Caloric restriction and lifespan extension in the fungus *Podospora anserina*: glucose levels and the shape of the reaction norm

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Caloric restriction and lifespan extension in the fungus *Podospora anserina*: glucose levels and the shape of the reaction norm

by

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Abstract

Calorie restriction (CR) has been known as a diet which allows lifespan extension. This effect was also observed in *P. anserina*, a filamentous short-lived fungus. This study tested various CR levels on three genetic variants of *P. anserina* to observe the switching threshold from normal to CR-affected condition and investigate the involvement of presumed senDNAs. Two wild-type strains of *P. anserina* were selected from each genetic variant: glucose-insensitive, moderately-glucose-insensitive and glucose-sensitive. They were cultured in race tubes at various glucose concentrations as the different level of CR. Three strains, one from each genetic variant were re-cultured at five consecutive time-points, and then analyzed for their wild-type and mutant mtDNAs (30 kb-senescent mtDNA, senDNA-α and -γ) relative copy number by quantitative real-time PCR (Q-PCR) technique. All strains showed lifespan-extension effect of CR and the response to glucose concentration series was strain-specific. The reaction norm shapes also indicated strain-specificity of switching ranges from normal to CR-affected condition and their threshold value. The switching provided further exploration on CR mechanism pathway studies, e.g. by stimulation of survival response as proposed in the hormesis hypothesis theory. During normal senescence, mtDNA rearrangements occurred and senDNA-α accumulation was observed as a prominent hallmark of ageing and an accelerator of senescence. In CR condition, the wild-type mtDNA was relatively stabilized in all strains. However, we found several unexpected senescent events in both conditions, which indicated that more regions in mtDNA are needed to be considered in mtDNA rearrangement analysis.

Keywords: senescence, glucose restriction, mitochondrial DNA, senDNA-α

List of abbreviations

mtDNA = mitochondrial DNA
CR = calorie restriction
Q-PCR = quantitative polymerase chain reaction
CRE = cyclicAMP responsive element

Preface

Looking back on the first day that I started this minor thesis, I was reminded of this quote "*Trust not what inspires other members of society to choose a career. Trust what inspires you.*" Meeting, sitting together with my supervisors and discussing *Podospora anserina*, a piece of miracle in science discovered in the less elegant spot in nature, for the past four months have always been inspiring ones and re-convinced me that becoming an artist-scientist is what indeed I want to pursue. Special thanks to Bas Zwaan and Fons Debets, for their guidance, advice and also for sharing their insights, enthusiasm and passion for science with me. I look forward to have many more discussions and keep on being inspired by you in the future.

To Marijke Slakhorst and Bertha Koopmanschap, for being very helpful during my lab work and also for your time and patience. As well as for information and support which have been given.

I would also like to thank my closest family and friends, who are rooting for me at all times. My family in Indonesia, Dave Salawan Bessie and family, and last but not least, my fellows sharing the same journey, Steisianasari Mileiva, Karen Puspasari, Yuniwaty Halim, Stephanie Sonya and Astriani Dewanto. For your unconditional supports which are priceless to me.

1. Introduction

1.1. *Podospora anserina* as model system for ageing

P. anserina is a filamentous ascomycete fungus naturally found in the dung of herbivores. Being easy to culture and brief in its life cycle, *P. anserina* has long been used as a model system, especially in ageing investigations. Unlike most fungi which are considered to be immortal, *P. anserina* is a short-lived one with a normal lifespan of 2-3 weeks, having to survive, grow and reproduce in an ephemeral substrate.

As reviewed by Lorin *et al.* (2006), during senescence or ageing processes, *P. anserina* undergoes several phenotypic changes: 1) progressive decrease of growth rate, 2) loss of fertility, first as the female function of producing fruiting bodies and then followed by the male function of producing spermatia, 3) increasing mycelial pigmentation, in which a lipofuscin band may appear before growth stop, 4) abnormal branching with a wavy shape of tips and swelling of hyphal tips ended with hyphal burst, which can be observed at the microscopic level. Some of the changes are described in Figure 1.
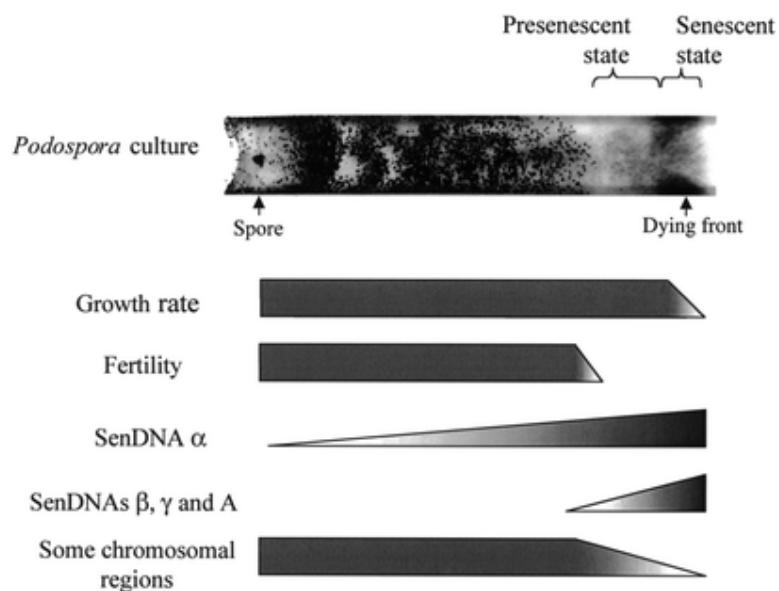
Figure 1. Changes of P. anserina during senescence on growth rate, fertility, extrachromosomal mitochondrial DNA amplification and mitochondrial chromosome modification. Source: Curr. Genet. , 2002. 40:365-373 [1]

P. anserina is known to be the model which links mitochondrial instability to ageing. It was revealed that mitochondrial DNA (mtDNA) rearrangements and deletion occurred during aging, which leads to loss of wild-type mtDNA. Kück *et al.* (1981) reported that in the senescent state, two distinct rearranged mtDNA develop from the wild-type mtDNA: a senescent mtDNA with 30 kb length and a circular DNA with approximate length of 2.4 kb (later on verified as 2,539 kb length) [2]. The circular

DNA which is now known as senDNA-α or pl-DNA, corresponds to the sequence of the intron α (first intron) of Cox1 (cytochrome c oxidase subunit 1) gene.

Several observations led to believe that senDNA-α was the major cause of aging in *P. anserina*. Accumulated senDNA-α was found present in all senescent wild-type strains and mitochondrial mutants that escape senescence have lost the intron [3, 4]. However later findings put this hypothesis in doubt. A mutant with affected cytosolic ribosomal protein function and another mutant with alteration of the grisea gene showed senescence development without senDNA-α accumulation [5, 6]. In another study, Begel *et al.* (1999) constructed a mutant without intron α of Cox1 and this mutant displays (delayed) senescence in spite of not having senDNA-α accumulation [4]. It was then suggested that senDNA-α does not play a role as a senescent determinant, but rather as an accelerator. As intron α was shown to be a mobile genetic element, which allows itself to transpose to different positions in mtDNA, senDNA-α is considered to contribute to mtDNA instability [4, 7]. Then senescence progressively strikes as the rearranged mtDNA resulted in impaired expression of mitochondrial genes.

1.2. Calorie restriction (CR) effect on *P. anserina*

Calorie restriction (CR) has been recurrently defined as a dietary regimen in which food intake is reduced without occurrence of malnutrition. CR has been shown to increase lifespan of many organisms, including *P. anserina* [8]. Van Diepeningen *et al.* (2010) showed that different wild type strains of *P. anserina* exerted different response to CR. In the study, PASM with 2% D-glucose was described as the normal condition at which all isolates have a relatively short lifespan, while CR condition used 0.2% and 0.02% D-glucose as mild CR and severe CR conditions. Some strains showed immediate lifespan-extension response to 10-fold change in glucose concentration (2% to 0.2% glucose), while other strains showed response only at 100-fold change or were completely not affected by the CR condition [9]. Figure 2 showed the reaction norm of several strains from the study of van Diepeningen *et al.* (2010). From this figure it is clear that all strains showed a strong effect at 0.02%, but differed in their response at 0.2% glucose: some strains (i.e. Wa76 and Wa84) showed the effect, others (Wa44 and Wa77) only had a modest CR effect and again others showed no lifespan-extension effect at 0.2% glucose (Wa50 and Wa67).
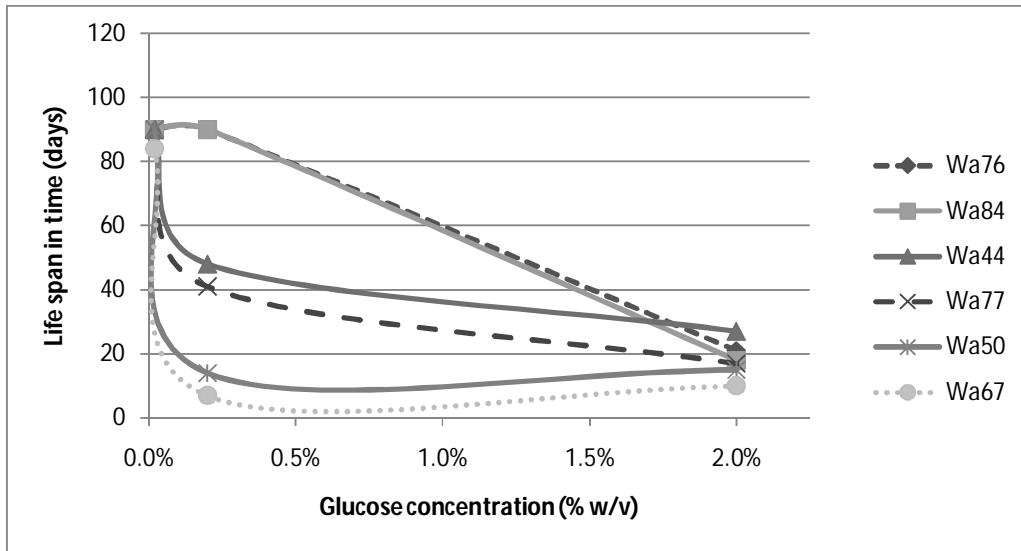
Figure 2. Lifespan reaction norm of wild type strains of P. anserina in respond to 2% (w/v) D-glucose as normal condition, 0.2% as mild CR condition and 0.02% as severe CR condition. Lifespan increases as glucose decreases as the effect of CR, but the shifting pattern is not parallel for each strain. Data were taken from van Diepeningen *et al.* (2010) [9].

The effect of CR on mtDNA rearrangements was also investigated. In the normal condition, cultures consistently showed an accumulation of senDNA-α. In CR condition of 0.2% glucose, observed accumulation of senDNA-α was comparable to normal condition. An exception was observed in one of the cultures where senDNA-α accumulation was less, but other senDNAs were shown to be present. The severe CR resulted in major alterations in the mtDNA rearrangement. SenDNA-α was not accumulated, even in the ultimately dying cultures, and amplification of other senDNAs, such as β, γ, ε, φ and ψ was observed. It was concluded that severe CR inhibits the accumulation of senDNA-α but yields other senDNAs amplification [8], however, the mechanism of how CR reduces the instability of mtDNA has not been clear yet.

1.3. Aim of experiment

The aim of this thesis is to investigate the genetic variation in CR response among wild-type strains of *P. anserina*. In the beginning we analysed six wild-type strains of *P. anserina* with presumed different sensitivity to glucose and CR effect at different glucose concentrations. A detailed reaction norm of the lifespan in response to different glucose concentration was determined. Next, we tested the strain-dependent effect of glucose concentrations on senDNA-α accumulation, as a molecular hallmark of senescence, and additionally wild-type mtDNA, senescent mtDNA and senDNA-γ copy number. Quantitative RT-PCR (Q-PCR) is applied to measure the dynamics of relative copy number.

2. Materials and Methods

2.1. Strains and culturing conditions

Six strains of wild-type *P. anserina* collected in Wageningen were selected to represent different responses to D-glucose concentration, based on the data from previous studies [9]. *P. anserina* synthetic medium (PASM) was used in the culturing of all strains with varying D-glucose concentrations as the CR treatment. PASM contained 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 1 g urea and 20 g agar with pH adjusted to 5.8 [10]. After sterilization, biotin and thiamine solution were added to the medium to a final concentration of 100μg/ml. Trace element solution which contained 500 mg citric acid·H_2O, 500 mg $ZnSO_4 \cdot 7H_2O$, 100 mg $Fe(NH_4)_2SO_4 \cdot 6H_2O$, 25 mg $CuSO_4 \cdot 5H_2O$, 5 mg $MsSO_4$, 5 mg H_3BO_3, and 5 mg $Na_2MoO_4 \cdot 2H_2O$ per 100 ml was also added for 1 ml per liter PASM. Double mating type spores were obtained from -80°C storage and grown on PASM with 2% D-glucose and ammonium acetate. After 3-day incubation in dark condition at 27°C, the cultures were ready as explants in the experiment.

2.2. Lifespan analysis

Varying glucose concentrations were used to determine how strains responded to gradual change from normal (2% glucose) to severe calorie restricted condition (0.02%). The variations were adjusted according to the anticipated crucial range of switch obtained from the previous study. Glucose concentrations of each group are described as below (Table 1). For each strain and condition, three replicas were made available.

Table 1. Glucose concentrations of PASM for each strain of *P. anserina* in lifespan analysis.

Glucose (% w/v)	Glucose-insensitive		Intermediate		Glucose-sensitive	
	Wa50	Wa67	Wa44	Wa77	Wa76	Wa84
0.02	X	X	X	X	X	X
0.04	X	X				
0.06	X	X	X	X		
0.08	X	X				
0.1	X	X	X	X		
0.12	X	X	X	X		
0.14	X	X	X	X		
0.16	X	X	X	X		
0.18	X	X	X	X		
0.2	X	X	X	X	X	X
0.4			X	X	X	X
0.6			X	X	X	X
0.8					X	X
1.0			X	X	X	X
1.2					X	X
1.4			X	X	X	X
1.6					X	X
1.8					X	X
2.0	X	X	X	X	X	X

Cultures were grown in race tubes of sterile 50 ml pipettes containing 20 ml medium and growth was marked every 2-4 days. Incubation was done at $26^{o}C$ and dark condition for 42 days as lifespan measurement was truncated for practical reason. Lifespan was defined in length (cm) and time (days) of growth until the first growth stop was observed. Maximum length of growth accommodated by the race tube was approximately 27.5 cm.

2.3. DNA isolation of cultures from five time-points

At the end of the lifespan experiment, three strains were selected to be investigated for the mtDNA and senDNA-α copy number changes in the course of time. Five time-points of *juvenile, middle-age-1 (ma-1), middle-age-2 (ma-2), middle-age-3 (ma-3)* and *old* were derived from normal condition of each strain. The time-points were defined chronologically based on the biological age of normal condition. The point where the inoculated explant grew was defined as *juvenile* time-point in both CR and normal conditions, but the first growth stop occurred in the normal condition was defined as *old* time-point for both conditions, which means CR's *old* time-point was not necessarily the point where CR culture had also stopped growing. Figure 3 describes where the *old* time-points were taken from the race tubes of each strain. Assuming that the cultures grew at a similar growth rate over time, three time-points were taken in between *juvenile* and *old* time-points.
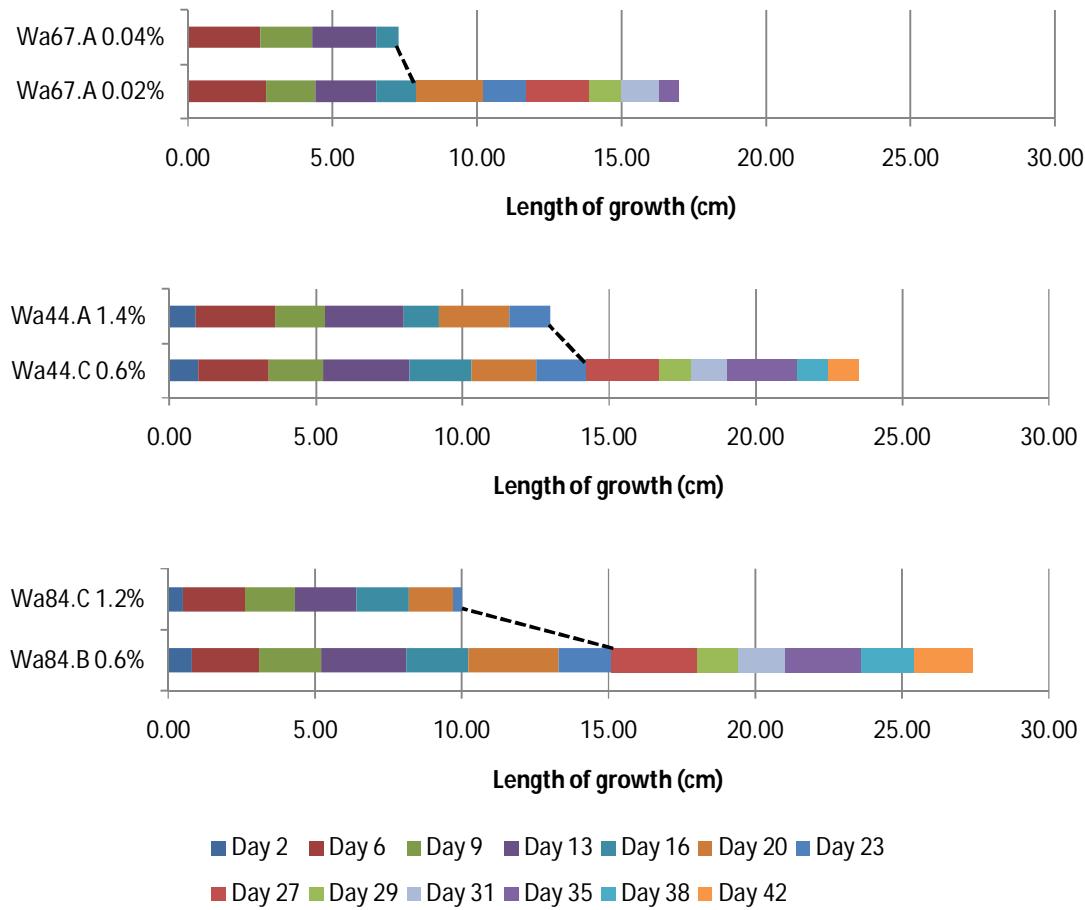
Figure 3. Growth scheme of selected three strains, Wa67, Wa44 and Wa84 (A, B and C, respectively), to be analyzed for their mtDNA and senDNA-α copy number changes in the course of time. In each strain, upper bar showed the growth in normal condition, while lower bar showed the CR condition. Black dashed line indicates the first growth stop in normal condition and the corresponding time-point in the CR condition. Glucose concentration and replica identity of each isolate were also indicated.

To harvest the mycelium for DNA isolation, each time-point of each strain was plated to PASM with corresponding glucose amount and incubated in the dark at 27°C. The medium was covered with cellophane to ease the harvesting of mycelium. After two days of incubation, mycelia were harvested, snap-frozen and ground with a bead beater (two times of 10s beating). DNA isolation was done using DNeasy Plant Mini Kit (Qiagen, Valencia, CA), following the standard protocol of the kit, except for the final DNA elution which was done twice (first and second elution were 50μl and 25μl of TE buffer, respectively). DNA concentrations were measured on the NanoDrop ND-2000 UV-Vis spectrophotometer.

2.4.   Mitochondrial DNA and senDNA-α copy number analysis

Quantitative real-time PCR was used to determine mtDNA and senDNA-α copy number. Primers which were specific to mtDNA and senDNA-α were designed using criteria and designing steps in Appendix A. Primer pairs' sequence are listed in Appendix B. All primers were self-designed, except

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for Lsu (a mitochondrial gene) and Gpd (a nuclear genome) genes which obtained from Sellem *et al.* (2007) [11]. Gradient temperature Q-PCR was first done to check the specificity of the amplification and also to determine the optimum annealing temperature and amount of DNA template. Q-PCR was performed in duplicate, on serial dilutions of the total DNA samples as follow: 1/1, 1/10, 1/40, 1/160, 1/640. Q-PCR was performed on the BioRad thermal cycler. The reaction mix consisted of 3µl of DNA template, 0.6µl of 10µM both forward and reverse primer, 5µl of SYBR Green from BioRad, and MQ water to reach total volume of 10 µl. 3µl of DNA template contained 5 ng of DNA, obtained from the dilution of isolated DNA.

3. Results

3.1. Strain-specific lifespan-extension effect of CR

In this study, we used *P. anserina* strains collected in Wageningen which had been studied previously by van Diepeningen *et al.* (2010). By using two level of CR, low CR (0.2% glucose) and severe CR (0.02% glucose), they found that the strains collected had an increased lifespan as CR effect and this response was strain-specific. The strains were then classified into four types of variants: 1) glucose-insensitive variants, which responded only to severe CR treatment, 2) moderately-glucose-sensitive variants, which response depended on the level of CR: modest response to low CR and large response to severe CR, 3) glucose-sensitive variants, which strongly responded even at low level of CR and 4) non-responder strain, which did not respond to both level of CR [9]. This classification was the starting point of the strains selection in this study, as they showed an intriguing genetic variation regarding their responses to CR. Since this study aimed to study the effect of CR, the non-responder strain was excluded from the study. The use of PASM, a synthetic medium with adjustable glucose concentration as the only carbon source, enabled this experiment to exclude the effect from different type of carbon source and specifically observe the effect from glucose, the catabolite-repressive sugar.

The survival response to series of glucose concentrations from the selected strains was presented in Figure 4. The glucose-insensitive strains showed increasing survival at diminutive glucose level, the moderately-glucose-sensitive responders at intermediate glucose level, and the glucose-sensitive strains at higher glucose level. Notably, one of the three replicates of the moderately-glucose-sensitive strain Wa44 appeared to be capable of maintaining survival even without the help of CR. In the normal and slightly glucose restricted condition (2%, 1.4% and 1% glucose), a replica of each concentration reached a lifespan of minimum 42 days (Figure 4B). Meanwhile, Wa67 was barely affected by CR condition, as it hardly survived even at very low glucose concentration (Figure 4A).

A Glucose-insensitive strains

B Moderately-glucose-sensitive strains

C Glucose-sensitive strains
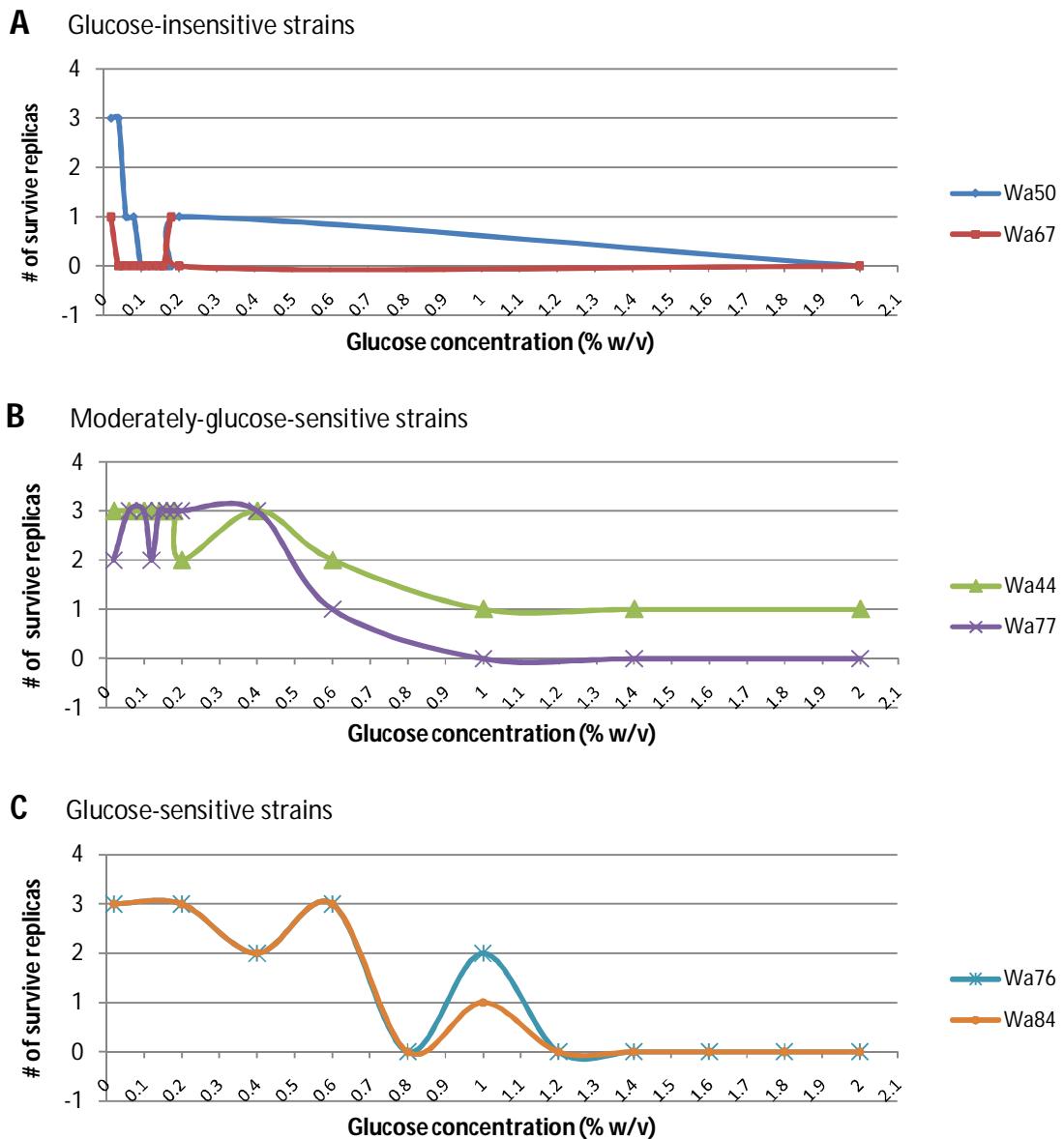
Figure 4. Survival of the three replicas of each glucose concentration at the end of experiment. Cultures were grown for 42 days, then the number of replicas without observed growth stop was plotted along the glucose concentration series. (A) The glucose-insensitive strains, Wa50 and Wa67. (B) The moderately-glucose-sensitive strains, Wa44 and Wa77. (C) The glucose-sensitive strains, Wa76 and Wa84.

The lifespan-extension pattern of the strains was further analysed with a lifespan plot in Figure 5 (lifespan in length, the plot of lifespan in time is supplemented in Appendix C). The use of glucose concentration series revealed that the shifting pattern of lifespan extension as effect of CR was distinct from one strain to another. Some strains had a wide glucose range of transition from normal to extended lifespan (Wa50, Wa44, Wa77, Wa76 and Wa84), while another was strictly narrow (Wa67). The plot also suggested the possibility of there is an optimum CR level to increase lifespan, as indicated by plots of Wa44, Wa77, Wa76 and Wa84. Here the lifespan went up as the glucose was decreasing, but at some point the lifespan-extension effect was slightly reduced, e.g. glucose concentration at 0.6% for Wa84, which stood out as the peak lifespan. Another observation was the

presence of stochastic CR response which was strongly indicated in Wa50 (at glucose concentration of 0.06%) and vaguely in other strains (1% of Wa44 and 1.2% of Wa76).
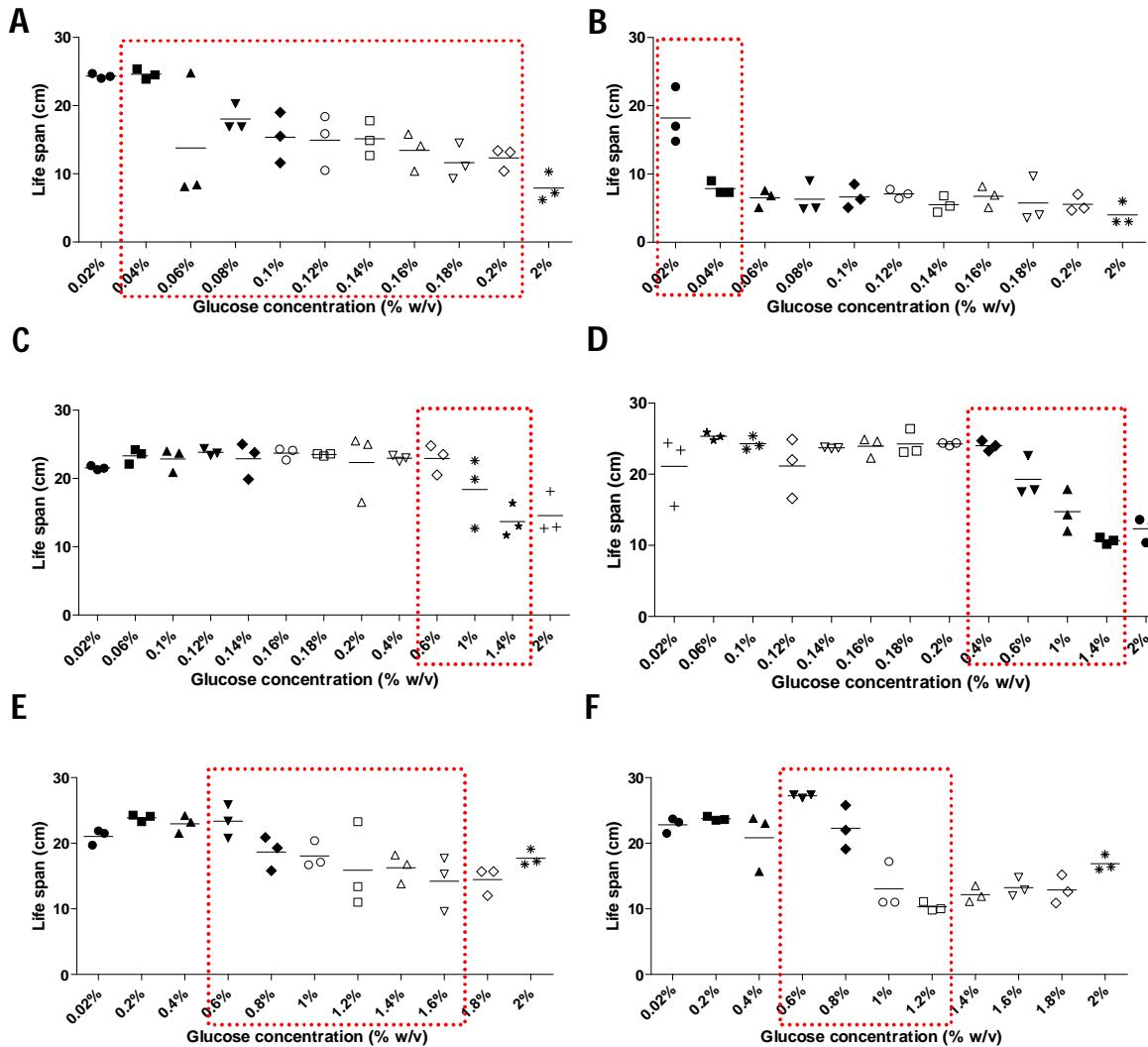
Figure 5. Lifespan (in cm of length) plot of each strain in the corresponding glucose concentration series. The plots of Wa50, Wa67, Wa44, Wa77, Wa76 and Wa84 are represented in A, B, C, D, E and F, respectively. Red dashed-brackets indicate the transitional range from normal to CR-affected condition. The growing culture observation was truncated at 42 days.

To proceed with the next analysis, we picked three strains, one from each glucose-classified group of strains and strains with narrow-ranged transition from normal to CR-affected condition were preferred. From each strain, two isolates of different glucose concentration were picked to represent normal and CR condition. Glucose concentrations picked were the ones with which replicates showed similar results. The criteria resulted on the selection of Wa67, Wa44 and Wa84, to represent glucose-insensitive, moderately-glucose-sensitive and glucose-sensitive strains, respectively.

3.2.   Mitochondrial DNA destabilization in senescence

In the next experiment, the mtDNA and its modification dynamics during senescence were studied by comparing their relative copy number. Relative copy number can be estimated by relative quantification of quantitative RT-PCR (Q-PCR) technique. Relative quantification means that the amplicon of interest is corrected by a control amplicon, which referred to an amplicon of a nuclear gene in this case. Several pairs of primers were designed to amplify specific regions in the mitochondrial genome and the nuclear genome, using the mitochondrial sequence from Cummings *et al.* (1990) and the nuclear sequence obtained from Nucleotide database of NCBI [12]. From the total of ten pairs of primers to analyze mtDNA, five of them amplified the specific target region and showed a proper reaction efficiency (refer to melt curve of primer pairs in Appendix B) and both pairs of primer to analyze nuclear genome worked properly. Four pairs of primers in mitochondrial genome were selected as they represented different DNA fragments during senescence: 1) Cox1-i7 to represent the wild-type mtDNA, 2) Cob to represent senescent mtDNA with the size of 30 kb, 3) Cox1-i1 to represent senDNA-α and 4) Lsu to represent senDNA-γ. There were three primer pairs which amplify Cox1-i1 and preliminary results showed that all pairs worked properly and had similar results. In the analysis, we used Cox1-i1-2 primer pairs. Figure 6 depicted the position of amplicons along the mtDNA and their corresponding senescent mutant mtDNA. A nuclear gene, Adcy (adenyl cyclase) was also analyzed as the reference gene.
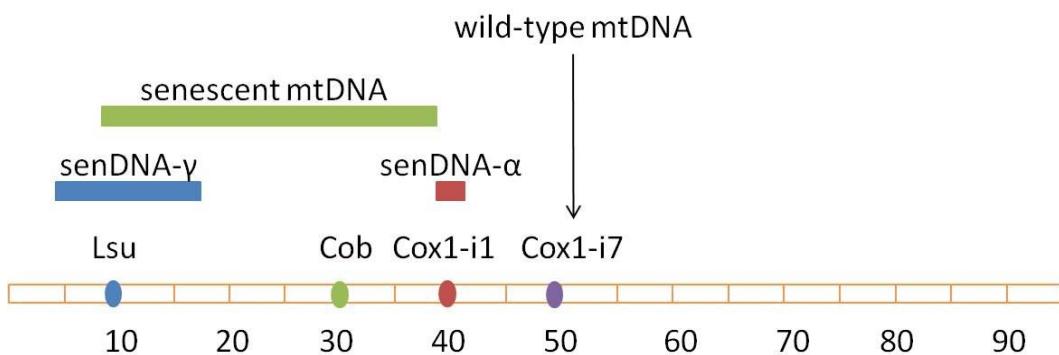

Figure 6. Position of analyzed Q-PCR amplicons and their corresponding senescent mutant DNA in the 94,198 kb wild-type mtDNA (originally circular DNA, but represented as linear here). Dots represented the amplicon, while bars represented the senescent mutant DNAs.

To examine the integrity of mtDNA, we made comparisons between the wild-type mtDNA of each strain, to the possible senescence by-products, the 30 kb senescent mtDNA, senDNA-α and -γ (Figure 7). Good maintenance of mtDNA stability would be indicated by the by-products' curves being parallel to wild-type's curve over time. In normal conditions, this was indeed the case in every strain until they reached *ma-2* time-point. Despite of the well-maintained stability, the level of mtDNA copy number differed in the way that strain Wa84 maintained the same level, Wa67 had a slight decrease and Wa44 had a major decrease after *juvenile* time-point.

In the next life stage, the stability of mtDNA started to be disrupted in a strain-specific manner. The most eminent change was the accumulation of senDNA-α which was shown by a small increase in strain Wa67, a progressive increase in Wa84, or a significant burst in Wa44. Intriguingly, the significant burst in Wa44 was encountered by a decline in the *old* time-point. Along with the accumulation of senDNA-α, there were also indications of decreasing wild-type mtDNA and slightly increasing senescent mtDNA, although these indications were not consistently observed at *old* time-points (e.g. at the *old* time-point, Wa67 had an increasing wild-type mtDNA instead of a drop). All strains did not indicate a significant accumulation of senDNA-γ, although it is worth to note that the relative copy number of Lsu was higher than wild-type mtDNA in strains Wa44 and Wa84 at the *old* time-point.

The instable events observed in normal condition were relatively absent under the effect of CR. The 30 kb senescent mtDNA, senDNA-α and -γ showed to have curves parallel to wild-type mtDNA. The only instability observed was the accumulation of senDNA-α in strain Wa44 at the *old* time-point. Unexpectedly, the level of accumulation was even higher than what observed in normal condition. This might indicate that CR affect metabolism so that the isolate might withstand such instability.

Next, the levels of mtDNA copy numbers were compared across the different strains (Figure 8). In both normal and CR condition, Wa44 appeared to have higher level of wild-type mtDNA copy number. However, Wa44 also appeared to be the least stable strain, due to the fluctuating mutant mtDNAs (30 kb senescent mtDNA, senDNA-α and –γ) copy numbers. It even accumulated senDNA-α in CR condition, while other strains were relatively stabilized. The glucose-insensitive strain Wa67 had the lowest level of wild-type mtDNA with a relatively good stability. It was the only strain which did not accumulate senDNA-α in both normal and CR conditions. Wa84 was the strain with results as expected, moderate stability in the early life, accumulation of senDNA-α at the point before growth stop and increasing senescent mtDNA over time.
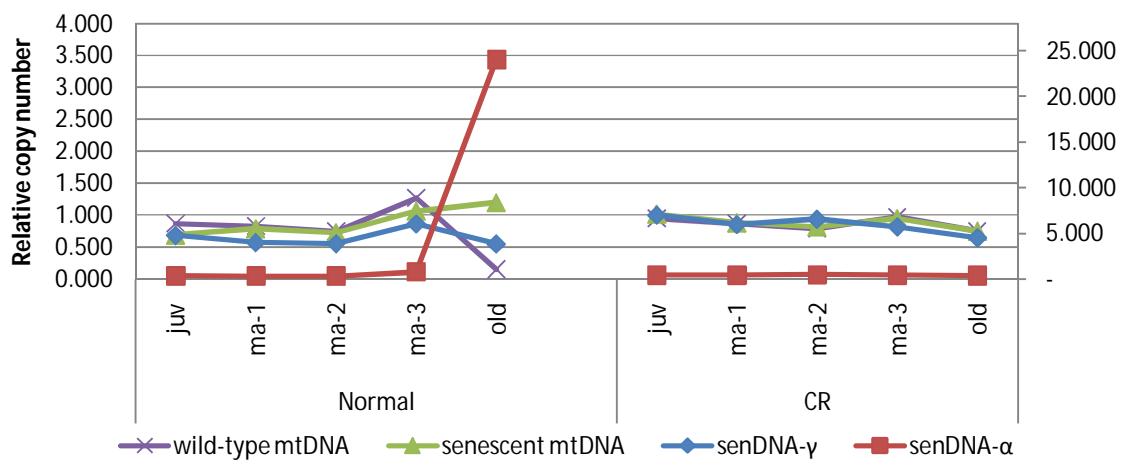
A Glucose-insensitive strain Wa67
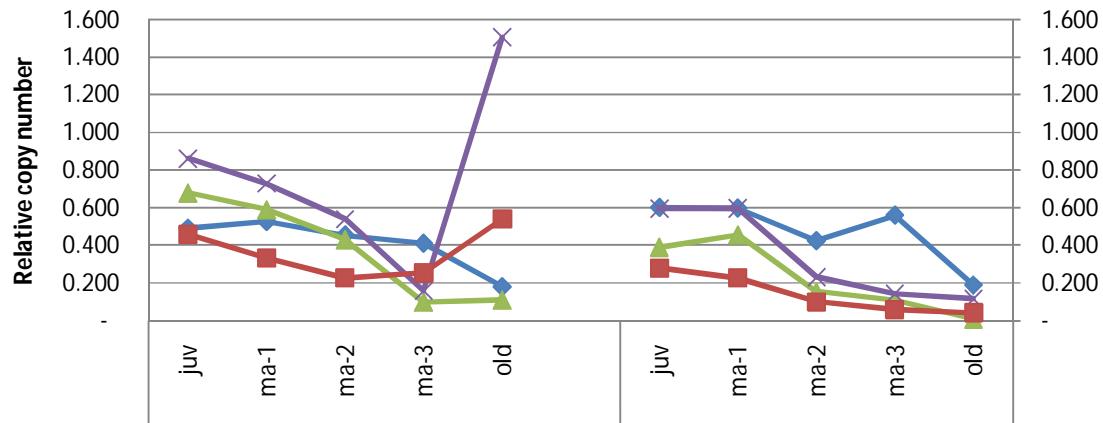
**B** Moderately-glucose-sensitive strain Wa44
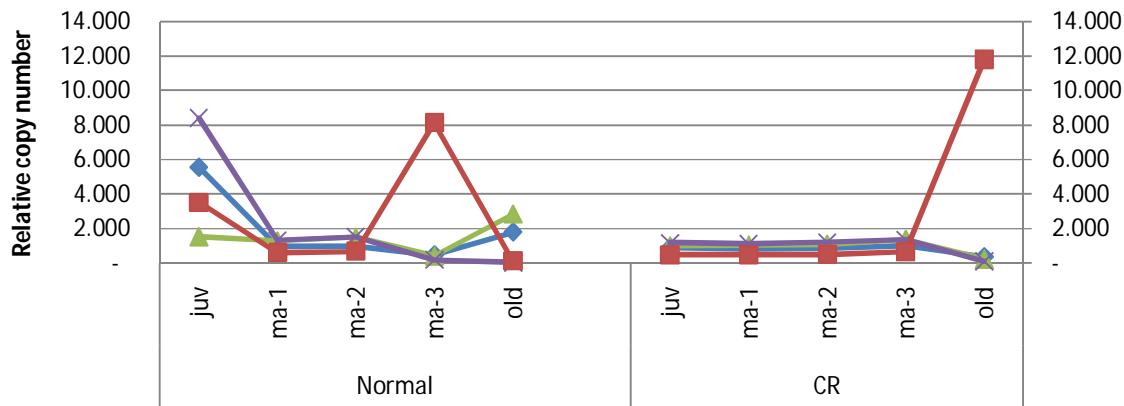
C Glucose-sensitive strain Wa84

Figure 7. Dynamics of wild-type mtDNA, senescent mtDNA, senDNA-α and –γ in mtDNA rearrangement during senescence in strains Wa67, Wa44 and Wa84 at normal and CR condition. Relative copy numbers were quantified by using Q-PCR technique. The wild-type mtDNA, senescent mtDNA, senDNA-α and –γ were represented by primer sets of Cox1-i7, Cob-i1, Cox1-i1 and Lsu, respectively. Relative copy number of senDNA-α was plotted to secondary vertical axis due to large scale differences with others. The normalization was done by using nuclear gene Adcy.

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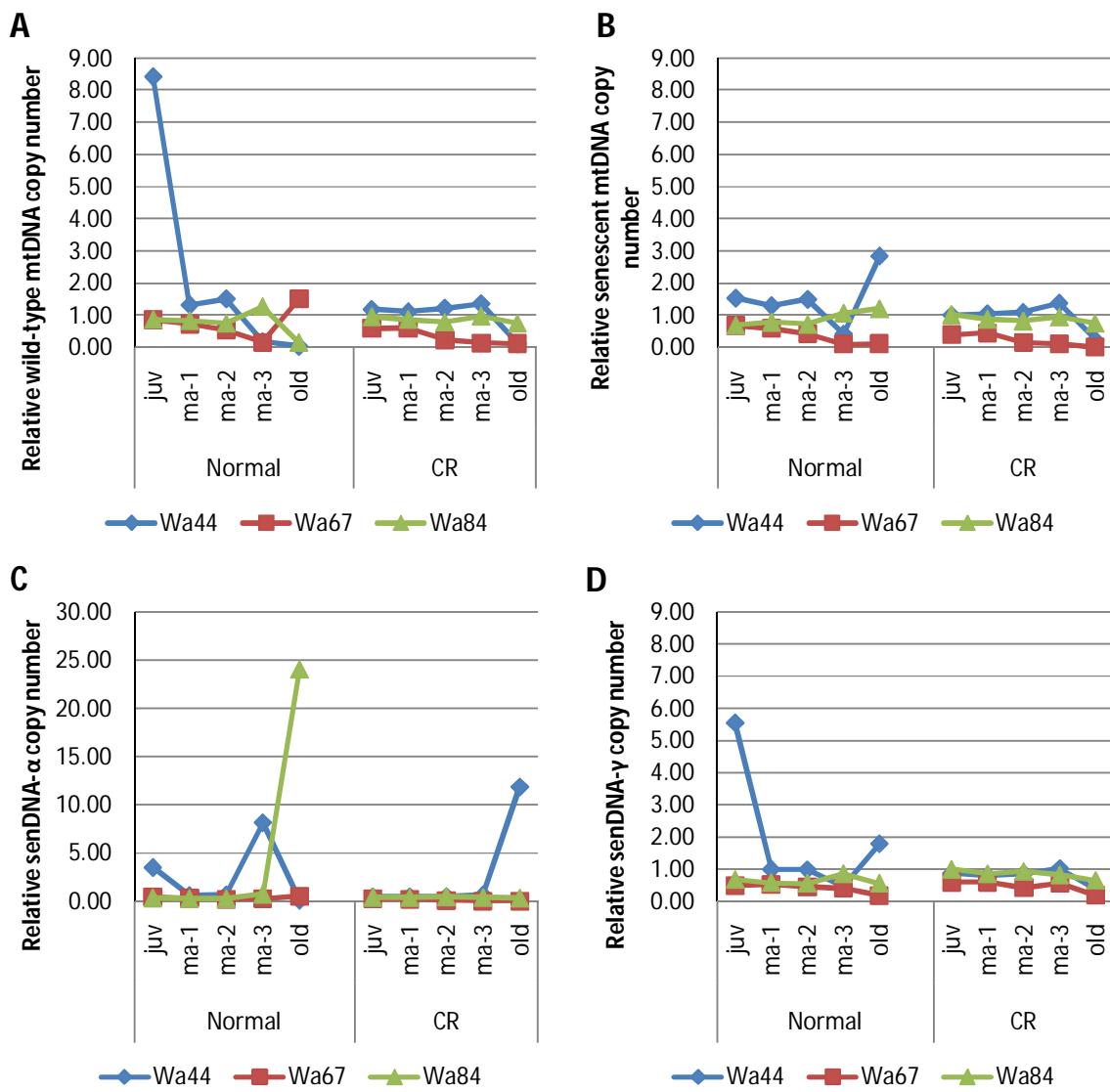
Figure 8. The relative copy number comparison between strains of wild-type mtDNA, senescent mtDNA, senDNA-α and –γ (represented by A, B, C and D, respectively).

4. Discussion

4.1. Genetic variant dependency of CR-effect

In this study, we showed that lifespan of all strains was extended with decreasing glucose availability, consistent with the result of previous study [9]. The reaction norm of different strains in response to CR condition was also analyzed in more detail. Lifespan appeared to be a plastic trait, which responded glucose-dependently. This indicated the presence of a system which is able to be "switched on" according to glucose availability to increase survival in food scarcity. The switch seemed to have a threshold value which acted strain-dependently. With the decreasing glucose concentration, lifespan progressively shifted in a certain strain-specific transitional range, either wide or narrow. In lower concentrations than the threshold, isolates kept the same value or slightly decreased. Stochastic response was observable in strains with wider transitional range.

Kimpel and Osiewacz (1999) proposed that PaGrg1, a glucose-repressible gene, to be significantly involved in lifespan control. A homologue of grg-1 gene of *Neurospora crassa*, PaGrg1 was shown to be up-regulated in strain *s* of *P. anserina* in response to carbon starvation condition. Further analysis on the upstream sequence of PaGrg1 revealed that it might be *cis*-regulated by transcription factors similar to CREB element (Cyclic AMP Responsive element), CREA and CRE1 [13]. These transcription factors were demonstrated as energy availability-related and glucose-responding regulators demonstrated in other filamentous fungi [14, 15]. This suggested that PaGrg1 or other unidentified genes which are involved in glucose- or carbon-dependent signaling pathway might play a role as the switch to shift from normal to CR-affected lifespan. PaGrg1 is particularly interesting, since it was thought to be a stress protein. PaGrg1 showed to be up-regulated during two events which commonly generate stress, carbon starvation and ageing process [13], while stress protein was proposed to be closely associated to CR mechanism in the hormesis hypothesis of CR [16]. When carbon starvation in CR induces a mild stress, the stress protein generated is thought to signal survival response(s), e.g. increasing stress resistance, metabolism alteration, etc. [17].

Therefore, in relation with the lifespan-extension effect of CR which was strain- and glucose-specific, we would like to propose a model, which is illustrated in Figure 9. With decreasing glucose availability, carbon-starvation stress is increased until it reaches *x*% glucose concentration, which starts to stimulate a beneficial survival response, for example an increased mtDNA resistance against age-related rearrangements. However, the beneficial survival response does not grant an optimum longevity until it reaches *y*% of glucose, the optimal glucose concentration to obtain lifespan-extension effect of CR. The optimum beneficial survival response continues until *z*% glucose and then the stress generated is too much and it loses the beneficial survival response. The transition range from normal to CR-affected condition, which was indicated in Figure 5 may be projected *x* to *y*% glucose, and this range is a subject to the strain's genetic variant.
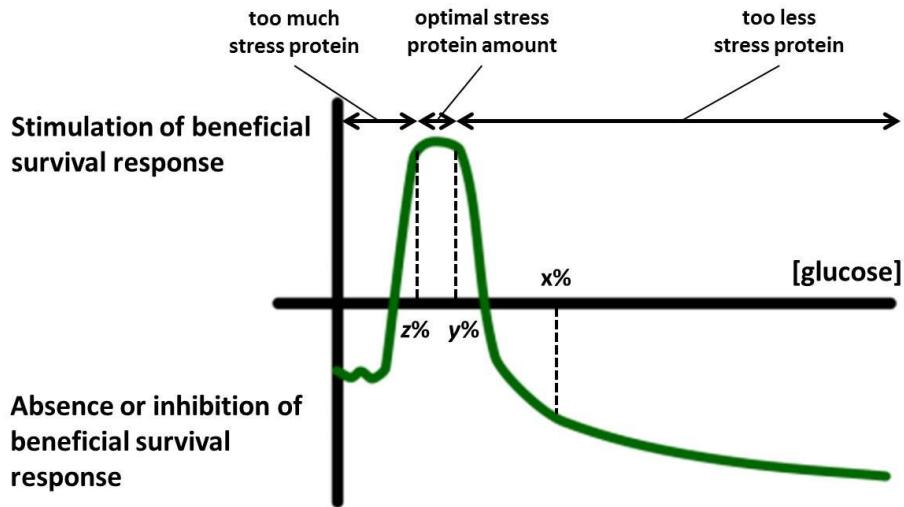
Figure 9. The proposed hormesis hypothesis of glucose restriction in *P. anserina*. The hormesis curve has a biphasic shape: 1) the phase above the horizontal axis, in which glucose concentration gives a mild carbon-starvation stress and stimulation of beneficial survival response and 2) the phase below the horizontal axis, in which no stress or too much stress generated from the glucose concentration and beneficial survival response is absent or inhibited.

Despite that the lifespan-extension effect of CR was similar to the result of the study from van Diepeningen *et al.* (2010), the effect of CR condition on lifespan in this study was much smaller than reported earlier, e.g. Wa67 showed larger respond to severe CR in the lifespan extension, as in the previous study Wa67 had the mean lifespan of more than 80 days, while in this study only one replica had the possibility to live more than 42 days. This might be due to a disadvantage from this type of lifespan experiment, which accuracy largely depends on the time interval of growth marking. *P. anserina* has the characteristic of being capable to re-grow after a growth stop, attributed to the possibility of the apical cells from different part continues to grow while the cells of visible growing tip has stop to grow. Therefore, a large interval of observation has higher chance to miss the observation of the first growth stop which then concealed by the re-growth.

4.2. Mitochondrial DNA destabilization in senescence

In this study, we observed that CR might stabilize mtDNA from mutation or mtDNA rearrangement as was shown in normal condition. The result complied with the previous study, which reported that senDNA-α accumulation as an indicator of mtDNA instability was inhibited in CR condition [8]. However, it was not the case for the moderately-glucose-sensitive strain Wa44, which accumulated senDNA-α while continued to grow. This might imply that apart from the mtDNA-destabilization effect, CR might have other ways to increase survival [17]. This finding also confirmed the discussion in previous reviews that senDNA-α's role is an accelerator in senescence, instead of a cause [18, 19]. It was observed in strain Wa67 at normal condition that senescence progressed and eventually the isolate died without the presence of highly accumulated senDNA-α.

In the case of senDNA-α significant burst in Wa44 which was followed by a decline in *old* time-point, there was probably a selection for hyphae with functional wild-type mtDNA which was allowed to continue its growth. Mitochondrial function indeed supports energy supply for hyphal tip elongation

and branching, so that higher number of functional mitochondria may support higher growth rate. In the meanwhile, the hyphae with senDNA-α accumulation was selected out of the growing culture and no longer found in the next time-point. Since the CR isolate still growing with high accumulation of senDNA-α, it is intriguing to investigate how the senDNA-α copy number would be in the CR condition after the *old* time-point.

The accumulation of senDNA-α in normal condition was started to be observed at later stage of *P. anserina* life, *ma-3* time-point. This was also suggested by Albert and Sellem (2002), that mtDNA was not rearranged until the beginning of senescent state. They also suggested that the amplification of various senDNAs occur at an exponential rate [1]. As it was observed in isolates accumulating senDNA-α in this study, senDNA-α was indeed amplified to large amount in a short time. Khrapko (2011) proposed this type of DNA mutation as a "late mutation, fast expansion" mechanism {Khrapko, 2011 #39}. This mechanism was enabled in senDNA-α case, due to its rapid liberation and amplification property of Cox1's intron α [20].

Apart from senDNA-α, the 30 kb senescent mtDNA copy number also showed a potential to be a senescent indicator. In the isolates which accumulated senDNA-α during senescence, with the decreasing wild-type mtDNA copy number, senescent mtDNA was shown to be increasing, although the level of increase was not as prominent as senDNA-α level. However, this fragment of rearranged mtDNA would still be interesting to be analyzed in future studies, especially in isolates which do not accumulate senDNA-α but having the symptoms of senescence.

Strain Wa67 was an intriguing case in this study. While being very short-lived and showing a very fast senescing condition, Wa67 did not show major signs of mtDNA instability. The relative copy number of wild-type mtDNA always looked parallel with the mutant mtDNA indicator and at the *old* time-point, the wild-type mtDNA was even increasing. However, the increment was not followed by other region of mtDNA, which might suggest that there was mtDNA rearrangement(s) occurred, but it was not a common one and the designation of Cox1-i7 amplicon as wild-type mtDNA was no longer correct.

In conclusion, we observed that glucose-dependent effect of CR on lifespan was strain-specific, indicating that certain genetic variant(s), such as PaGrg1, might be involved in the CR mechanism pathway. CR was indeed destabilized mtDNA from various possible rearrangement, for instance senescent mtDNA, senDNA-α and -γ. In the future studies, it is recommended to extend the time-points of CR cultures in order to observe the mtDNA dynamics alteration from normal condition and also to include more senDNAs in the analysis.

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Appendix A-1. Primer designing criteria and software used

Criteria	Settled by
50-60% GC content	Primer3Plus
18-24 bases long	Primer3Plus
Limit G or C stretch longer than 3 bases	Primer3Plus
Place C and G on ends of primers, but no more than 2	Primer3Plus
Limit secondary structure	IDT DNA
Ideal amplicon size: 75-150 bp	Primer3Plus
No stable interactions between primers (primer-dimer)	Beacon designer
Annealing temperature between 58-60C	Primer3Plus
No specificity to nonspecific sequences	BLAST

Appendix A-2. Primer designing steps

1. Obtain the target sequence: http://www.ncbi.nlm.nih.gov/nuccore
2. Upload target sequence in Primer3Plus: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi
   Use your own setting by uploading the setting in "General settings"
3. Check primers' self dimer, heterodimer, hairpins:
   http://www.premierbiosoft.com/qpcr/index.html
   Discard primers with ΔGs more negative than -3.5 kcal/mol.
4. Check amplicon's secondary structure in UNAfold:
   http://eu.idtdna.com/Scitools/Applications/unafold/
   Change the temperature to $60^{o}C$ and Mg concentration to 3 mM. Discard primers if Tm of amplicon exceeds $60^{o}C$.
   If the link does not work (suspected to be expired after around 10 times of usage) use:
   http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/
   Choose hairpin function, change the temperature and Mg concentration, click update. If the link does not work either, use:
   http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form
   Change folding temperature and Mg concentration as the above, and also adjust the Na concentration to 50 mM. Tm information is available in Thermodynamic details. However, results of IDT and Albany slightly differ.
5. Additional: blast primers to check for specificity to non-specific sequences:
   http://podospora.igmors.u-psud.fr/blast.php

Appendix B-1. List of primers for senDNA-α (Cox1-i1)

Name	Forward (5′ to 3′)	Reverse (5′ to 3′)	Amplicon size
Cox1-i1-1	GATCCGTAGTAGTAGGTGTG	GATACATTGCCCTCTCTC	143 bp
Cox1-i1-2	GGGAAGATGAGACCACTA	CCATGTGAGCTACTGTGA	113 bp
Cox1-i1-3	CTTTGATGGAGAGCCGTA	AAGCAGCCAGTCCTTAGTC	96 bp

Appendix B-2. List of primers for mtDNA genes

| Name	Forward (5′ to 3′)	Reverse (5′ to 3′)	Amplicon size
Cox1-e1-1	GTGAATCGAGAGATGAATG	GAGAACGCTGTACCTAGTAA	99 bp
Cox1-e4-1	GTCATACGGCACTTCATC	AACCTCTGGTCCTTTATCTT	139 bp
Cox1-i7-1	GGATAGCAAGAACCCTCA	CAAAGACGCCTACAACCT	143 bp
Cox1-i8-1	AGCACGCAAGTAGGAACT	GCAAAGCTCGTTCATCTC	199 bp
Cob-i1-1	GGGACAATCAGCAGGTAAG	GGTGTCAAGTGTGTAGTCTCTG	82 bp
Cob-i2-1	AGACTGGGTCACTAATGG	GAAGAGCTTGCACCTAAC	75 bp
ATPase6-i1	ACTTCTTACCTGGCCTCA	ACCGTCTACTCGTTGCTC	135 bp
Lsu	GGGTACGACTGTTCGTCGAT	CCCCATGTTCCTTTCGTACA	129 bp

Appendix B-3. List of primers for nuclear genes

Name	Forward (5′ to 3′)	Reverse (5′ to 3′)	Amplicon size
MKK1	TGGGAGAGTTGAGGATAG	ATCGTACCAGATCACCAC	141 bp
Adcy	CTCAGTAGTGGTGGCTCT	CTCGGTATTCTTCAGGTC	93 bp
Gpd	GTGAGCTCAAGGGCATTCT	CCGTTGAGGTCAGAGGAGAC	60 bp

Appendix B-4. Melting curve of primers listed

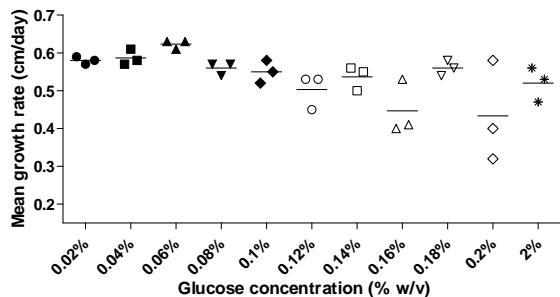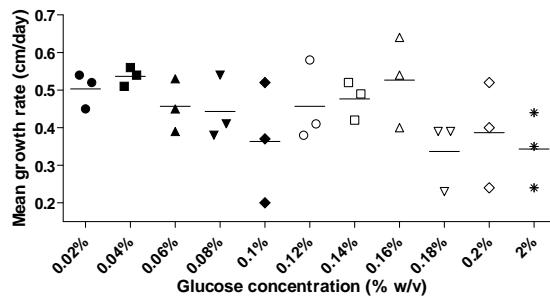| Cox1-i1-1	Cox1-i1-2	Cox1-i1-3
Cox1-e1-1	Cox1-e4-1	Cox1-i7-1
Cox1-i8-1	Cob-i1-1	Cob-i2-1
ATPase6-i1	Lsu	
MKK1	Adcy	Gpd
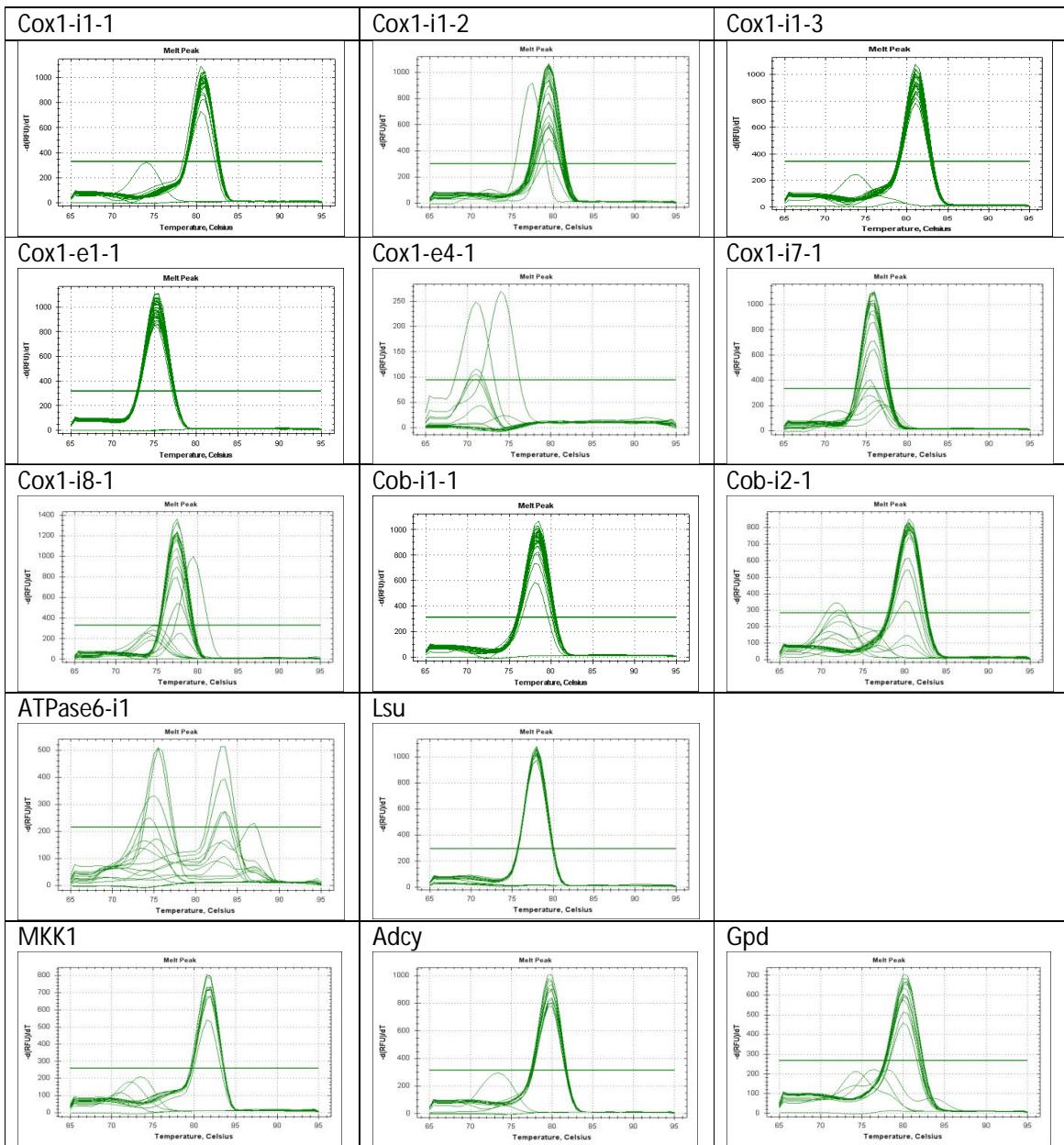
Appendix C. Lifespan (in days) plot of each strain in the corresponding glucose concentration series. The plots of Wa50, Wa67, Wa44, Wa77, Wa76 and Wa84 are represented in A, B, C, D, E and F, respectively. The growing culture observation was truncated at 42 days.
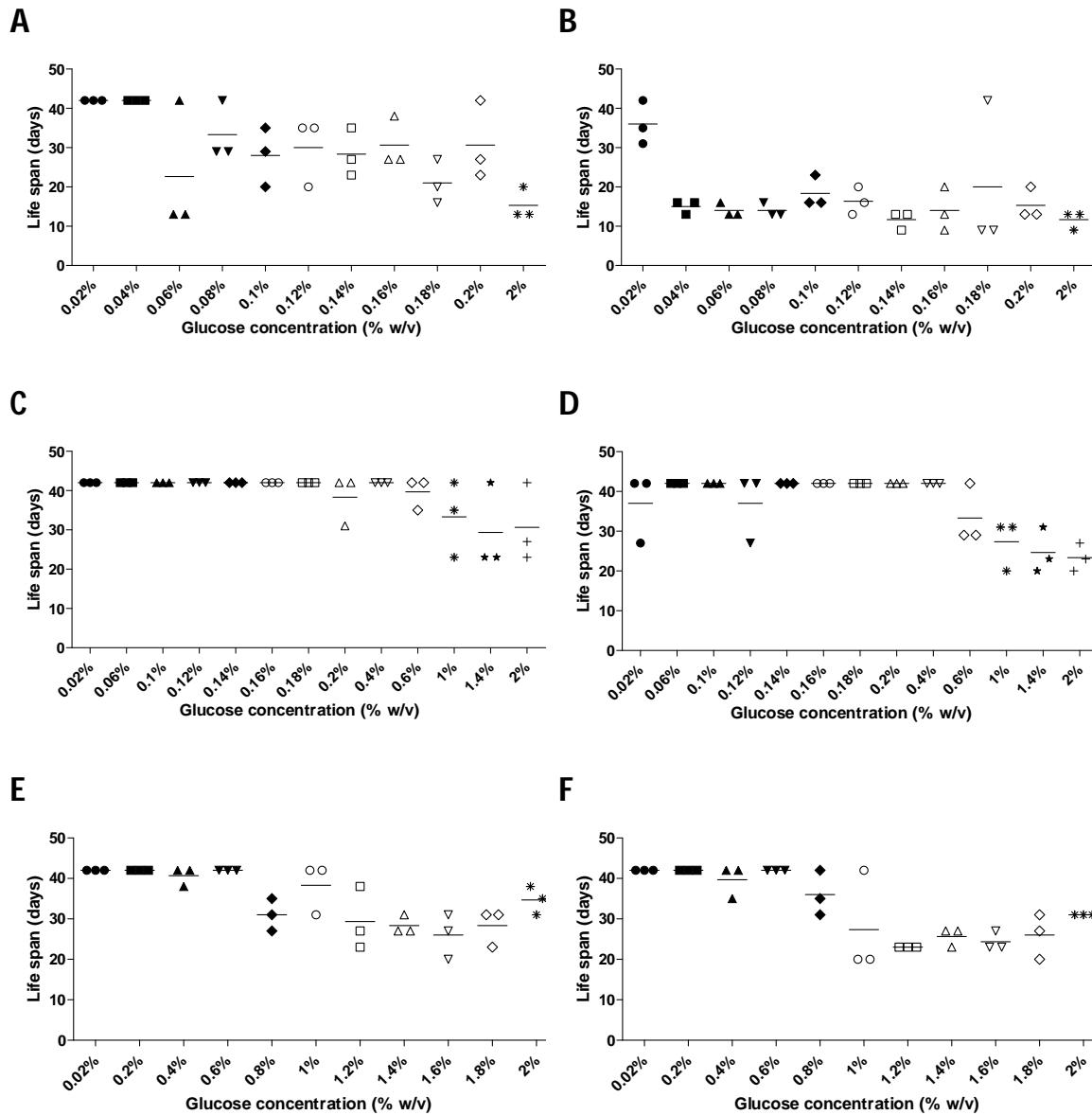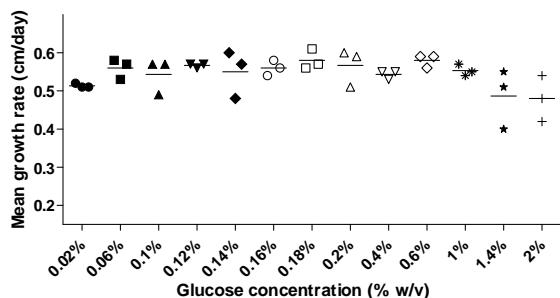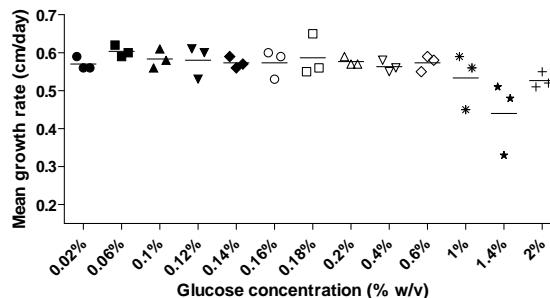
Appendix D. Growth rate plot of each isolate of each strain in the corresponding glucose concentration series. The plots of Wa50, Wa67, Wa44, Wa77, Wa76 and Wa84 are 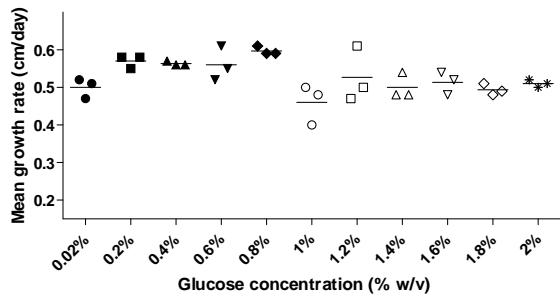represented in A, B, C, D, E and F, respectively.