EFFECTS OF MICRONUTRIENTS ON SURVIVAL AND MITOCHONDRIAL VIABILITY IN HONEY BEES (APIS MELLIFERA)

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Abstract

Old age is a phase in life that many of us fears. This fear is often related to the impaired quality of life caused by diseases old age brings along. Several micronutrients, often in plant extracts, have promise to significantly affect survival and aging.

The production of reactive oxygen species (ROS), a byproduct of cellular respiration in mitochondria, was often linked to aging and senescence. Compounds that can inactivate ROS, so-called antioxidants, are suggested to increase lifespan. The plant extract *Rhodiola rosea,* claimed to possess antioxidant properties*,* will therefore in this sense, be able extend lifespan. However, for several animal models as well as for humans, life extending effects are disputed and the possible mechanisms through which *Rhodiola rosea* acts are not clear. In addition, the role of antioxidants in aging is highly disputed.

Honeybees serve as an invertebrate model to better understand the emergence of large interindividual differences in aging with likely relevance to human population as well.

Here I show that *Rhodiola rosea* affects lifespan in the honeybee (*Apis mellifera carnica*) in a dose-dependent matter. *Rhodiola rosea* appears to increase the oxygen consumption in solution. Together, concentration dependent lifespan effects and oxygen consumption by *Rhodiola rosea* may be interpreted as follows: high doses cause the bees to die from hypoxia due to lowered availability of oxygen in the cells. Low doses of *Rhodiola rosea,* however, may increase the lifespan due to ROS scavenging and hence an antioxidant mode of action. In summary, this study suggests that lifespan effects by *Rhodiola rosea* may be linked to oxidative stress.

Research on micronutrients can be valuable, because it can provide insights to the puzzling mechanism behind aging. Being able to understand the mechanisms behind the intriguing lifespan of honeybees can be helpful to understand the lifespan in humans.

Sammendrag

Det å bli gammel er noe flere av oss frykter. Denne frykten er ofte relatert til en nedsatt livskvalitet som følge av sykdommene alderdommen bringer med seg. Flere næringsstoffer, og ofte de i planteekstrakter, sies å påvirke levetid og aldring.

Produksjonen av reaktive oksygen derivater (ROS), et biprodukt av cellulær respirasjon i mitokondrier, ble ofte satt i sammenheng med aldring og levetid. Antioksidanter, som kan inaktivere ROS, er kjemiske stoffer som foreslås å kunne øke levetiden. *Rhodiola rosea* er et planteekstrakt som hevdes å øke levetiden ved å inneholde antioksidanter. Denne effekten på levetid, både hos forsøksdyr og mennesker, er imidlertid mye diskutert og på hvilken måte *Rhodiola rosea* virker er uklart. I tillegg er det uenigheter når det kommer til hvilken rolle antioksidanter faktisk har når det kommer til aldring.

Honningbier er virvelløse dyr mye brukt som modellorganisme for å forstå de store interindividuelle forskjellene i aldringsprosessen, som også er like relevant hos mennesker.

Jeg dokumenterer at *Rhodiola rosea* påvirker levetiden til honningbier (*Apis mellifera carnica*) avhengig av dose. Det ser videre ut til at *Rhodiola rosea* øker oksygenopptaket i løsning. Disse to resultatene, en dose-avhengig effekt på levetid og økt oksygenopptak, kan tolkes på følgende måte: høye doser fører til at biene dør på grunn av hypoksi, og lite oksygen i cellene. Lavere doser, på den andre siden, kan være fordelaktig og øke levetiden ved at *Rhodiola rosea* virker som en antioksidant og bekjemper ROS. Kort oppsummert; dette indikerer at påvirkningen *Rhodiola rosea* har på levetid er relatert til oksidativt stress.

Forskning på næringsstoffer kan være verdifullt, ved å gi innsikt i mekanismene bak den gåtefulle aldringsprosessen. Å forstå mekanismene bak aldringsprosessen hos honningbier kan være nyttig for å forstå de samme mekanismene hos mennesker.

List of abbreviations

ROS reactive oxidative species

ETS electron transport system

ETC electron transport chain

ATP adenosine triphosphate

DNA deoxyribonucleic acid

EGTA Ethylene glycol tetraacetic acid

BSA bovine serum albumine

KOH potassium hydroxide

DTT dithiothreitol

SOD superoxide dismutase

NAD^+ Nicotinamide adenine dinucleotide

FAD Flavin adenine dinucleotide

OCT octanoyl carnitine

FCCP carbonylcyanide p-trifluoromethoxyphenylhydrazone

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1. Introduction

Micronutrients can exert significant effects on survival and aging. Called micronutrients because they are nutrients required by humans and other organisms throughout life in micro amounts, enables the body to produce enzymes, hormones and other substances essential for proper growth and development [1, 2, 3]. Today, a great variety of micronutrients – often plant extracts - are sold freely on the marked, promising a better health and a longer lifespan. Yet, often it is not clear if health claims are valid or if micronutrients come without any risk, when used at different dosages or over longer periods.

The global population aged 60 and above has increased from 200 million in 1950 to around 760 million today. By 2050, it is projected to reach 2 billion [4]. One reason for this is a general increase in life expectancy. Some argue that the increased life expectancy are attributed to cultural adaptions (such as changes in medical treatments and knowledge as well as advances in both nutrition and sanitation practices), rather than genetic evolution [2, 6]. Biologists may raise doubts about the limits to human longevity, while economists worry about how to finance pension and health programs [7]. Although we live longer, we do not necessarily enjoy more years of health; in this prospective, micronutrients and plant extracts could be beneficial, vital and essential.

In order to defend themselves, plants have the ability to synthesize a wide variety of chemical compounds [8]. Many of these compounds, or phytochemicals, have beneficial effects on long-term health when consumed by humans, and can be used to effectively treat human diseases [9]. Phytochemicals are chemical compounds that occur naturally in plants [10]. It is defined as "Nonnutritive bioactive plant substance, considered to have a beneficial effect on human health" [11]. Many phytochemicals have antioxidant activity [12], and these antioxidants help the plant to defend their structures against reactive oxygen species (ROS) produced during metabolism. The human body is as well exposed to reactive oxidant species, and we have also evolved an effective antioxidant system. However, this defense system is not flawless and

dietary addition of plant-based antioxidants may be beneficial [13]. Looking at the evolution, humans may have evolved in a relatively hypoxic environment. And eating plant-based, antioxidant-rich foods makes sense, and did in fact traditionally form the major part of the human diet [13].

Rhodiola rosea is promoted as an adaptogen, which is defined as a substance that "Allow an organism to counteract adverse physical, chemical, and biological stressors increasing non-specific resistance" [14]. "Statens legemiddelverk" in Norway decided in 2003 that *Rhodiola rosea* was no longer to be classified as a medicinal product, and the herb is now marketed and sold freely [15]. *Rhodiola rosea* is also called Roseroot, Rose Root, Golden Root, Siberian Golden Root, and King's Crown [15]. The involvement of plant adaptogens in the prevention of diseases associated with age and aging has been studied intensively. Studies has shown that *Rhodiola rosea* extracts were able to extend the lifespan of fruit fly *Drosophila melanogaster* and nematode *Caenorabdidis elegans* in a dose-dependent manner [16, 17]. There are several different bioactive compounds in the plant *Rhodiola rosea*, and its activity is primarily attributed to p-tyrosol, salidroside and three salidroside-like glycosides (rosarin, rosavin, rosin) [18]. A review article [18] summarizes the pharmacological effects of *Rhodiola rosea* as follows:

- adaptogenic and stess- protective (neuro-cardio and hepato- protective) effects
- cardioprotective effects
- antioxidant effect
- stimulating effect on the central nervous system including effects on cognitive functions such as attention, memory and learning
- anti- fatigue effect
- antidepressive and anxiolytic effects
- endocrine activity normalizing
- lifespan increasing effect.

Another review article [19] evaluated different studies that found positive effects of *Rhodiola rosea* on human subjects. The evaluation showed that all but one of the studies was unreliable. The review article concludes that a potential adaptogenic effect of *Rhodiola rosea* is yet to be sufficiently documented in a scientifically satisfying way.

Human trials are indeed disputed, while model studies seem to repeatedly have confirmed an effect.

Research on animal models can provide critical biological knowledge on factors that might have a negative or positive influence on the health of human beings. Since bees have a short lifespan, and since the genome of the honeybee seems more similar to humans than other insects sequenced thus far [20], the honey bee is widely used as a model organism. The honey bee's social behavior makes it an important model for understanding how genes regulate behavior through the development of the brain and central nervous system. The nests of honey bees are sophisticated social architectures [21], where there is a reproductive division of labor between "Anatomically distinguishable primary reproductive and usually less reproductively-capable workers" [22]. Within the worker caste there is a further division of labor. What is interesting is that the activities and work that are performed by an individual often change with age, a form of behavioral development known as age polyethism [22]. Adult female workers go through an age-related task schedule, which requires them to change between nurse, forager, and winter stages. This age-dependent performance of tasks, together with the intriguingly ability of worker bees the reverse this normal ontogeny [23], makes the honey bee an interesting model organism for particularly study aging.

The cytoplasm of nearly all eukaryotic cells contains mitochondria. They are especially abundant in cells and parts of cells that are associated with metabolically demanding activities (for example the flight muscle in honeybees). Their most immediate function is to produce adenosine triphosphate (ATP). A problematic by-product of the cellular respiration, and oxidative phosphorylation, is reactive oxygen species (ROS) [24]. ROS production ranges from 0.1 to 4% of the oxygen consumed [25]. Molecular damage induced by mitochondria-derived ROS is considered as a general mechanism of aging that is active across most if not all species, as also stated in the mitochondrial theory of aging, promoted by Denham Harman in the 50s [26]. A further increase in ROS production is the adverse effect when ROS generates damage that leads to respiratory chain dysfunction. Increased ROS production can then cause, for example an exponential increase of mitochondrial DNA mutations over time, resulting in aging and

associated degenerative diseases [27]. Thus natural compounds with antioxidant actions such as vitamins and minerals, polyphenols, and other non-nutrient compounds of plants, which inhibit generation of ROS, or which scavenge free radicals, are may be beneficial for human [28].

From the late 1990s until the mid-2000s the free-radical theory of ageing was the dominant mechanistic idea as to why we age and die [29]. But in later years, several studies show no relationship, or a reverse relationship between increased production of ROS, and decreased lifespan [27, 30]. Some of these studies suggest that, contrary to the free-radical theory, an adaptive response in the mitochondria to increased free radical levels is necessary to up regulate the defense system within the cell, which in turn will improve the health. But either way, oxidative stress seems to have a correlation to aging, and especially in relation to increased risk of age-related diseases (such as various cancers, cardiovascular disease and neurodegeneration) [31].

Addressing the hypothesis of the ability of plant extracts and micronutrients to affect survival and mitochondrial viability, bees will be tested for survival using established feeding and incubation protocols. Mitochondrial function can be assessed by measuring the rates at which they consume oxygen on different substrates and how exposure to reagents such as toxins affects oxygen consumption. With the second set of experiments I will ask if survival affects by *Rhodiola rosea* can be linked to mitochondrial activity or to other oxygen related mechanisms in the honeybee.

2. Materials and method

2.1 Subjects

The experiments were performed in 2012 and 2013 at the Norwegian University of Life Sciences, Ås, Norway. Bees used in these experiments were sampled from colonies consisting of 8000 – 10000 *Apis mellifera carnica* winter bees. Winter bees were used to represent a homogenous group of bees- with similar worker phenotypes and physiology. To control for hive specific effects, individuals from different colony sources were used.

2.2 Lifespan and survival assay

The winterbees where collected from two different hives at the facilities of the UMB. Bees for the first round of experiments where collected Friday 20th of January 2012. Bees for the second round of experiments where collected from the same two colony sources, Monday 27th of February 2012.

The bees were placed in 10 cages, with maximum 58 and minimum 50 bees in each. The plastic cages measured 7. 5 cm x 8. 5 cm x 12. 5 cm, with two feeding holes at the top, one for a tube containing water and one for a tube containing food (see Fig.1). The one side is covered with beeswax, to make the environment as natural as possible for the bees. One side is made of lattice making it possible to observe the bees. Near the bottom there is a hole in which the dead bees can be removed.

Fig. 1: one of the cages used to house the bees

The cages were placed in an incubator (Hera cell 150 – Thermo scientific) that held a temperature of 30.0°C, an O_2 level of 21.5 %, and a CO_2 level of 1.4 % (all of which were monitored daily). The cages were randomly placed on the same shelf.

2.2.1 Rhodiola Rosea and the concentrations

The *Rhodiola Rosea* used in these experiments where bought from a local pharmacy. It is produced by Nutraceutical Corp for Solaray, Utah, USA. The pure *Rhodiola Rosea* powder are stored in capsules, 500 mg of powder in each (>15 mg rosavins and >5 mg salidrosides/per capsules).

The different concentrations for each round where made by first making a stock solution of 100 mg/ml. From this stock solution the other concentrations were made, by additionally adding 50 % of Bifor® (a standardized food given to bees, produced by Nordic sugar member of Nordzucker Group), 1 % of lipid mixture for insect cell culture (Sigma L5146), 2 % of Grace's amino acid mixture for insect cell culture (Sigma G0148) and distilled water. All of the solutions were stirred with a stirring machine until the powder was dissolved. This "high carbohydrate" diet resembles the natural diet of bees, and is copied from Nick Baker [5]. The stock solution and the different concentrations where stored in test tubes in the freezer at -18 °C.

2.2.2 General setup

Five different concentrations of *Rhodiola rosea* were given the bees (0.025 mg/ml, 0. 25 mg/ml, 2. 5 mg/ml, and 25 mg/ml). In each round of experiment each concentration was given to two cages. The experiment was done in two rounds with 2 cages per concentration. Thus, each concentration was tested in 4 replicate boxes with a total of about 200 individuals per treatment. Two cages served as control; i.e. given no *Rhodiola rosea* at all (see Fig.2). The first two days the bees were only given sugar solution with amino acids and lipid mixture, but without *Rhodiola rosea*. This was done to make sure that the bees that died later in the experiment, did so because of the treatment effects, and not as a cause of initial handling stress induced during the sampling.
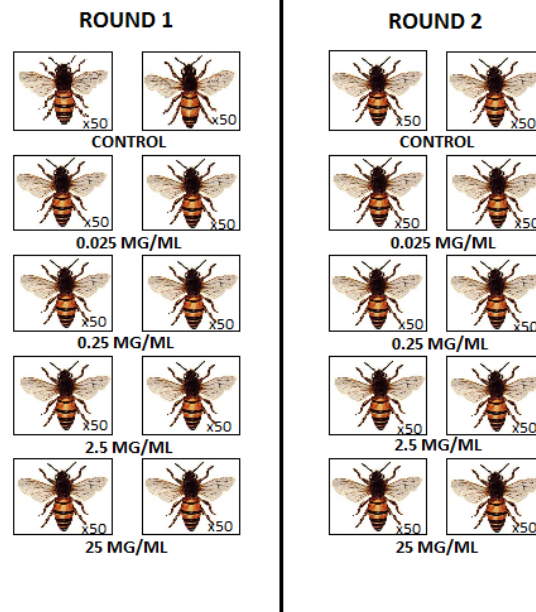
Fig. 2: Overview of the experimental set-up in the survival assay

The test tubes, with 4 small pinholes at the bottom, were placed standing through the top of the cage ensuring the bees' *ab libitum* access to the food and water, and replaced every other day. The numbers of dead bees were counted daily, and removed from the cages, to reduce potential pathogen load.

2.3 Statistics

A total of 1079 bees were used, and Kaplan-Meier survival analyses [32] were calculated based on the number of deaths recorded. A level of 5 % ($p < 0.05$) were assessed as significant. Post-hoc analysis, in terms of two sample test, was performed using the Cox's F-test.

2.4 OCR measurements

The winter bees were collected from two different hives the same day as the experiment, and stored in a dark room with constant supply of food (30% sucrose solution) and water until the dissection. As the mitochondrial yield is high in the active flight muscle of the bee, this muscle was dissected out and used. Oxygraph-2k (OROBOROS® Instruments, Innsbruck, Austria) provides the instrumental basis for

measuring mitochondrial respirometry, and hence mitochondrial function. Each titration protocol used [33] was specific to the examination of individual aspects of respiration control in permeabilized muscle fibers. The different compounds were added within the same time window to make the different runs comparable. The titration protocol was completed within one hour. All experiments were performed in mitochondrial respiration medium (MiR05), consisting of 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, 1 g/l BSA essentially fat free, 60mM lactobionic acid, adjusted to pH 7.1 with 5 N KOH.

Thorax flight muscles were kept in ice-cold relaxing medium (BIOPS) while dissected and separated into small bundles of muscle fibers under a microscope. BIOPS contains 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1 (as recommended by Oroboros Instruments, Innsbruck, Austria [33]). For permeabilization of the cell membrane 20 µL of Saponin solution (5 mg of Saponin powder in 1 ml BIOPS) were added. After shaking by gentle agitation for 30 min, the bundles were washed in MiR05 solution, and further shaken for 10 min. After the washing procedure, wet weight of the fiber bundles was assessed (between 1.5 mg and 4 mg of biopsy material), and the biopsy material was subjected to high resolution respirometry.

The two chambers of the Oxygraph-2k were run in parallel, one served as the test chamber whilst the other as the mock control chamber. Each single biopsy was divided into the two chambers and both samples processed independently. The test chamber was added mitochondrial substrates together with *Rhodiola rosea,* the control chamber was added mitochondrial substrates together with MiR05 buffer (in equal volume to what was added to the test chamber). The experiment started with flux stabilization without any substrates.

Oxygen concentration is recorded at 0.5 Hz and converted from voltage to oxygen concentration using a two-point calibration. Respiration rates (O2 flux) are calculated as the negative time derivative of oxygen concentration [34]. The software DatLab (Oroboros Instruments, Innsbruck, Austria) was used for data acquisition. The results

were copied and pasted into a table of the Excel template "O2k-Analysis_Cell-PCP.xls", for further analysis.

2.4.1 Rhodiola rosea and the concentrations

Concentrations of 0.025 mg/ml, 0.25 mg/ml and 2.5 mg/ml of *Rhodiola rosea* were used. The lowest concentration was obtained by adding 0.025 ml of *Rhodiola rosea* dissolved in respiration medium (2 mg/ml), to the chamber.

By adding 0.025 ml of 18 mg/ml to the chamber, the concentration of 0.25 mg/ml was made.

By adding 0.05 ml of 90 mg/ml *Rhodiola Rosea* solution to the chamber, 2.5 mg of *Rhodiola rosea* per ml was made.

2.4.2 Respiration by mitochondria incubated with Rhodiola rosea

The first aim with the experiments is to test the general effect of *Rhodiola Rosea* on mitochondria from honey bee muscle tissue.

Substrates to NADH dehydrogenase (Glutamate to complex 1) and Succinate dehydrogenase (Succinate to complex 2) are injected in both chambers, that results in feeding electrons into their respective complexes, and down the electron transport chain of the mitochondria (state 2 respiration). Malate also contributes with electrons to complex 1, through Kreb's cycle and NADH. Electron supply from the β-oxidation was added with Octanoyl carnitine (OCT). Adding ADP, which binds to the ATP synthase, causes the electron transport system to proceed at a maximum speed, only restricted by the chemiosmotic gradient (state 3 respiration).
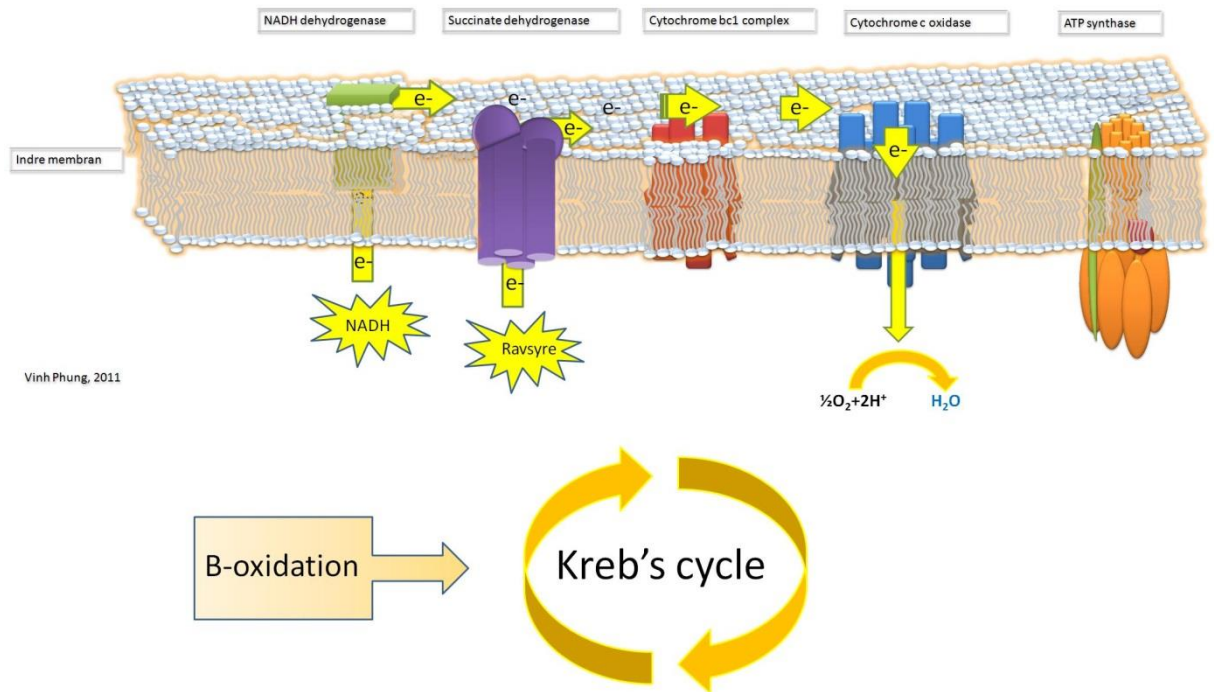
Fig. 3: the electron transport chain (ETC).

Rhodiola Rosea is added in a series of increasing concentration to the test chamber, the same volume of MiR05 was added in the control chamber (to keep the volume in the two chambers equal). Antimycin A is given to both chambers at the end of the titration regime, which has an inhibitory effect on mitochondrial respiration (i. e. complex 3). Antimycin A allows non-mitochondrial respiration to be measured as it inhibits all ETS activity by uncoupling complex 3 from complex 4 [34]. See table 1 for overview of chemicals added to the two chambers.
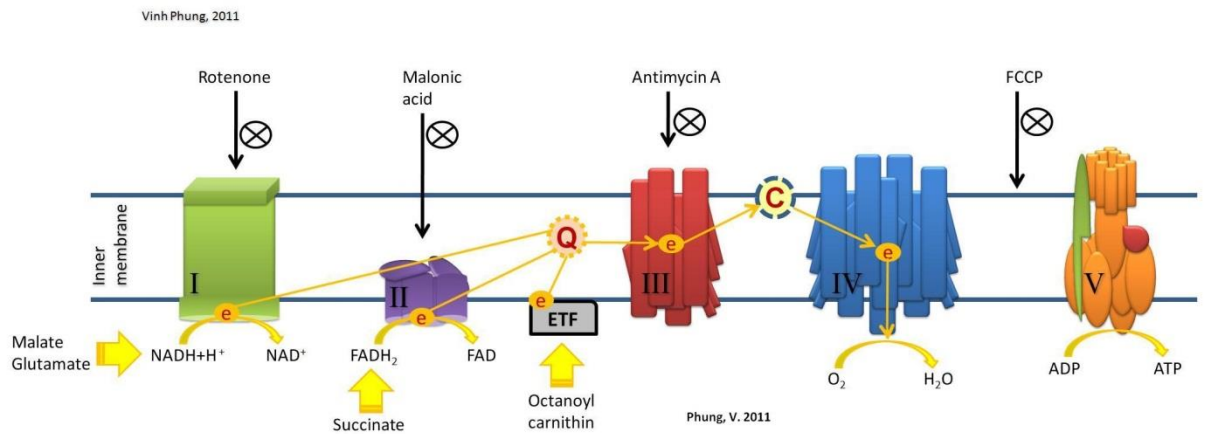
Table 1: Overview of chemicals injected to the test chamber and control chamber

The chemicals are added in the order as displayed

TEST CHAMBER		CONTROL CHAMBER	
Chemicals	Amount µl	Chemicals	Amount µl
Glutamate	10	Glutamate	10
Malate	10	Malate	10
Succinate	20	Succinate	20
OCT	10	OCT	10
ADP	10	ADP	10
Rhodiola rosea	25 (0.025 mg/ml)	MiR05	25
Rhodiola rosea	25 (0.25 mg/ml)	MiR05	25
Rhodiola rosea	50 (2.5 mg/ml)	MiR05	50
Antimycin A	3	Antimycin A	3

2.4.3 Respiration by mitochondria in different states incubated with Rhodiola rosea

In this experiment I asked on which mitochondrial respiratory chain elements *Rhodiola rosea* may act on; first by testing the effect of *Rhodiola rosea* on complex 1, second by testing the effects on complex 2, and finally by testing effects after uncoupling the electron transport chain. The concentration with the most apparent effect (in terms of oxygen consumption), according to the results obtained from the previous experiment, was used in this experiment (2.5 mg/ml).

Fig. 4: the electron transport chain with its substrates, inhibitors and uncouplers

The two complexes were activated separately by injecting the appropriate substrates, before injection of *Rhodiola rosea*. Inhibitors specifically for each of the complexes were finally added (see table 2).

Table 2: Overview of chemicals injected to the test chamber and control chamber

The chemicals are added in the order as displayed

COMPLEX 1				COMPLEX 2			
TEST CHAMBER		CONTROL CHAMBER		TEST CHAMBER		CONTROL CHAMBER	
Chemicals	Amount (µl)	Chemicals	Amount (µl)	Chemicals	Amount (µl)	Chemicals	Amount (µl)
Glutamate	10	Glutamate	10	Succinate	20	Succinate	20
Malate	10	Malate	10	ADP	10	ADP	10
ADP	10	ADP	10	Rhodiola rosea	50 (2.5 mg/ml)	MiR05	50
Rhodiola rosea	50 (2.5 mg/ml)	MiR05	50	Malonic acid	5	Malonic acid	5
Rotenone	5	Rotenone	5	Antimycin A	3	Antimycin A	3
Antimycin A	3	Antimycin A	3				

Rotenone is an inhibitor which blocks NADH dehydrogenase (complex 1), preventing electrons from being passed from one carrier to the next. Malonic acid is a competitive inhibitor, and acts against succinate dehydrogenase (complex 2).

To obtain uncoupled mitochondria FCCP is added after substrates and ADP (see table 3).

Table 3: Overview of chemicals injected to the test chamber and control chamber

The chemicals are added in the order as displayed

TEST CHAMBER		CONTROL CHAMBER	
Chemicals	Amount µl	Chemicals	Amount µl
Glutamate	10	Glutamate	10
Malate	10	Malate	10
Succinate	20	Succinate	20
OCT	10	OCT	10
ADP	10	ADP	10
FCCP	1	FCCP	1
Rhodiola rosea	50 (2.5 mg/ml)	MiR05	50

FCCP permeabilizes the inner mitochondrial membrane, and dissipate the chemical gradient, allowing the electron transport system (ETS) to run at max speed. *Rhodiola rosea* is finally added. Uncoupled mitochondria could yield information if *Rhodiola rosea* was working as an uncoupler by itself - by lowering the overall oxygen consumption without any use of inhibitors.

2.4.4 Oxygen consumption by Rhodiola rosea without mitochondria

Without any mitochondria or other substrates in the chamber (only respiration buffer; MiR05), the same concentrations used in the first part of the experiment were injected in increasing order, to a final concentration of 0.025 mg/ml, 0.25 mg/ml and 2.5 mg/ml, respectively. The other chamber served as a control, injected with respiration medium only.

3. Results

This result sections consists of two main parts. To test for general effects of *Rhodiola rosea* on lifespan in honey bees, I first tested how the root extract affected survival in a survival assay. The second set of experiments studied if *Rhodiola* rosea can specifically affect mitochondria, first in general and then more in specific.
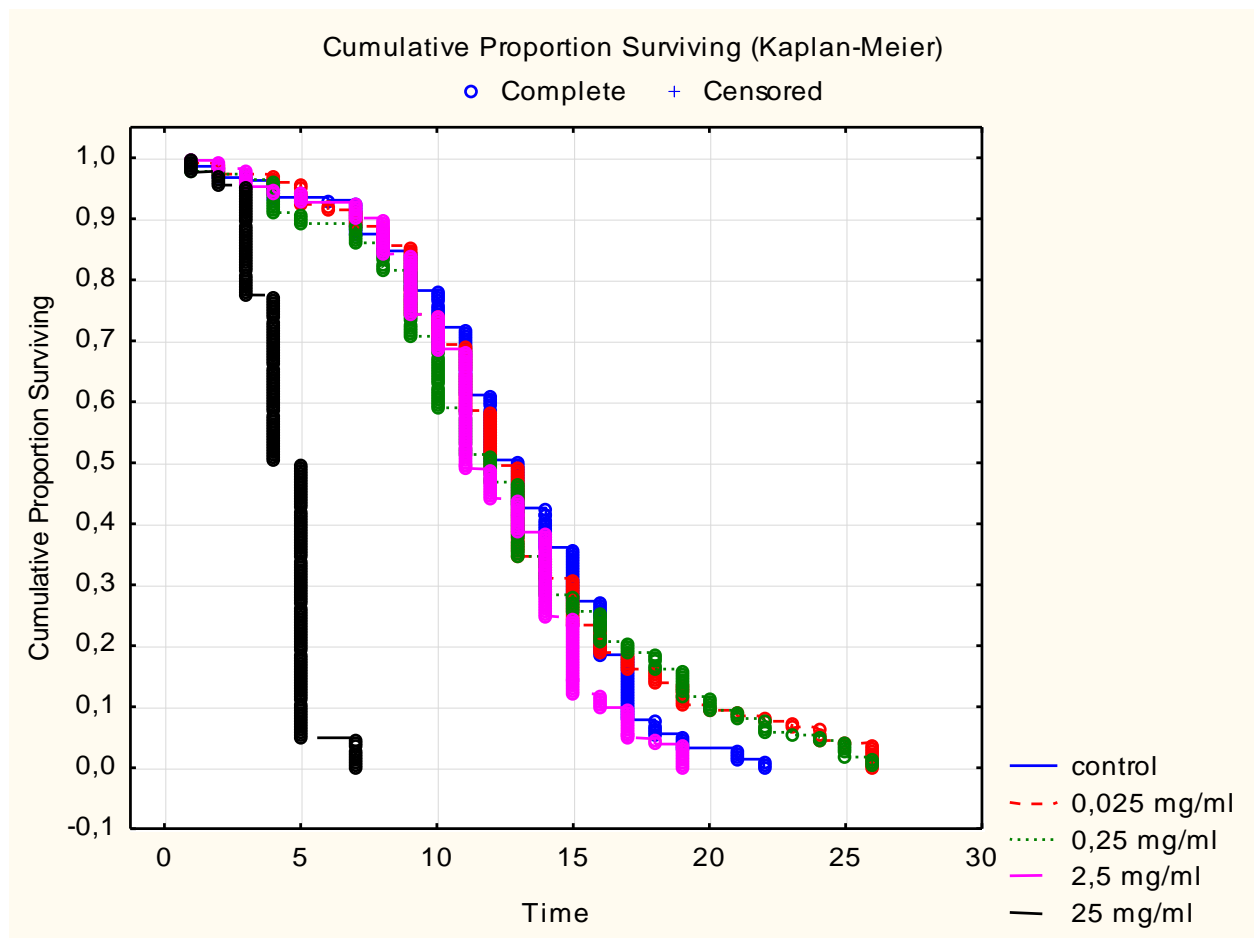
3.1 The effects of *Rhodiola rosea* on survival.

Effects of *Rhodiola rosea* on survival were studied by feeding *Apis mellifera carnica* winter bees with different concentrations of *Rhodiola rosea.* The tested concentrations were 0.025 mg/ml, 0.25 mg/ml, 2.5 mg/ml and 25 mg/ml, with control group that was fed a sugar solution. Each concentration was tested on >200 individuals, kept in 20 boxes, housed in four replicate boxes, with two boxes tested in each of the two replicate rounds.

The data pooled for all animals, all replicate boxes and replicate rounds are shown in Fig. 5. The general shape of all the curves looks very similar, and like typical, classical, exponential, rectangular shaped [22] Kaplan-Meier survival curves. The curves start off with being flat, before the bees die off and the curves are declining with a constant pace, until the end when the curves flatten out.

Starting with the most apparent result; the survival for the highest concentration (25 mg/ml) differs from the other curves in that its slope is much steeper. All the bees were dead after being alive for only 33 % of the lifespan in the control. After four days half of the bees were dead, compared to control where approximately 95% is still alive after four days, and where the median survival time is 13 days. This result suggests that high doses of *Rhodiola rosea* are lethal. The 2.5 mg/ml concentration shows a minimal decrease in survival, whereas the two lowest concentrations, 0.025 mg/ml and 0.25 mg/ml, show a slightly increased survival.

Similarly, the Kaplan-Meier statistics for survival shows an overall effect of *Rhodiola rosea (*survival (chi-square$_{216/215/202/233/209}$=401,75, *df*=4, *p*<0,01)*.* As compared to the

control survival was significantly less with the two highest concentration (2.5 mg/ml, p<0.005, and 25 mg/ml, p<0.01 given by the Cox's F two sample test). In contrast the prolonged survival I observed for the two lowest concentrations were not significant (p>0.05, and p>0.05).

Figure 5: survival, measured as cumulative proportion surviving, after given different concentrations of *Rhodiola rosea*. The curves give the percentages of how many bees that is still alive after a given day. An overall effect of *Rhodiola rosea* on survival is shown (chi-square$_{216/215/202/233/209}$=401,75, *df*=4, *p*<0,01).

Yet, test for replicate effects revealed that there are differences between the two runs (p<0.01). Because results in the two replicate differed significantly, these differences may mask more subtle concentration dependent effects. I therefore analyzed the data of the two runs separately, and found that the lifespan enhancing effect of the two lowest concentrations was highly significant (p<0.005) in each of the runs.

When comparing the two hives, there was a significant difference between them as well (p<0.01). Analyzing the data of the two hives separately show similar results as before but for two concentrations (0.25 mg/ml and 2.5 mg/ml) a significant effect was not detected (p>0.05).

As a summary I detected a life shortening effect for the two highest concentrations (2.5 mg/ml and 25 mg/ml). For the two lowest concentrations (0.025 mg/ml and 0.25 mg/ml) I detected a life prolonging effect, although only when the two replicate rounds were analyzed separately. This may indicate a more subtle effect, as compared to higher concentrations.

3.2 The effects of *Rhodiola rosea* on mitochondria.

In this section I tried to find underlying effects that could explain the impact of *Rhodiola rosea* on lifespan observed in the survival assay. Micronutrients often exert its effects on survival through interactions with mitochondria [35], so here I will present the result I got when I tested the effect of *Rhodiola rosea* on mitochondria from the flight muscle of the bee. Effects of *Rhodiola rosea* on mitochondria were studied by high-resolution respirometry, which allows for the measurement of respiration in response to titrated compounds. In each section I will present the results from the one animal that showed the effect most clearly.

3.2.1 The effects of different concentrations of Rhodiola rosea on mitochondria

The tested concentrations of *Rhodiola rosea* were 0.025 mg/ml, 0.25 mg/ml and 2.5 mg/ml. The highest concentration used in the survival assay was not used here, as it was impossible to dissolve the *Rhodiola rosea* enough to be injected (into the chambers) by the syringes. The concentrations was added in increasing order, and the effect was monitored and compared to control, which did not receive any *Rhodiola rosea*. Each concentration was tested on six animals. The results indicate that reduced survival is in one way or another, directly or indirectly, caused by increased oxygen consumption induced by *Rhodiola rosea*. The differences between consumption after the highest concentration are added, to either the control, the consumption when no *Rhodiola rosea* is added, or the consumption when lower concentrations is added, is highly noticeable in all of the six animals. In the test animal presented below 2.5 mg of

Rhodiola rosea pr. ml. increased the O_2 consumption. The O_2 consumption after *Rhodiola rosea* is added compared to before, is 10.48 pmol/(s*ml) per mg of *Rhodiola rosea.* The other concentrations (0.025 mg/ml and 0.25 mg/ml) are not showing any overall consistency (in the six animals tested) when it comes to O_2 consumption.
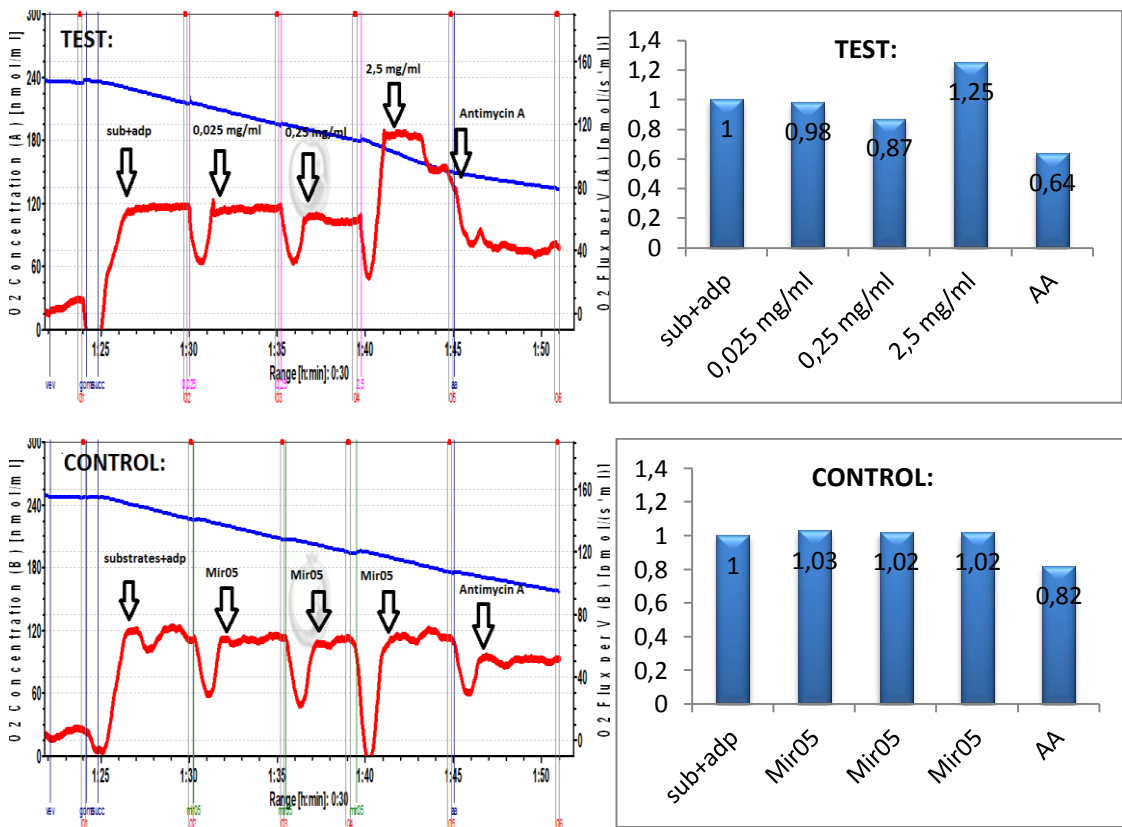
Figure 6: Oxygen concentration ([μM] blue line) and oxygen flux per ml ([pmol/s*ml]red line) in O2k test chamber and control chamber, with permeabilized fibers from honeybees with the standard titration protocol. In the bar chart; the flux control ratio. The ratio is in comparison to the starting value. Two representative examples for a total of n= 12 test runs. As shown in the test chart, the concentration with most effect is 2.5 mg/ml.

Octanoyl carnitine, Glutamate, Malate, and Succinate was added to fuel electrons from both complex 1 and complex 2 into the respiratory chain. This parallel substrate input corresponds to mitochondrial substrate supply in vivo, thereby reflecting maximum capacity of the phosphorylation system. This, together with added ADP causes the coupled mitochondria to run at its maximum, saturated level, meaning that the system cannot be up regulated any further. My results do indeed show an increase in oxygen

consumption after adding *Rhodiola rosea*; it may suggest that components of the *Rhodiola rosea* extract are able to consume oxygen.

I further observe that Antimycin A is only partly inhibiting the mitochondrial respiration. The addition of Antimycin A at the end of the titration regime reveals any nonmitochondrial contribution to cellular oxygen consumption. This observation increase the likelihood that there are some substances in *Rodiola rosea* that is consuming oxygen.

3.2.2 The effects of Rhodiola rosea on complex 1, complex 2, and under uncupled conditions

In this section I wish to go more in detail about the effects of *Rhodiola rosea* on mitochondria. By adding different substrates and inhibitors in specific sequences different segments of the electron transport system can be investigated.

I first tested complex 1 by applying glutamate, malate and ADP. Representative examples are shown in Fig. 3. The figures indicate an increase in oxygen consumption after *Rhodiola rosea* (2.5 mg/ml) is added (15.2 pmol/(s*ml) per mg of *Rhodiola rosea*).
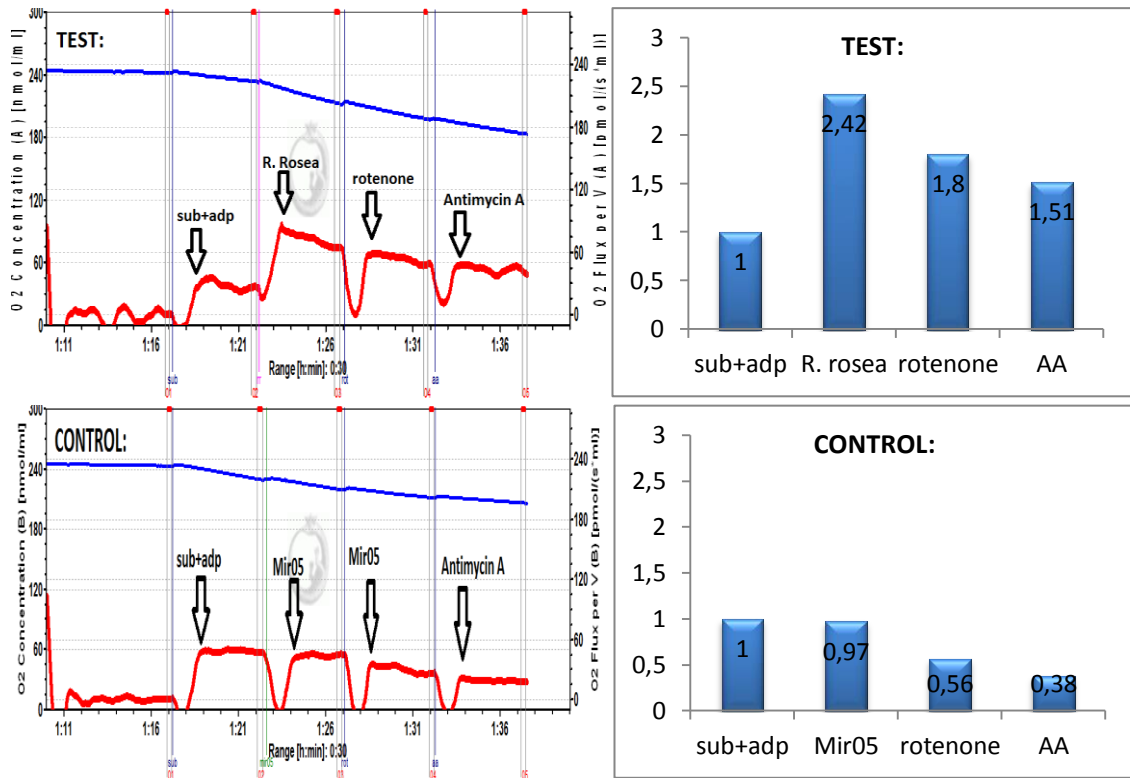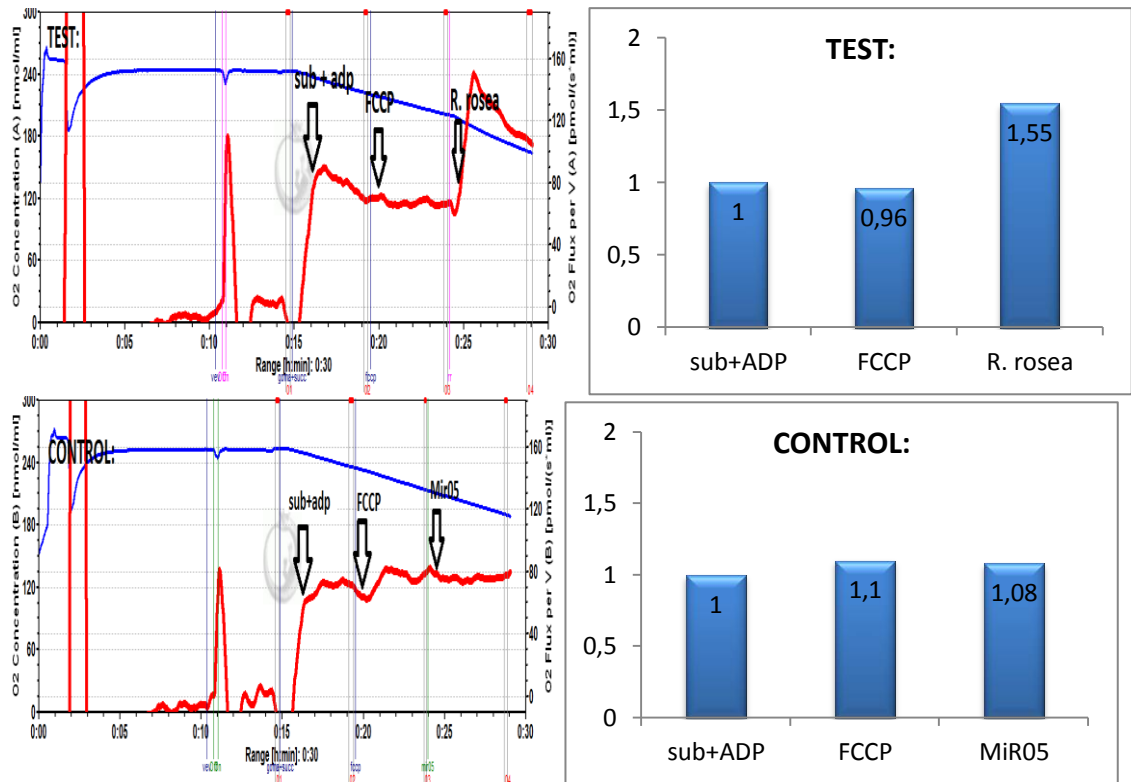
Figure 7: Oxygen concentration ([μM] blue line) and oxygen flux per ml ([pmol/s*ml]red line) in O2k test chamber and control chamber, with permeabilized fibers from honeybees with the standard titration protocol. In the bar chart; the flux control ratio. The ratio is in comparison to the starting value. Two representative examples for a total of n= 12 test runs. As shown in the test chart, *Rhodiola rosea* is able to increase the O_2 consumption after activation of complex 1.

Complex 2 was tested by adding succinate and ADP, and I observed an increase in oxygen consumption after injection of *Rhodiola rosea* (36.08 pmol/(s*ml) per mg *Rhodiola rosea*). See Fig. 4. However, I did not add Rotenone together with Succinate to prevent reverse electron flow back to complex 1, so that might contribute to the flux as well.
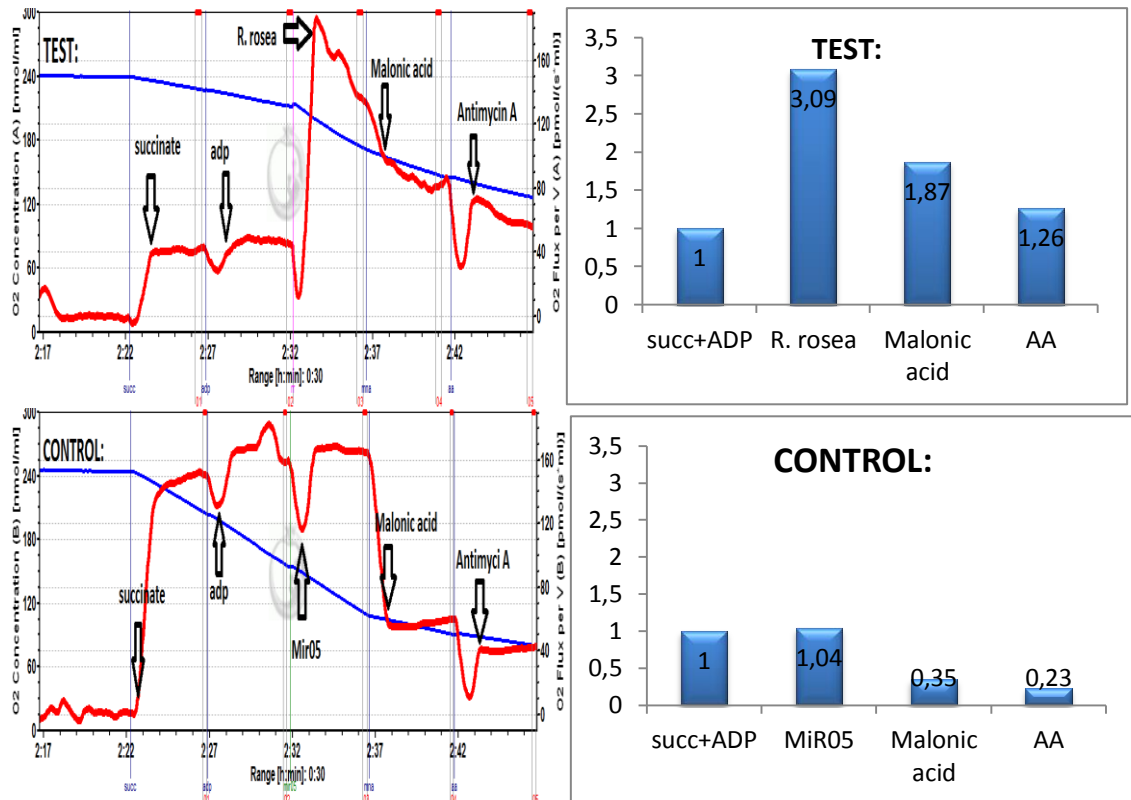
Figure 8: Oxygen concentration ([μM] blue line) and oxygen flux per ml ([pmol/s*ml]red line) in O2k test chamber and control chamber, with permeabilized fibers from honeybees with the standard titration protocol. **In the bar chart; the flux control ratio.** The ratio is in comparison to the starting value. Two representative examples for a total of n= 12 test runs. As shown in the test chart, *Rhodiola rosea* is able to increase the O_2 consumption after activation of complex 2.

The effect of *Rhodiola rosea* under uncoupled conditions was tested by uncoupling the mitochondria using FCCP. I observe an increase in oxygen consumption, as shown in Fig. 5 (16.08 pmol/(s*ml) per mg *Rhodiola rosea*).

Figure 9: Oxygen concentration ([µM] blue line) and oxygen flux per ml ([pmol/s*ml]red line) in O2k test chamber and control chamber, with permeabilized fibers from honeybees with the standard titration protocol. In the bar chart; the flux control ratio. The ratio is in comparison to the starting value. Two representative examples for a total of n= 12 test runs. As shown in the test chart, *Rhodiola rosea* is able to increase the O_2 consumption after uncoupling the mitochondria.

In the above experiments I observed similar response curves during *Rhodiola rosea* application. *Rhodiola rosea* was able to increase the oxygen consumption in all sets of experiments, which is a bit confusing considering the different protocols used, and different segments tested.

Antimycin A is not sufficient to decrease the oxygen consumption (Fig. 3 and Fig. 4). Rotenone, a complex 1 inhibitor, which blocks respiration with all NAD-linked substrates, does not seem to shut down the electron transport chain either (fig. 3). Ideally rotenone should be able to decrease the O_2 consumption to the level before adding substrates/ADP. Malonic acid which acts against succinate dehydrogenase in complex 2 is also unable to decrease the oxygen consumption, as much as expected (fig.4). Since the inhibition of complex 1, 2 and 3 is not capable of decreasing the

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respiratory rates to baseline levels, and since the overall appearance of the graphs looks the same, this cannot provide evidence for an effect of *Rhodiola rosea* in the mitochondria. However; interpreting the results is difficult. Although the inhibitors reduce oxidation only partial, there is an inhibition. So what I see might be a combination of reduced oxidation of *Rhodiola rosea* compounds and complex 1- and complex 2- substrate oxidation, respectively. But I do not know the extent of each.

FCCP is an uncoupling agent, which dissipate the chemical gradient causing an increase in electron transport to its maximum level. In this state, *Rhodiola rosea* is able to increase the oxygen consumption even further (fig. 5). All of these results led me to do an additional experiment to test the hypothesis of *Rhodiola rosea* alone being able to consume oxygen.

3.2.3 The effect of Rhodiola rosea in the absence of mitochondria from muscle tissue

Concentrations of 0.025 mg/ml, 0.25 mg/ml and 2.5 mg/ml were added the oxygraph chamber without bee muscle tissue present, to see if *Rhodiola rosea* is able to consume oxygen without the muscle as a substrate. *Rhodiola rosea* is able to increase the oxygen consumption, without any muscle as substrate (Fig. 6). This may be indicative of *Rhodiola rosea* containing antioxidants, and thus acting as an oxygen consumer. The highest concentration (2.5 mg/ml) increased the consumption with 32.2 pmol/(s*ml) per mg *Rhodiola rosea,* whereas 0.25 mg/ml increased the oxygen consumption with 13.6 pmol/(s*ml) per mg *Rhodiola rosea.*
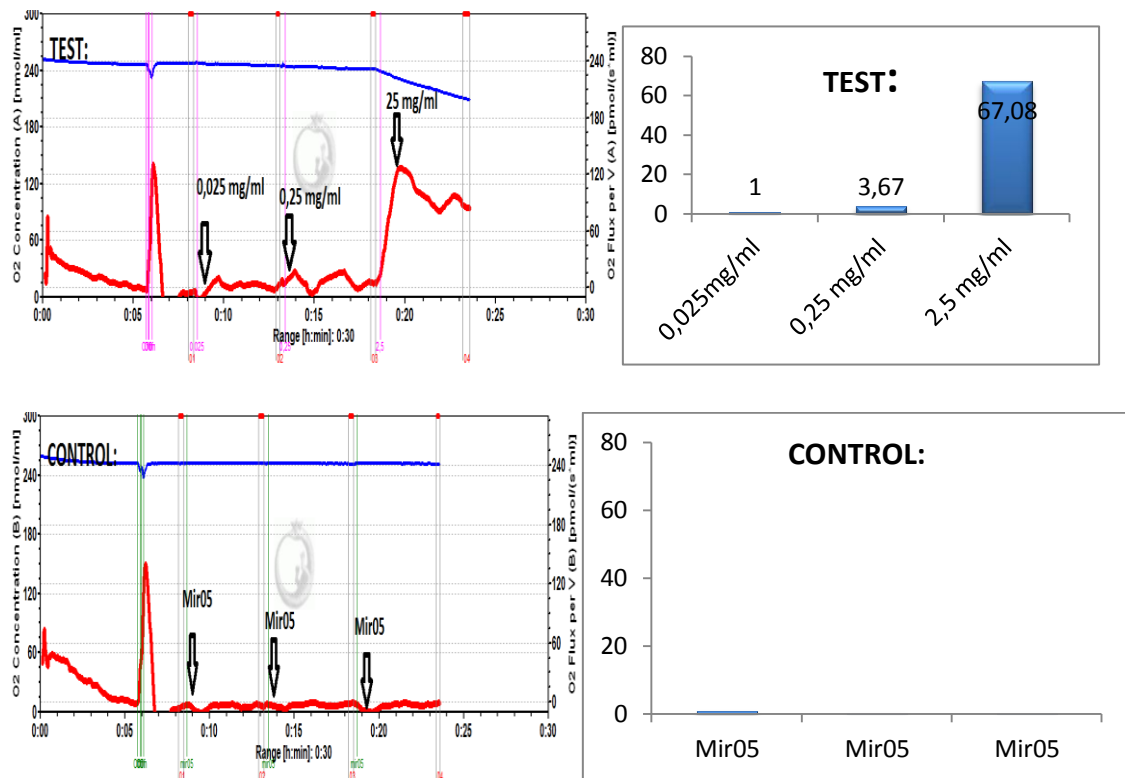
Figure 10: Oxygen concentration ([μM] blue line) and oxygen flux per ml ([pmol/s*ml]red line) in O2k test chamber and control chamber, with permeabilized fibers from honeybees with the standard titration protocol. **In the bar chart; the flux control ratio.** The ratio is in comparison to the starting value. Two representative examples for a total of n= 6 test runs. As shown in the test chart, concentrations of 0.25 mg/ml and 2.5 mg/ml of *Rhodiola rosea* are able to increase the O$_2$ consumption.

4. Discussion

My study evaluates if the herb *Rhodiola rosea* affects survival. This study also wishes to find out in more specific details where the herb exerts its effect. Since mitochondria are assumed critical for controlling survival I am searching for an effect in the mitochondria, in terms of oxygen consumption.

4.1 The effects of *Rhodiola rosea* on survival

My paper shows that *Rodiola rosea* does affect survival in a dose dependent manner. A strong negative correlation was found between the concentration of *Rodiola rosea* and lifespan. The mean lifespan was decreased by 8 days (61.5%) in the group receiving the highest concentration (25 mg/ml). Decreasing the concentration by a tenfold (2.5 mg/ml) decreased the lifespan with one day (7.7%). The maximum lifespan was decreased by 15 and 3 days (68 % and 14 %) respectively. The two lowest concentrations did not show an effect before later in the life, but increased the maximum lifespan with 4 days, or 15%. This result suggests a modulation of the ageing process by *Rodiola rosea.* Thus lower doses seems to be beneficial in terms of life expectancy, whereas higher doses are not.

This is in accordance with studies performed on *Caenorhabditis elegans* [16]*,* and *Drosophila melanogaster* [17]*. C. elegans* supplemented with the highest concentration of *R. rosea* had an adverse effect and shortened lifespan significantly (8.82 % in mean lifespan and 15.6 % in maximum lifespan). The most beneficial concentrations of *Rhodiola rosea* did not only extend the mean lifespan (15.4%), but were also able to increase the maximum lifespan (12.4 %). *Rodiola rosea* fed to *Drosophila melanogaster* extended both mean (24% in both sexes) and maximum (16% in males and 31% in females) life span when compared to control.

In order to explain these effects on lifespan proved by the survival assay just described, I looked for an effect in the mitochondria in terms of oxygen consumption. Oxygen

consumption can provide and yield useful information about mitochondrial function and/or dysfunction, during appropriative titrations of substrates, inhibitors and uncouplers [36].

4.2 The effects of *Rhodiola rosea* on mitochondria

The first tests on flight muscle tissue suggested that *Rodiola rosea* may increase O_2 consumption in mitochondria (Fig. 2). However, when examining possible action by *Rodiola rosea* on specific mitochondrial targets with selected substrates, increased oxygen consumption was detected in all additional tests (Fig 3 - 5). This lack of specificity was unexpected and prompted a further control experiment, where *Rodiola rosea* was tested without muscle tissue as a substrate. I found that *Rodiola rosea* alone at a concentration of 2.5 mg/ml showed substantial oxygen consumption (Fig 6). While the latter experiment cannot completely rule out that *Rodiola rosea* can have minor effects on mitochondria, they suggest that direct oxygen consumption by *Rodiola rosea* components might be its primary mode of action in affecting bee's lifespan.

There are different hypothesizes about the mechanisms behind the effects conferred by *Rhodiola rosea*, and two of the explanations is whether it works as an antioxidant or pro-oxidant, in which a beneficial effect of the pro-oxidative action is usually explained in terms of an hormetic fashion, with an induction of anti-oxidative endogenous defense mechanisms [16]. Hormesis is a term used by toxicologists to refer to a biphasic dose response to an environmental agent characterized by low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect [37]. The characterization of *Rodiola rosea* as an antioxidant, or as a pro-oxidant, are not completely distinct though, overlapping each other at a certain degree (i.e. antioxidants are also included among hormetic agents) [38]. And in either way, the protection by *Rodiola rosea* against oxidative stress is a possible mechanism explaining its beneficial effects. The importance of oxidative stress, reactive oxygen species (ROS) and free radicals in human disease states has attracted increasing attention over the past decade [28]. ROS can initiate a wide range of toxic oxidative reactions and are related to many degenerative diseases [35]. To control the impact of oxidative stressors, cells are equipped with antioxidant enzymes and small molecules to detoxify

excess ROS and maintain intracellular ROS at appropriate levels. These antioxidant enzymes/molecules include superoxide dismutase, catalase, glutathione peroxidase, glutamate cysteine ligase, glutathione synthetase, and reduced glutathione [28]. But this defense system is unable to scavenge all reactive oxygen species, and therapeutic strategies thus should aim at reducing free-radical formation and scavenging free radicals. Natural compounds with antioxidant actions such as vitamins and minerals, polyphenols, and other non-nutrient compounds of plants, which inhibit generation of ROS, or which scavenge free radicals, may be beneficial for human.

My data is supportive only for the hypothesis that *R. rosea* may work as an antioxidant, through the action as an oxygen consumer.

Oxygen consumption by *Rodiola rosea* might be beneficial at low concentrations, and harmful at higher concentrations. In particular increased oxygen consumption might lead to oxygen depletion in the cells, which in turn might inhibit cell respiration, deplete the organism of oxygen, and finally killing it. In this sense *Rodiola rosea* may kill the species due to hypoxia. At low concentrations *Rhodiola rosea* can be beneficial for the organism because it consumes oxygen and as a direct cause of that produces less harmful ROS. In this sense *Rodiola rosea* may act as a ROS scavenger.

My results from the high-resolution respirometry showed that the highest concentration of *Rodiola rosea* (2.5 mg/ml) increased the oxygen consumption, and my lifespan data show a significant effect on lifespan. These results put together indicate that *Rodiola rosea* is able to modulate lifespan through its action as an oxygen consumer. A reasonable explanation is that the longevity at lower concentrations is due to ROS scavenging, the decreased life expectancy is due to depletion of oxygen.

In support of my findings, a study done by Schriner et al. [17] found a decreased ROS production in isolated mitochondria from Drosophila melanogaster, without an altered mitochondrial function. Since oxygen consumption by mitochondria and ROS production is inversely related, it is likely that *Rodiola rosea* acts as a scavenger. Schriner et al. also demonstrated that *Rodiola rosea* increased mean and maximum lifespan in Drosophila melanogaster in a dose dependent manner, without altering the antioxidant defense system. They concluded that the root may work as an antioxidant.

Rodiola rosea working as an antioxidant is also supported by a study done on yeast *Saccharomyces cerevisiae* [14], which suggests that an elevation of activity of the major antioxidant enzymes is not required for the observed life-extending action of *Rodiola rosea*. Similar results in yet another study [39] demonstrated the protective role *Rodiola rosea* on human cells against oxidative stress without activating the antioxidant defenses.

Several papers argue that the decrease in endogenous superoxide (O_2^-) levels is the plausible explanation for the protective effect observed by *Rodiola rosea* [17, 39]. Superoxide is a key oxidant because it is produced constantly and unavoidably in the mitochondria from electron leakage during their passage along the respiratory chain. It is hypothesized that *Rodiola rosea* can act in the same way as superoxide dismutase (SOD), which is an important antioxidant defense in nearly all cells exposed to oxygen, due to its scavenging effect on O_2^-. A study done on cultured human keratinocytes incubated with *Rhodiola rosea* [40] indicate that *Rhodiola rosea* may act as SOD. A study on cardiomyocytes from rats designates salidrosides as the potential component in *Rhodiola rosea* to act in similar ways as superoxide dismutase [41].

In line with my survival assay and the bidirectional effect of *Rodiola rosea*, Wiegant et al. [16] showed that *Rhodiola rosea* has an impact on lifespan. But in contrast to my hypothesis, Wiegant et al. proposes that the mechanism behind the effect on lifespan results is an up regulation of the cellular defense mechanism (i. e. *Rodiola rosea* working as a pro-oxidant).

More than 140 compounds are isolated from roots and rhizomes of *Rodiola rosea* [18], and it could be useful to look at the different compounds separately in the search for the mechanisms behind the observed effects of *Rhodiola rosea*. With that being said, the effect of all the different compounds might be synergic, and also be due to the reciprocal distribution in a quantitative manner [40].

Schriner et al. have performed two experiments with *Rhodiola rosea* on *Drosophila melanogaster* in terms of survival [17, 42.] They used *Rodiola rosea* extract from two different companies, and both experiments showed a lifespan enhancing effect, but the latest being more pronounced (24% vs. 13%). They suggest that these results are

attributed to the differences in compounds of *Rodiola rosea* from the two different companies.

With its many components, it is also possible that *Rhodiola rosea* may have potentially different effects on different parts of the organism. And in terms of survival particular pharmaceuticals or botanicals that affect aging may do so through multiple pathways. But most importantly, it is not appropriate to simply assume that a compound that affects adult survival *only* has such effects.

In this study *Rhodiola rosea* increases the oxygen consumption. After adding 2.5 mg/ml of *Rhodiola rosea* without bee muscle as a substrate, the response seen is substantial and points towards a response that is not specific for mitochondria. However, the ability of *Rhodiola rosea* to consume oxygen can still have an impact on mitochondria, which the addition of 0.25 mg/ml of *Rhodiola rosea* in the last experiment may indicate (see Fig. 10). In this test 0.25 mg/ml of *Rhodiola rosea* is also able to increase the O_2 consumption.

According to the Nutraceutical Corp for Solaray, Utah, USA the daily recommendations for an adult human being are 500 mg of roseroot each day. If I were to extend this recommendation to apply to honey bees as well, this would mean a daily dose of 1.35 mg. In this thesis I am showing a lethal effect of roseroot at 0, 4638 mg. The health recommendations for humans are almost 3 times as much as what I prove to be lethal for honey bees. Further; I am showing a beneficial effect when the doses are 100 and 1000 times less than the recommendations.

Schriner et al. [17] showed a highly significant increase in lifespan at doses of 25 mg/ml and 125 mg/ml in *Drosophila melanogaster*. The size of *Drosophila melanogaster* is about 6 times less than the size of *Apis mellifera carnica,* so doses proven to be beneficial in one species may not have such effects in other species.

5. Conclusion and outlook

My study shows that *Rhodiola rosea* has a dose dependent effect on longevity in honeybees, and that there is increased oxygen consumption in solutions with *Rhodiola rosea.*

There are different theories about what makes us age, and hence affects our lifespan. The one theory my experiments lean towards is the free radical theory of aging (Harman, 1956). This theory states that accumulations of free radical damage is the mechanism behind aging, and that antioxidants (which neutralize free radicals) is able to slow down and delay this process. My results indicate that *Rhodiola rosea* may work as an antioxidant, in a dose dependent matter.

This dose-dependence tendency of *Rhodiola rosea* is an important point to remember, as it may explain contradictory results to whether *Rhodiola rosea* has an effect or not. Furthermore, different preparations of the *Rhodiola rosea* may have different distributions of its components (i. e. the observed effect of *Rhodiola rosea* may not only be due to the different components, but also the reciprocal distribution). Also; the different components may not only affect survival.

While honeybees are good models for aging research (short lifespan), effects may be different in other models. Yet, as long as we do not completely understand effects in mice and humans, and as long results in these more complex systems are ambiguous – the invertebrate model approach is very promising.

My study shows that the plant extract from the root *Rhodiola rosea* has an impact on survival and may affect aging. Knowing that *Rhodiola rosea* can have such an effect, could potentially provide answers to the puzzling mechanisms behind aging. Together with future findings on what compounds *Rhodiola rosea* constitutes that modulate the aging process, anti-aging strategies could be worked out.

Does *Rhodiola rosea* comes without any risk then? Based on my study, I would probably not recommend a daily supply of *Rhodiola rosea* before more research is

undertaken. *Rhodiola rosea* clearly has an effect, and it is worth continued investigation.

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