure external standard of both CoQ_9 and CoQ_{10}, kindly donated by Kaneka (Kobe, Japan), previously prepared in ethanol and stored at −80 °C, showing a good correlation (R2 > 0.999) in a range between 0.07 and 2.25 μg/mL (Fig. 1A and B). The results were expressed as μg/g.

An example of a HPLC-ECD of CoQ chromatography obtained in a biological sample of Drosophila is shown in Fig. 2.

2.7. Statistical analysis

Each experiment was performed at least in three replicates, and data are presented as means ± SD. Two-way ANOVA was used to compare differences among groups followed by Tukey's test (Prism 8, GraphPad Software, San Diego, CA). Values of $p \leq 0.05$ were considered statistically significant. Survival curves were constructed by Kaplan-Meier survival estimator and analysed using the OASIS2 software [39].

3. Results

3.1. Coenzyme Q content and distribution varied from larval stages to aging

Drosophila melanogaster was analysed for its CoQ content and distribution from the final larval stage through to aging, with specific attention to differences across various body segments (head, thorax, and abdomen) in the two sexes.

Samples of L3, pre-pupa, and pupa stages were collected, and after flickering, males were analysed at 0, 2, 3, 4, 6 weeks, while females at 0, 2, 4, 6, 8 weeks (Fig. 3A). It was not possible to analyse males at 8 weeks because the survival rate was lower than 10 % (Fig. 3B). The survival rate of males at 6 weeks (66 %) was comparable to the survival rate of females at 8 weeks (58 %) (Fig. 3B).

In the three larval stages, CoQ_9 content was significantly higher than CoQ_{10} content and, with the progress of larval stages, only the content of CoQ_9 significantly increased (Fig. 3C).

In the adult phase, similarly to what was observed in the larval stages, CoQ_9 content was markedly higher than CoQ_{10} content in both males and females in all the three body segments (Fig. 3D–G). In particular, the content of CoQ_9 (Fig. 3D and E) was higher in the thorax compared to the other segments. In the thorax, CoQ_9 reached the maximum at 4 and 3 weeks in females (235.1 ± 43.2 μg/g) and males (235.2 ± 40.4 μg/g), respectively but only in males it significantly decreased in the following weeks. In the head of females, the maximum content of CoQ_9 was measured at 6 weeks (148.6 ± 12.7 μg/g) and was almost double compared to the maximum level reached in males (87.0 ± 10.2 μg/g at 3 weeks). The abdomen had the lowest CoQ_9 content compared to the other segments, but an

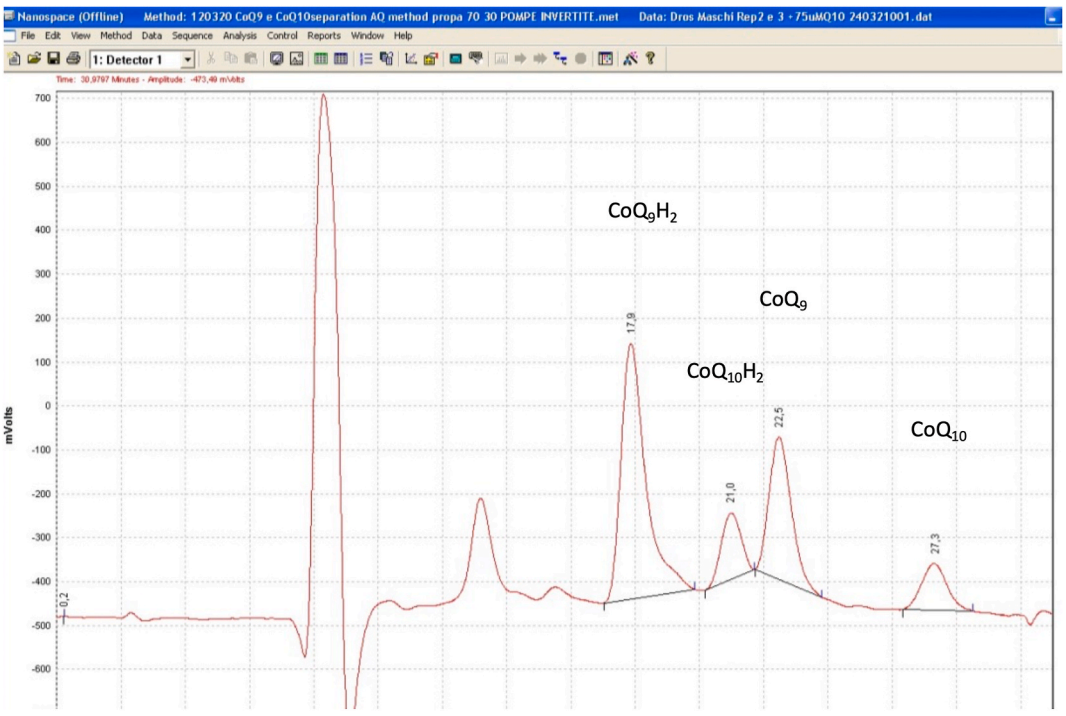

Fig. 2. Example of HPLC-ECD of CoQ in a biological sample of Drosophila (flies head). Both isoforms and redox forms are present displaying four peaks CoQ_9/COQ_9H_2 and $CoQ_{10}/COQ_{10}H_2$.

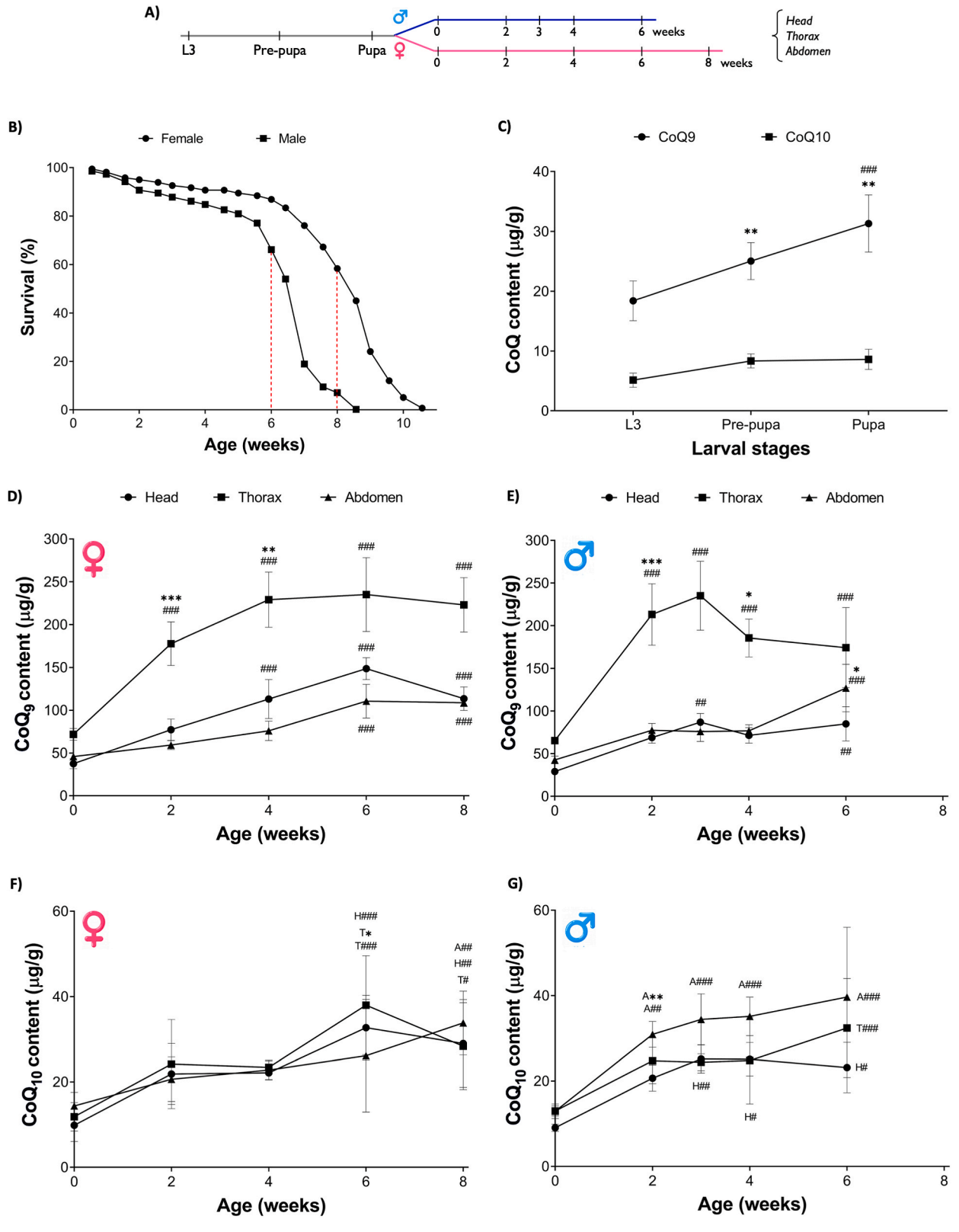

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Fig. 3. Survival curves and CoQ_9 and CoQ_{10} content and distribution across various life stages of *D. melanogaster*. A) Stages of life at which samples were taken. (B) Percentage of survival of female (●) and male (■) flies (n = 520 and n = 550, respectively) as a function of time (in weeks). C) CoQ_9 (●) and CoQ_{10} (■) in larval stages (n = 15 larvae in four replicates at each larval stage). D-G) CoQ_9 (in females: D, and males: E) and CoQ_{10} (in females: F, and males: G) content in the three body segments (● head, ■ thorax and ▲ abdomen; three pools for each sex and age of n = 30 heads, n 20 thorax, n = 20 abdomen) in different life stages. Data were compared to L3 stage or 0-week-old flies (#p ≤ 0.05; ##p ≤ 0.01; ###p ≤ 0.001) or to the previous time point (*p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001). H: Head; T: Thorax; A: Abdomen.

increase has been observed in both sexes at 6 weeks.

The content of CoQ_{10}, in all body segments in both males and females, showed an increasing trend. At 6 weeks CoQ_{10} content was significantly higher compared to the previous weeks in the thorax and head of female flies, while in the abdomen only at 8 weeks the content of CoQ_{10} was significantly higher compared to the previous weeks (Fig. 3F). In males, CoQ_{10} content significantly increased at 2 weeks in the abdomen, at 3 weeks in the head and at 6 weeks in the thorax (Fig. 3G).

In Fig. 4 the total content of CoQ (CoQ_9 + CoQ_{10}) of male and female flies is reported. Of note the total CoQ content in both male and female flies was comparable up to the fourth week. However, by the sixth week, the total CoQ content in females was significantly higher than in males. This observation is particularly intriguing considering that females generally live longer than males. Perhaps the higher CoQ content in females could represent a greater antioxidant defense, enabling them to better counteract oxidative stress, which is suggested as one of the causes of aging.

Summarizing, the data showed that CoQ_9 represents the predominant CoQ isoform in every phase of *D. melanogaster* life cycle in both males and females.

CoQ_9/CoQ_{10} ratio was about 3 in the larval stages (Fig. 5A), while in adult females it varied from 3.0 ± 0.8 in the abdomen at 2 weeks to 9.6 ± 1.7 in the thorax at 4 weeks (Fig. 5B). In males the values were similar but slightly lower in the abdomen and head (Fig. 5C). However, the only interesting variation was observed in the thorax of males where the ratio increased with age up to the maximum at 3 weeks and then decreased reaching at 6 weeks a level comparable to the starting one (Fig. 5C).

These variations in the CoQ content were not associated with alterations in the oxidative status of both isoforms, which remained constant throughout all tested life stages except for the transition from larval L3 to pre-pupa stage. L3 stage exhibited lower CoQ oxidation (CoQ_9: 15.5 ± 0.8 %; CoQ_{10}: 27.0 ± 1.2 %) which approximately doubled at pre-pupa stage (CoQ_9: 37.3 ± 1 %, $p < 0.001$; CoQ_{10}: 54.6 ± 0.6 %, $p < 0.001$) (Fig. 6A) and remained constant throughout all tested life stages (Fig. 6B–E).

3.2. *CoQ supplementation affects CoQ content but not the lifespan*

To find the best supplementation strategies to increase the endogenous CoQ content, 2 weeks old *D. Melanogaster* flies were supplemented for 2 weeks with two different concentrations (15 μM and 75 μM) of both CoQ_9 and CoQ_{10}.

The supplementation induced a tissue-specific CoQ uptake that was greater in female fruit flies in respect to males. In fact, in females CoQ_9 content was increased by both CoQ_9 and CoQ_{10} supplementation in all body segments compared to the control except for thorax, where 75 μM CoQ_9 and 15 μM CoQ_{10} did not show any effects (Fig. 7B). Differently, in males only 75 μM CoQ_{10} treatment was able to modify CoQ_9 endogenous levels in the abdomen (Fig. 7C). The same CoQ_{10} concentration was able to increase the endogenous CoQ_{10} content in all body segments in females (Head: 181.5 ± 22.0 %, $p < 0.001$; Thorax: 201.8 ± 39.9 %, $p < 0.001$; Abdomen: 167.7 ± 70.1 %, $p < 0.001$) (Fig. 7D) while the lower concentration did not modify endogenous CoQ_{10} levels. In males, 75 μM CoQ_{10} led to a significant increase of CoQ_{10} content in thorax (76.5 ± 3.3 %; $p < 0.001$) and abdomen (87.0 ± 23.5 %; $p < 0.001$). Interestingly, CoQ_{10} was significantly increased in the abdomen also after 15 μM CoQ_9 and 15 μM CoQ_{10} supplementation (Fig. 7E).

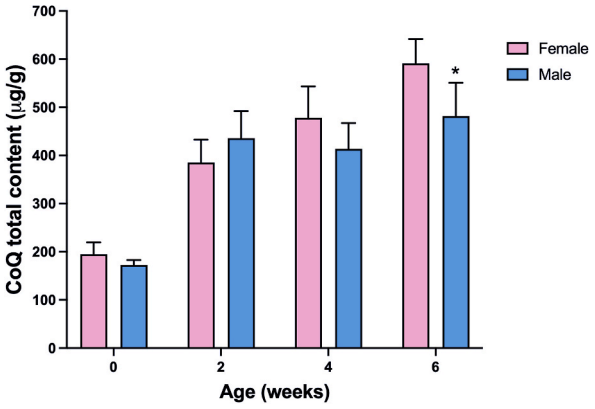

Fig. 4. Total content of CoQ (CoQ_9+CoQ_{10}) across various life stages of male and female *D. melanogaster*.
The bars represent the sum of the content of CoQ_9 and CoQ_{10} in the head, thorax and abdomen of male and female flies in different life stages. Two-way ANOVA was used for statistical analysis. Šídák's multiple comparisons test was used for multiple comparisons of total CoQ of males and females at each age. *$p < 0.05$ male *vs* female at 6 weeks.

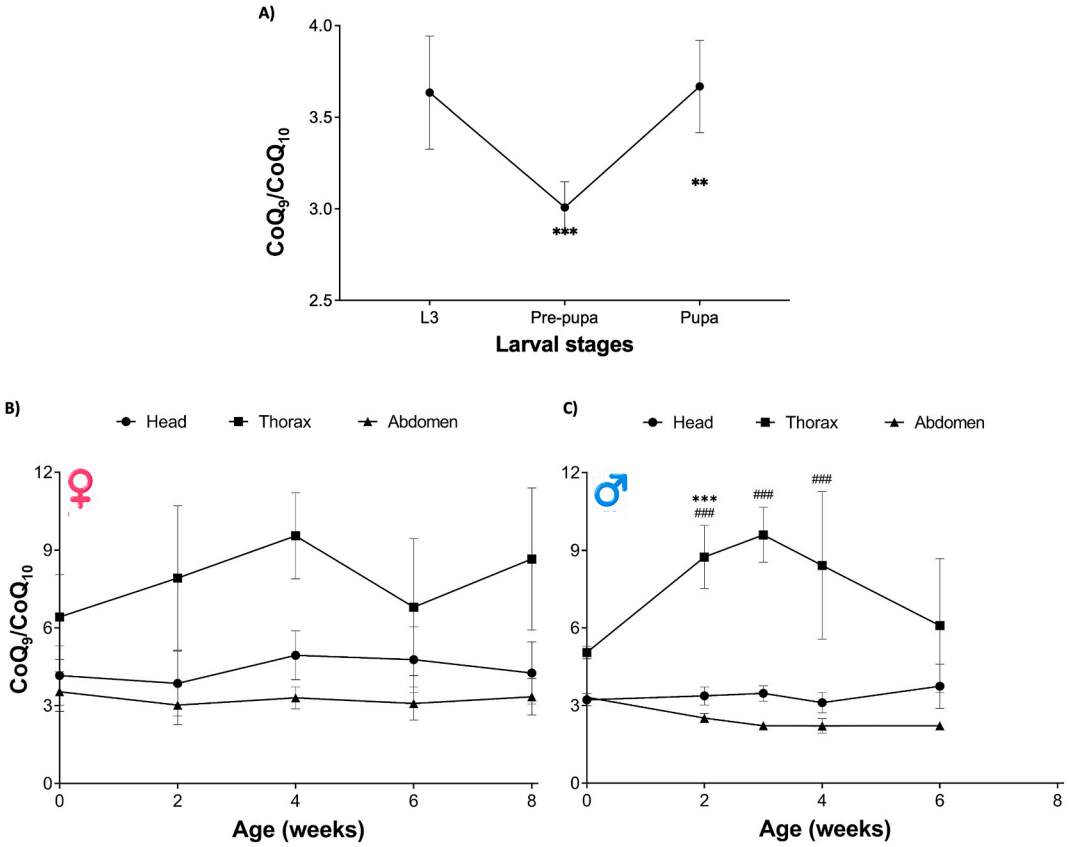

Fig. 5. CoQ_9/CoQ_{10} **ratio across various life stages of *D. melanogaster*.** CoQ_9/CoQ_{10} ratio in different larval stages (A). In adult flies CoQ_9/CoQ_{10} ratio was evaluated in head (●), thorax (.) and abdomen (.) of female (B) and male (C) *at different ages. Data were compared to 0-week-old flies (###p ≤ 0.001) or to the previous time point (**p ≤ 0.01, ***p ≤ 0.001).*

The various supplementations also affected the CoQ_9/CoQ_{10} ratio. In particular, CoQ_9/CoQ_{10} ratio was decreased in females in all body segments after 75 μM CoQ_{10} supplementation, while in males only in thorax with both CoQ_{10} tested concentrations (Fig. 8A and B).

Contrary to what was expected, the supplementations were unable to decrease the percentage of oxidation of both CoQ_9 and CoQ_{10}, and in some cases this parameter was even increased in the head and in the thorax, but not in the abdomen. In particular, CoQ_9 oxidation was significantly increased only in thorax after 15 μM CoQ_9 supplementation in females and after 15 and 75 μM CoQ_{10} supplementation in males (Fig. 8C and D). While CoQ_{10} oxidation was increased in the head of females and males by 75 μM or 15 μM CoQ_9 supplementation, respectively, and in the thorax of both sexes after the supplementation of 15 μM CoQ_9 and 75 μM CoQ_{10}; in males also the supplementation with the lowest CoQ_{10} concentration was able to increase CoQ_{10} oxidation in the thorax (Fig. 8E and F). It should be noted that Crem appears to affect the oxidation state of both forms of CoQ. Indeed, both CoQ_9 and CoQ_{10} show a lower percentage of oxidation compared to the control values reported in Fig. 6D and E.

To exclude possible effects of CoQ_9 or CoQ_{10} supplementation on food intake, the CAFE assay was used (Fig. 9A). Supplementation of CoQ_9 and CoQ_{10} at both tested concentrations did not influence food intake. However, males showed higher food intake in respect to females. To confirm these results, flies' body weights were measured at 0 and 4 weeks (Fig. 9B). No differences in body weights were observed between treatments, although males exhibited different trends from females.

To verify the potential effect of CoQ supplementation on longevity, two weeks old male and females flies where supplemented with 75 μM CoQ_{10} for the duration of their lifespan. 75 μM CoQ_{10} was chosen because showed the greatest effect in increasing the endogenous level of CoQ in both sexes. Fig. 10 shows that CoQ_{10} supplementation does not affect the longevity of Drosophila, as evidenced by the overlapping longevity curves of flies treated with the vehicle and those supplemented with CoQ_{10}, for both females and males. However, it is noteworthy that the vehicle, Cremophor RH 40 (Crem), appears to have a negative effect on longevity. Compared to the control flies, those treated with the vehicle exhibit a lifespan reduction of approximately one week.

4. Discussion

In this study, for the first time, the content of CoQ_9 and CoQ_{10} in *Drosophila melanogaster* was analysed both in larval stages and in the three body segments (head, thorax, and abdomen) of adult male and female flies at various times in their life cycle. The results

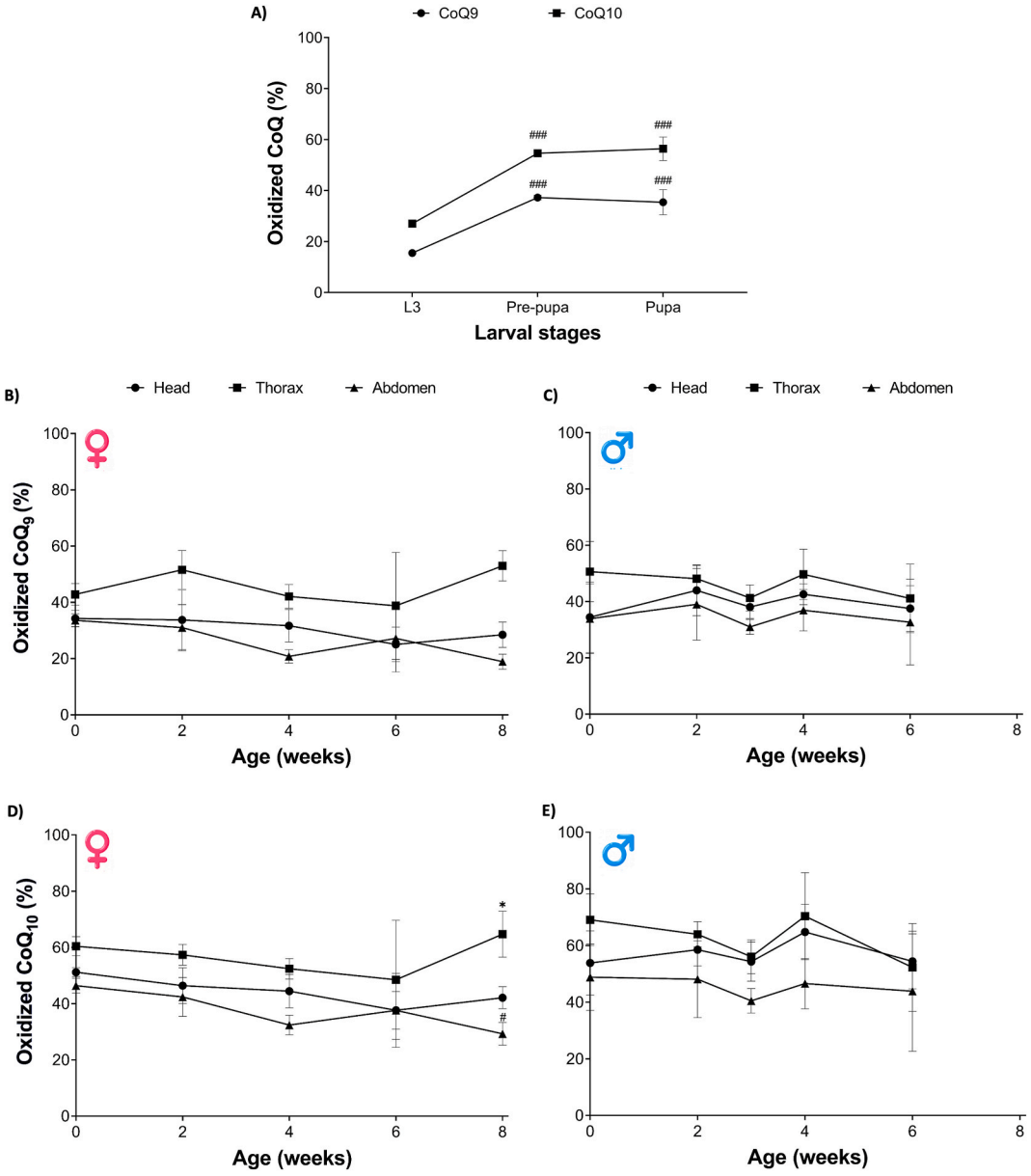

Fig. 6. Oxidative status of CoQ_9 and CoQ_{10} in different life stages of *D. melanogaster*. A) Percentage of CoQ_9 (●) and CoQ_{10} (.) oxidation in different larval stages. B-E) Percentage of oxidized CoQ_9 (in female: B, and male: C) and oxidized CoQ_{10} (in female: D, and male: E) in head (●), thorax (.) and abdomen (.) of flies in different life stages. Data were compared to L3 stage or 0-week-old flies (#p ≤ 0.05, ###p ≤ 0.001) or to the previous time point (*p ≤ 0.05).

obtained show that the content of CoQ_9 and CoQ_{10} differs between the sexes and confirm that CoQ_9 is the predominant form of CoQ in all body segments.

In particular, important morphological changes occur during the development of the larva and pupae, accompanied by a high energy demand and a strong oxidative stress, respectively [41]. This agrees with the observed increase of CoQ_9, involved in ATP production, and with the oxidation of both CoQ_9 isoforms analysed in the last larval instars, particularly between L3 and pre-pupa. The observation that CoQ_9 levels significantly increase during Drosophila development, while CoQ_{10} levels remain unchanged, suggests that CoQ_9 is the predominant functional form of CoQ in the electron transport chain in Drosophila.

Interestingly, the content of CoQ_9 in the thorax is much higher than that measured in the other body segments. In the Drosophila thorax the CoQ_9 content increased rapidly in the first two weeks until it reached its maximum at 3 weeks in males and at 4 weeks in females. One possible explanation for the higher levels of CoQ_9 observed in the thorax compared to other body segments could be its crucial role in cellular bioenergetics. CoQ is predominantly localized in the mitochondria, which are abundant in muscle tissues. These

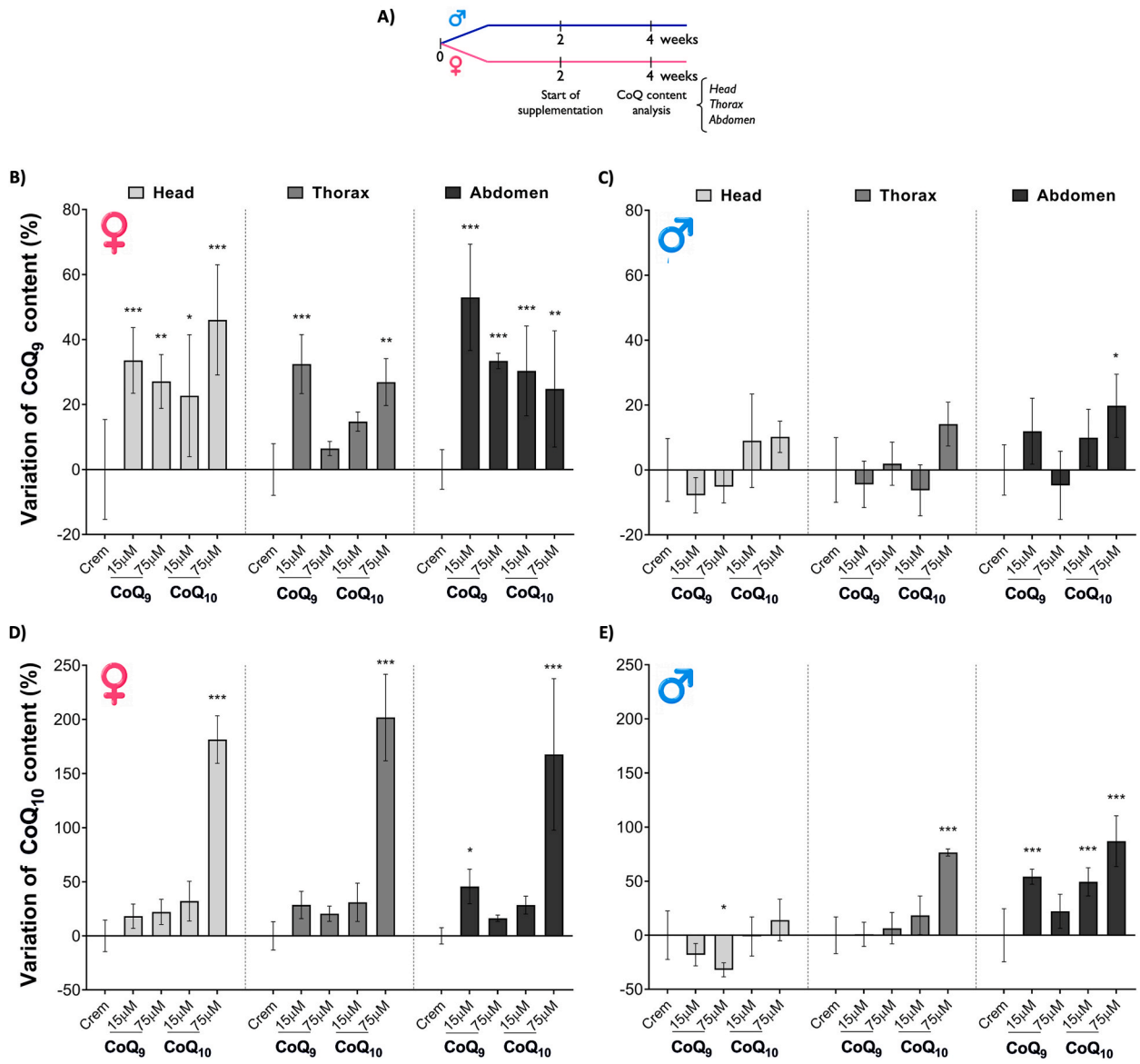

Fig. 7. CoQ_9 **and** CoQ_{10} **endogenous content in *D. melanogaster* supplemented with** CoQ_9 **and** CoQ_{10}**.** Two weeks old flies were supplemented with two concentrations (15 and 75 μM) of CoQ_9 and CoQ_{10} or with the vehicle (Crem) for two weeks (A; n = 80 flies for each condition). The percentage of variation (100*(CoQXsuppl-CoQXbaseline)/CoQXbaseline; CoQXsuppl indicate the CoQ_9 or CoQ_{10} content (μg/g of weight) in a body segment after specified supplementation, and CoQXbaseline represent CoQ_9 or CoQ_{10} content (μg/g of weight) of control flies in the same segment) of CoQ_9 (in female: B, and male: C) and CoQ_{10} (in female: D, and male: E) content was calculated for each body segment (head, thorax, abdomen) and sex. Data were compared to flies supplemented with the carrier (Crem). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

muscles, especially in the thorax where the wing and leg muscles are located, require significant energy for contraction, thereby necessitating higher concentrations of CoQ_9 to meet these demands [15]. Consistent with this hypothesis, in rats a correlation has been identified between muscle mass and the content of CoQ_{10} [42]. However, it should not be forgotten that the abdomen also contains organs that require elevated levels of energy, and therefore further studies will be necessary to confirm this hypothesis.

Sex specific differences in CoQ content during growth and senescence were also observed. In fact, while ubiquinone content increased significantly in both males and in females over the first weeks of adult life, the pattern of these increases was different depending on the sex. While CoQ_9 content in females reached a plateau, in males CoQ_9 content decreased with aging. This could be the result of reduced mobility that occurs with aging, in fact *Zhong* et al. [43] observed that the climbing ability of female flies was slightly higher than that of male flies after 4 weeks of age. A similar trend was obtained in terms of CoQ_9/CoQ_{10} ratio since CoQ_{10}, despite an increasing trend in concentration (in particular, in male flies) showed variations of much lower extent compared to CoQ_9. Therefore, summarizing CoQ_9 is not only the most abundant form but, its content is modulated throughout the life cycle of Drosophila to a much

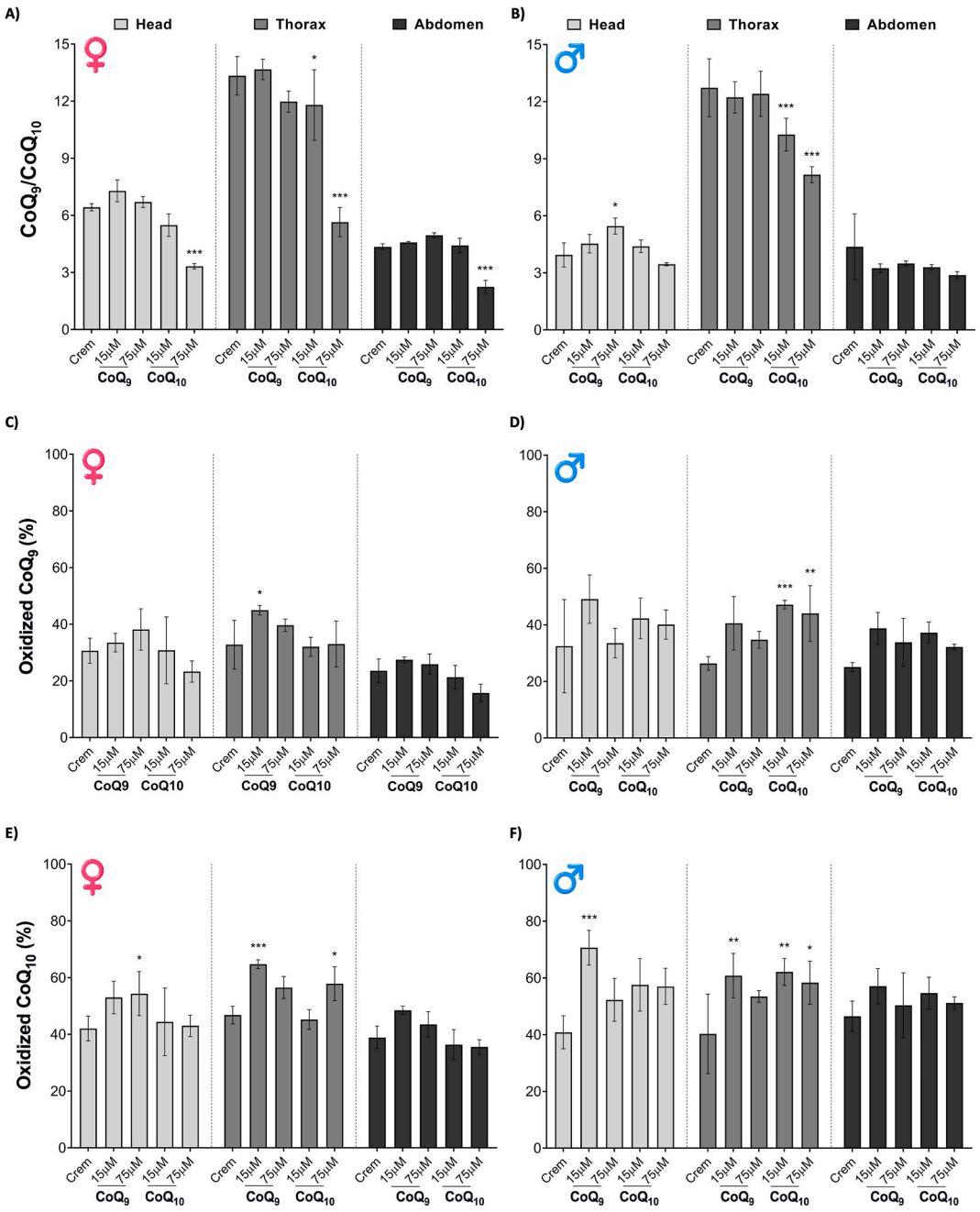

Fig. 8. CoQ_9/CoQ_{10} **ratio and** CoQ_9 **and** CoQ_{10} **oxidative status in *D. melanogaster* after** CoQ_9 **and** CoQ_{10} **supplementation.** Two weeks old flies were supplemented with two concentrations (15 and 75 μM) of CoQ_9 and CoQ_{10} or with the carrier (Crem) for two weeks. CoQ_9/CoQ_{10} ratio (in female: A, and male: B), oxidized CoQ9 (in female: C, and male: D) and oxidized CoQ_{10} (in female: E, and male: F) were evaluated for each body segment (head, thorax, abdomen) and sex. Data were compared to flies supplemented with the carrier (Crem). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

larger extent compared to CoQ_{10} in the thorax of organisms of both sexes, suggesting a potential critical function of this isoform also in relation to muscle activity. Further studies will be necessary to confirm this hypothesis.

On the contrary, head and abdomen segments showed a very different content and ratio of the two CoQ isoforms in respect to thorax. In both sexes the two isoforms showed a continuous increasing trend.

In the head, the main difference between the two sexes was the content of CoQ_9, which reached higher values in females, while the CoQ_9/CoQ_{10} ratio remained constant.

Overall, CoQ_9 and CoQ_{10} are not evenly distributed in the bodies of fruit flies. Specifically, over the course of their lifespan, levels of both CoQ isoforms increase with age and tend to stabilize at an advanced age, except in the thorax of males. These data contradict the

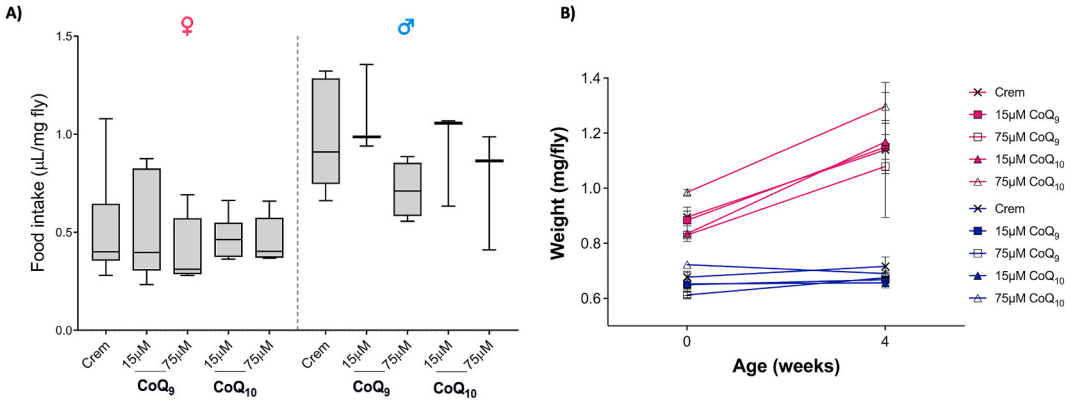

Fig. 9. Food intake and body weights of *D. melanogaster* supplemented with CoQ_9 and CoQ_{10}. (A) Food intake evaluated by CAFE assay of 2 weeks old female and male flies supplemented for 3 h with two concentrations (15 and 75 µM) of CoQ_9 and CoQ_{10} (five pools of n = 8 flies for each condition). (B) Body weights of flies at 0 and 4 weeks, i.e. after two weeks of supplementation of 2 weeks old flies with two concentrations (15 and 75 µM) of CoQ_9 and CoQ_{10} (five pools of n = 20 flies).

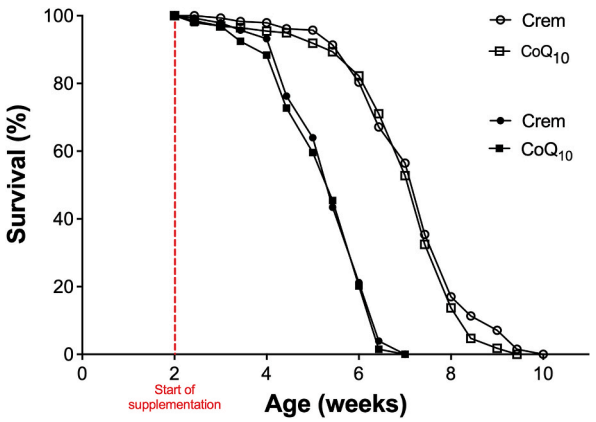

Fig. 10. Survival curves of *D. melanogaster supplemented with CoQ_{10}*. Percentage of survival of female (o) and male (●) flies as function of time (in weeks).

theory that suggests a decrease in CoQ level during aging. However, CoQ_9 levels were also found to increase with advancing age in *C. elegans,* with the highest concentrations of CoQ observed in the oldest worms [18]. The short life cycle of these organisms may not be sufficient to manifest a CoQ deficiency. Moreover, our data reported highlight substantial variations in CoQ content between the two sexes, which is expected considering the pronounced sexual dimorphisms in insects, suggesting the importance of studying males and females independently in future investigations. On the contrary, the sex-related differences seem to be less pronounced in vertebrates, in particular in humans where at plasma levels no significant differences were observed between male and females [44], while diet has been highlighted as a major confounding effect compared to gender [45].

Furthermore, in our *Drosophila* model, CoQ_{10} appears to exhibit a higher percentage of oxidation compared to CoQ_9 with oxidation increasing only during the larval and pupal stages, after which it remains relatively constant throughout the adult phase. Therefore, in contrast to other published reports, in the present study CoQ oxidative status does not seem to accurately reflect adult Drosophila tissues redox status. In fact, it has been reported that in *D. melanogaster* ROS levels increase with age as a consequence of loss of respiratory competence at complex I in the mitochondria [46]. However, these mtROS, while playing a role in signal ing pathways [47], do not seem to promote CoQ oxidation in our experimental model.

An additional objective of our study was to evaluate the effect of dietary CoQ_9 and CoQ_{10} supplementations on the tissue content of these two isoforms in male and female Drosophila.

Our results demonstrate that although CoQ_9 is the predominant isoform in Drosophila, when dissolved in a micellar solution containing Cremophor® RH 40 and glycerol and administered at concentrations of 15 and 75 µM for two weeks to 2-week-old flies, it had no effect on the content of both CoQ isoforms in all body segments of males, except in the abdomen, where the CoQ_{10} resulted increased. On the contrary, in females, CoQ_9 supplementation was associated with an increase of the content of the same isoforms (i.e. CoQ_9) in all body segments suggesting a higher bioavailability of CoQ_9 in females despite the food intake being lower than in males. Interestingly, the lower dose of CoQ_9 was more effective in increasing the levels of this coenzyme isoform. We hypothesize that the

higher concentration of CoQ_9 may have increased the endogenous content of CoQ_9 to a level that activates a sort of feedback mechanism, where the product itself inhibits the pathway leading to its synthesis. In contrast, the lower dose of CoQ_9 may not have been sufficient to trigger this feedback mechanism, thus contributing solely to an increase in CoQ_9 levels, together with an increased abdomen CoQ_{10} content. Another hypothesis is that an excess of dietary CoQ9 might have triggered the degradation of CoQ9 itself rather than inhibiting its synthesis. In our opinion, this aspect deserves further research.

Females also showed enhanced capacity to incorporate CoQ_{10} as well as CoQ_9. Notably, when CoQ_{10} was supplemented at 15 and 75 μM, increases of both CoQ_9 (which remained unchanged in males) and CoQ_{10} tissue content were observed. In particular, in females 75 μM CoQ_{10} was required to ensure a significant increase of CoQ_{10} isoform, together with a greater increase of CoQ_9, in all segments. The same concentration was effective, although to a lesser extension, in the thorax and abdomen of males. In agreement with our findings, Beaulieu et al. [47] observed that CoQ_{10} supplementation increases endogenous CoQ_{10} levels more significantly in women than in men. This aspect is particularly important because it suggests that different dosages may be required to achieve comparable results in both sexes. Future studies will be necessary to elucidate these observations and to determine the underlying mechanisms responsible for this difference.

The observed rise in CoQ_9 levels in the thorax may be linked to the higher demand for CoQ, driven by the abundance of mitochondria typically found in muscle tissue, a phenomenon previously demonstrated in both mice and rats [29]. On the other hand, the abdomen, characterized by the presence of the reproductive system and adipose tissue, could retain ubiquinone. Given its lipophilic nature, the distribution of Co Q could also be influenced by the lipid content of the tissue [14]. Due to the absence of the blood-brain barrier in *Drosophila* head, resulting in a lower selectivity to the passage of molecules, the increase in CoQ was of the same magnitude to that observed in other segments, which contrasts with findings in mice [29] and rats [27], where the brain has the lowest capacity for CoQ uptake. However, in our study, the fly head segment did not exclusively consist of brain tissue. Hence, investigating the distribution of CoQ in the head would be intriguing. If a specific increase in brain tissue does occur, it could provide an engaging model for exploring CoQ in the context of neurodegenerative diseases.

The potential interconversion between the two CoQ isoforms is of interest. In other words, when supplementing CoQ_{10}, an increase of endogenous CoQ_9 was also observed in female flies. However, the increase in endogenous CoQ_{10} only occurs when the same isoform was administered, except in the male abdomen, where CoQ_9 was also effective in boosting endogenous CoQ_{10} levels. Since currently no enzymes capable of converting one form of CoQ into another are known, although their existence cannot be ruled out, it can be hypothesized that the ability of a specific CoQ isoform to increase the level of another CoQ isoform could occur as a result of the modulation of biochemical pathways involved in their synthesis or catabolism. In particular, since CoQ_9 is the major quinone form synthesized by Drosophila, it is possible that CoQ_{10} supplementation drives biological changes likely associated with metabolic and mitochondria function that might influence CoQ_9 endogenous content. In this respect in future studies, it would be interesting to investigate mitochondria biogenesis and its modulation by CoQ_9 and CoQ_{10}.

While CoQ functions as a potent antioxidant, its supplementation as ubiquinones did not enhance the oxidative status of the CoQ pool, as evidenced by the increase in the absolute amounts of oxidized CoQ_9 and CoQ_{10}, rather than a decrease. One explanation could be that the endogenous reductases, which are necessary to reduce both endogenous and exogenous ubiquinone, were insufficiently effective, leading to an accumulation of CoQ in its oxidized form rather than to its prompt reduction.

Although 75 μM CoQ_{10} showed the highest uptake, when supplemented throughout the entire adult life of flies starting at two weeks of age, it did not affect lifespan. This might be attributed to the general observation that *D. melanogaster* does not exhibit a significant decrease in CoQ content with age, so the supplementation was not performed in a condition of CoQ deficiency [48,49]. Our data are not in agreement with the results *of Spindler* et al. [50] that supplemented male flies with CoQ_{10} and observed a decrease in mean lifespan and, surprisingly, a reduction in endogenous CoQ_{10} content. However, the supplementation was carried out using an inaccurate method; in fact, 10 μL of CoQ_{10} 5 mM dissolved in DMSO were spread on 5.5 mL of solidified food. This led to the creation of areas with different concentrations of CoQ on the surface of the food. On the other hand, positive effects have been observed with the supplementation of three isoforms of CoQ (CoQ_4, CoQ_9, CoQ_{10}; 50 μg per g of food) during the larval stages, capable to restore the neuronal development altered by the mutation of the *qless* gene, involved in the synthesis of CoQ [34].

Considering these data and the variations in the distribution of endogenous CoQ among the body segments of fruit flies over time and by sex, it is also conceivable that the absorption and subsequent efficacy of exogenous CoQ could vary significantly and be highly tissue specific. This has already been demonstrated in studies involving mice [29] and rats [26,27].

In conclusion, sex-related differences were recorded in the content of CoQ_9 and CoQ_{10} within the abdomen, thorax, and head. Variations were also observed in the temporal distribution of CoQ_9 and CoQ_{10} across these body segments, as well as in the absorption of the two isoforms when supplemented from 2 weeks of age until the age of 4 weeks. Additionally, supplementing 2-week-old Drosophila with CoQ_{10} throughout their lifespan had no effect on longevity. On these bases, future investigations on the impacts of CoQ in *D. melanogaster* will need to address the sexes separately, given their distinct traits and differential responses to CoQ supplementation. It should be noted that the vehicle used for supplementation, a procedure that in our cell culture experiments provided optimal stability and solubility, in the present experimental setting slightly alters the experimental conditions by reducing the oxidation level of endogenous CoQ and decreasing the longevity of the Drosophila by one week. Future studies will need to be conducted using alternative vehicles. Moreover, our results cannot be generalized to other strains of Drosophila. Future studies will be necessary to verify whether the observations made in the Canton-S strain will also be confirmed in other strains. The absence of functional assays represents another limitation of our study, and future research will be necessary to better understand the implications of our findings in relation to the physiology of both male and female *Drosophila melanogaster*.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have read and consent for publication.

Availability of data and materials

Original data that support the findings of this study will be made available upon request.

CRediT authorship contribution statement

Ilenia Cirilli: Writing – original draft, Methodology, Investigation, Formal analysis. **Patrick Orlando:** Writing – review & editing, Methodology, Investigation. **Silvana Hrelia:** Writing – review & editing. **Fabio Marcheggiani:** Methodology, Investigation. **Luca Tiano:** Writing – review & editing, Conceptualization. **Daniela Beghelli:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation. **Cristina Angeloni:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

List of abbreviations

CAFE	CApillaryFEeder
CoQ	Coenzyme Q
Crem	Glycerol-Cremophor® RH 40 mix

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