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Cite this article: van Diepeningen AD, Engelmoer DJP, Sellem CH, Huberts DHEW, Slakhorst SM, Sainsard-Chanet A, Zwaan BJ, Hoekstra RF, Debets AJM. 2014 Does autophagy mediate age-dependent effect of dietary restriction responses in the filamentous fungus *Podospira anserina*? *Phil. Trans. R. Soc. B* **369**: 20130447.

<http://dx.doi.org/10.1098/rstb.2013.0447>

One contribution of 9 to a Theme Issue 'What cost mitochondria? Maintenance and evolution of mtDNA'.

Subject Areas:

evolution, health and disease and epidemiology, genetics

Keywords:

dietary restriction, carbon and nitrogen restriction, ageing, autophagy, lifespan extension, *Podospira anserina*

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Electronic supplementary material is available at <http://dx.doi.org/10.1098/rstb.2013.0447> or via <http://rstb.royalsocietypublishing.org>.

Does autophagy mediate age-dependent effect of dietary restriction responses in the filamentous fungus *Podospira anserina*?

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Autophagy is a well-conserved catabolic process, involving the degradation of a cell's own components through the lysosomal/vacuolar machinery. Autophagy is typically induced by nutrient starvation and has a role in nutrient recycling, cellular differentiation, degradation and programmed cell death. Another common response in eukaryotes is the extension of lifespan through dietary restriction (DR). We studied a link between DR and autophagy in the filamentous fungus *Podospira anserina*, a multicellular model organism for ageing studies and mitochondrial deterioration. While both carbon and nitrogen restriction extends lifespan in *P. anserina*, the size of the effect varied with the amount and type of restricted nutrient. Natural genetic variation for the DR response exists. Whereas a switch to carbon restriction up to halfway through the lifetime resulted in extreme lifespan extension for wild-type *P. anserina*, all autophagy-deficient strains had a shorter time window in which ageing could be delayed by DR. Under nitrogen limitation, only PaAtg1 and PaAtg8 mediate the effect of lifespan extension; the other autophagy-deficient mutants PaPspA and PaUth1 had a similar response as wild-type. Our results thus show that the ageing process impinges on the DR response and that this at least in part involves the genetic regulation of autophagy.

1. Introduction

Calorie or dietary restriction (DR) refers to a dietary regimen in which an organism is subjected to a reduced food intake—either in calorific value or in specific food compounds—without causing malnutrition. DR has been shown to increase lifespan in a wide variety of organisms [1,2], even if applied temporarily [3,4]. The DR response is adaptive because it allows surviving lean periods by shifting the energy investment from germ line towards soma. As a result, reproduction is postponed until more favourable conditions arrive [5,6]. Despite this clear adaptive value of DR, little is known about the genetic and molecular bases that lead to the DR response. The lifespan extension effect can be viewed as postponement of ageing, but the age-independency of this response in, for instance, *Drosophila* refutes such a direct link to ageing mechanisms [7].

Both ageing and the presumed postponement of ageing by DR have been related to mitochondrial function. Harman's free radical theory focuses on the production of reactive oxygen species by the mitochondria, and their damaging effect on macromolecules as the major cause for ageing, and that as a result

of DR the production of reactive oxygen species is reduced and hence lifespan is extended [8]. Alternative views focus on other DR mechanisms such as lowering the levels of glycation-mediated protein damage [9] or emphasize the multi-causal nature of ageing and suggest a genome-wide regulated response to DR [10].

One way to deal with age-related damage and possibly to reallocate resources in time of scarcity is autophagy. Vacuolar/lysosomal autophagic degradation is the major pathway for continuous turnover of damaged and obsolete macromolecules and organelles, and the involved genes have a high level of conservation throughout all eukaryotes [11,12]. Autophagy supposedly has a dual role, one linked to nutrient deprivation and recycling and to increase cellular lifespan, and the other linked to programmed cell death [12,13]. It has been shown that upon nitrogen restriction, genes involved in the autophagic pathway as well as genes involved in cellular proteolytic activity are induced [14–17].

Podospora anserina has been a model system for ageing since the 1950s [18]. Ageing in *Podospora* and some other filamentous fungi probably evolved due to the ephemerality of its habitat [19]. That is, *P. anserina* grows on herbivore dung and all natural isolates have a lifespan of only two to three weeks [20,21]. The senescence phenotype is characterized by a loss in reproductive potential, a reduction in growth rate and aerial hyphae production, abnormal branching and swelling of hyphal tips, and the accumulation of a dark lipofuscin ageing pigment [22–24]. Ageing in *P. anserina* is systemic, highly suppressive and infectious, and is driven by mitochondrial function and metabolism (for a review, see [25]). Major rearrangements of the mitochondrial genome—often extra-chromosomal amplifications—are associated with ageing in *P. anserina*; many of these are caused by regions inside the mitochondrial genome, known as the senDNAs [26–28]. In addition, extra-genomic elements such as the invertron-like mitochondrial pAL2-1 plasmid and homologues can interfere with lifespan, seemingly independent of other intrinsic and external lifespan-influencing conditions [21,29].

DR in the form of glucose restriction or other carbon sources such as fructose or acetate leads to extension of the vegetative lifespan in *P. anserina*, both in semi-synthetic and synthetic media [21,30]. This prolongation in lifespan of *P. anserina* can vary from two weeks up to 3 years [21,30–32], and while no reproduction takes place during this period, sexual and asexual reproduction are restored when normal nutritional conditions are available [31,32]. During carbon and nitrogen starvation the same genes in *P. anserina* are expressed as under heterokaryon/vegetative incompatibility reactions when fusion between incompatible genotypes leads to a programmed cell death response [15]. Fungal incompatibility reactions are characterized by a strong increase in the total cellular enzymatic proteolytic activity [14], but also in genes involved in the autophagic pathway [15,17]. Although a direct link to autophagy has not been established, given the similarities in the starvation and incompatible reactions it seems probable that autophagy could play a role.

The objectives of this study were to determine the effect of autophagy and age on the DR response in *P. anserina*. We used a well-defined synthetic medium with carbon and nitrogen restrictions to realize the DR regimes. We used a set of wild-type strains as well as a selection of laboratory strains

and four knock-out mutants hampered in different genes involved in different stages of the autophagy pathway (Δ PaAtg1, Δ PaAtg8, Δ PaPspA and Δ PaUth1). We report here that in *P. anserina*, limitation of either carbon or nitrogen source results in a lifespan-extending DR effect in juvenile strains. However, in (pre)senescent (halfway through life and older) wild-type strains, this lifespan extension proved lost, in contrast to, e.g. effects observed in *Drosophila melanogaster* where DR reduces the short-term risks of death at least until the age of four weeks [3]. Although juvenile autophagy-deficient strains generally show a normal lifespan extension upon DR, the time period in which DR can extend lifespan proved to be severely reduced in the different autophagy-deficient mutants.

2. Material and methods

(a) Strains, mutants and genes

For testing the effect of reduction in either carbon or nitrogen sources on lifespan, we used the standard laboratory strains *s* and *Cs* [18] and several wild-type isolates (*Wa32*, *Wa50*, *Wa70*, *Wa76* and *Wa77*; [20,21]).

For our studies on autophagy and mitophagy, we used *P. anserina* strains derived from the original strain *s/Cs*, mutated in genes that based on homologies and previous studies are involved in the autophagic degradation of mitochondria. The examined genes act at four different stages of the autophagy route.

- (i) PaUth1 putatively codes for a mitochondrial outer membrane protein that is part of the SUN superfamily. The homologous protein in *Saccharomyces cerevisiae* is UTH (YKR042W) which is (one of) the label(s) to mark mitochondria for mitophagy and is involved in the oxidative stress response [33,34]. A knock-out *P. anserina* strain blocked in the production of PaUTH1 (CAP67803) was made based on this sequence as described for other genes by El-Khoury *et al.* [35] in a strain lacking heterologous recombination thanks to the *Ku* mutation.
- (ii) PaAtg1 codes for a serine/threonine protein kinase that is involved in the cytoplasm-to-vacuole transport and for the formation of autophagosomes [17]. Its product ATG1 is the homologue of yeast ATG1 (YGL180W).
- (iii) PaAtg8 also has a similar-named homologue in yeast (YBL078C) and codes for a lipid-conjugated ubiquitin-like protein that connects to phosphatidylethanolamine, leading to the tethering and hemifusion of membranes of autophagosome and vacuole [16,36].
- (iv) PaPspA codes for a subtilisin-like serine protease and is an orthologue of the vacuolar protease B PRB1 of *S. cerevisiae* (YEL060C), necessary for degradation within the vacuole [16].

Δ PaAtg1, Δ PaAtg8 and Δ PaPspA strains have kindly been provided by Dr Pinan-Lucarré and Dr Clavé from the Centre de Génétique Moléculaire of the Université de Bordeaux 2, in France. The four genes together are thus expected to cover several key processes in (mitochondrial) autophagy.

(b) Culture conditions and lifespan analysis

Podospora anserina synthetic medium (PASM) [21] was used in these experiments with as standard carbon and nitrogen sources, 2% (w/v) D-glucose and 0.1% (w/v) urea. D-glucose concentrations were varied from 0.02, 0.1, 0.2, 1, 2 to 4% (w/v), respectively, with a urea concentration of 0.1%. For the tests with different nitrogen amounts, 2% D-glucose was used

in combination with 0.1, 0.01, 0.001 or 0.0001% (w/v) urea, respectively. The pH of all these media was set to 6.4. For germination of spores, PASM was supplemented with 0.06 M ammonium acetate; to induce reproduction and spore formation, PASM was supplemented with 0.5% (w/v) dried and ground horse dung. All incubations were done at 27°C in the dark, except for fertility tests that were done with a 12 hour L/12 D circadian rhythm.

Lifespans and growth rates were measured in days of growth with a continuous linear growth rate using 30 cm glass race tubes with 20 ml PASM medium. Tubes were inoculated using explants derived from 2- to 3-day-old cultures grown from spores on PASM with acetate. Growth was marked in 1- to 3-day intervals, and lifespans and linear growth rates were determined using at least five replicas per strain per condition. When race tubes were grown full, explants were cut from the mycelium at the distal ends of the tubes and transferred to new ones. For practical reasons, lifespan measurements were truncated after 90 days. Differences in lifespan and growth rates were analysed using a generalized linear model (GLM) in R. Subsequent pairwise interactions of the GLM models were done with the `glt`-function from the `multcomp` package. *P*-values reported from these pairwise interactions were adjusted for multiple comparison using the Tukey method.

(c) The effect of age on the response to carbon restriction

The different (wild-type and autophagy-deficient) strains were grown on Petri-dishes with standard PASM with 2% (w/v) D-glucose and 0.1% (w/v) urea. At different stages during life, explants were transferred from normal media to severe carbon-restricted conditions (PASM with 0.02% (w/v) D-glucose). Lifespan was measured in fivefold as described before. The experiments were truncated after 90 days which was set for the analyses as the maximum lifespan-extending effect.

(d) Microscopic analysis of the $\Delta PaUth1$ mitophagy mutant

To study the effect of the $\Delta PaUth1$ mutation on mitochondrial dynamics, we crossed the $\Delta PaUth1$ mutant with a transformant strain, in which green fluorescent protein (GFP) is targeted to the mitochondria thanks to the mitochondrial-targeting sequence of *Neurospora crassa* ATP9 [37], and selected recombinant single-mating type offspring. The mitochondrial morphology was then visualized using confocal laser scanning microscopy (LSM 510, Zeiss, Germany).

3. Results

(a) Lifespan-extending effects of nitrogen restriction

Previously it has been shown that glucose restriction leads to a significant increase in lifespan [21,30–32]. For a constant starting concentration of 0.1% urea (w/v), our results indicate that in fact the relationship between lifespan and glucose concentration on a log scale is an inverse logarithmic function (figure 1a). To establish the effects of reduced concentrations of nitrogen on the lifespan of *P. anserina*, strain *s* was grown at concentrations of urea ranging from the standard 0.1 to 0.0001%. Unlike the inverse logarithmic relation found in glucose, a parabolic relationship between lifespan and the logarithm of the urea concentration was found (figure 1b). We observed that lifespan significantly increased for concentrations lower than 0.1% urea (figure 1b; $\chi^2 = 14.154$, d.f. = 3,

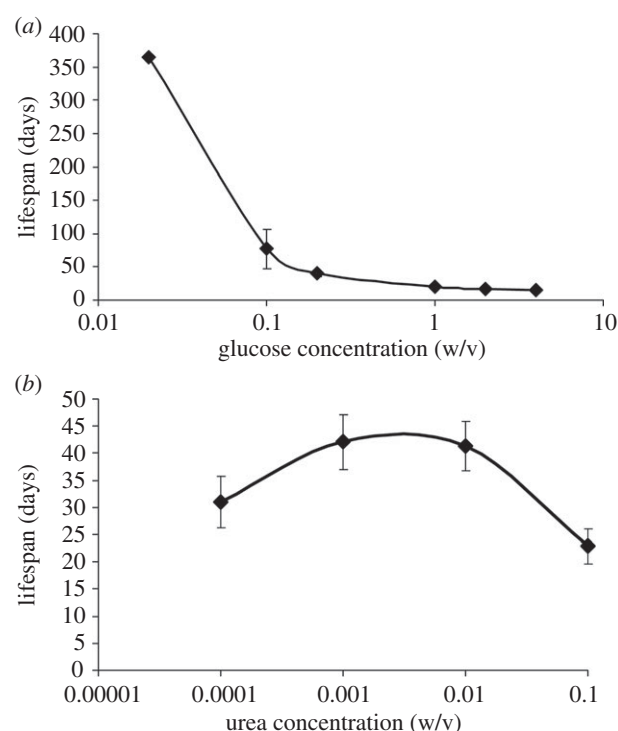


Figure 1. Effects of (a) D-glucose and (b) urea concentrations on the lifespan of *P. anserina* strain *s*. For the different D-glucose concentrations, the urea concentration was kept at 0.1% w/v; for the different urea concentrations the D-glucose level was kept at 2% w/v. The measurements of the five replicate lines growing on 0.02% glucose w/v and 0.1% urea w/v were truncated after 1 year of continuous growth, with all lines still alive.

$p = 0.003$). Lifespan of *P. anserina* peaked at both 0.01 and 0.001% urea, with a doubling in lifespan, whereas at 0.0001% lifespan only increased by 50% compared with the original 0.1% concentration (not significant after post hoc testing). Nitrogen restriction in *P. anserina* resulted also in several other phenotypic changes. Firstly, a significant 15% decrease in the growth rate was observed for the concentrations 0.01 and 0.001% urea compared with 0.1% urea (data not shown; $\chi^2 = 20.757$, d.f. = 3, $p = 0.001$). Secondly, mycelium density is decreased, while at the same time a strong increase in dark pigmentation is observed (figure 2). Thirdly, reduced fertility or even sterility was observed at the lower concentrations.

(b) Nutrient restriction in wild-types

Most previous work on DR in *P. anserina* has focused on a single strain, which has been maintained for many years in the laboratory. To establish whether these observations are not the result of adaptation to laboratory conditions, several wild-type strains were tested for their response to reduced concentrations of both glucose and urea (figure 3a,b, respectively). The lifespan of the wild-type strains increased in response to reduced glucose (figure 3a; $\chi^2 = 5269.7$, d.f. = 2, $p < 0.001$) and reduced concentrations of urea (figure 3b; $\chi^2 = 108.4$, d.f. = 2, $p < 0.001$) suggesting that the response to nutrient restriction universally applies to *P. anserina* strains. However, interestingly, the extent to which lifespan increased differed significantly between strains for glucose (figure 3a; $\chi^2 = 272.0$, d.f. = 4, $p < 0.001$) as well as urea restriction (figure 3b; $\chi^2 = 73.17$, d.f. = 3, $p < 0.001$). This is indicative of genetic variation for the DR

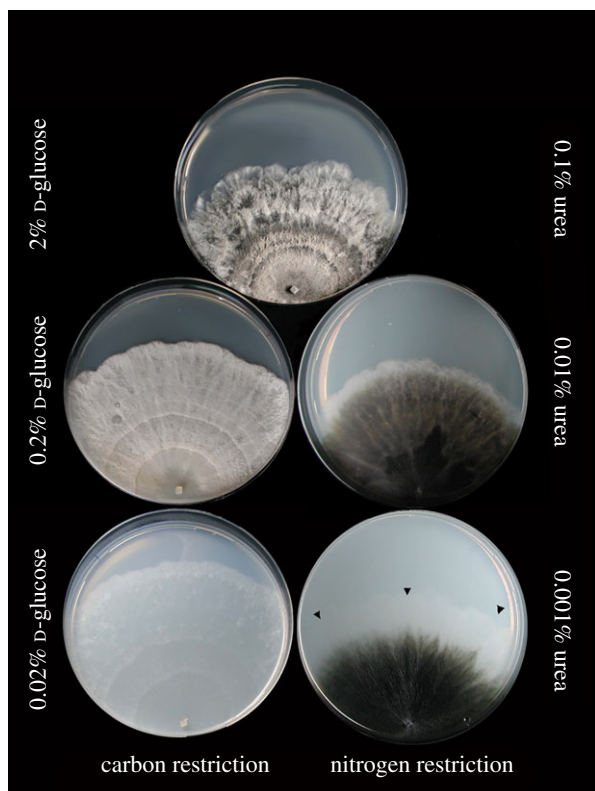


Figure 2. Phenotype of *P. anserina* strains on carbon (D-glucose; left) and nitrogen (urea; right) restriction, respectively. Growth front in the 0.001% (w/v) urea conditions marked with triangles. (Online version in colour.)

responses. Indeed, we found a strong interaction between the lifespan of the strains and the glucose (figure 3a; $\chi^2 = 532.7$, d.f. = 8, $p < 0.001$) and urea (figure 3b; $\chi^2 = 33.56$, d.f. = 5, $p < 0.001$) environment.

(c) The role of autophagy in lifespan extension upon carbon and nitrogen restriction

To test whether the process of autophagy is important for the lifespan extension by carbon and/or nitrogen restriction, we tested several existing autophagy mutants (table 1). In addition, we created a knock-out mutant for the homologue of *Uth1* from yeast involved in mitophagy and the oxidative stress response [34]. Phenotypically, the $\Delta PaUth1$ is comparable to the wild-type when grown in a Petri dish, and even the ageing pigment lipofuscin is formed as normal prior to cell death; only the growth rate is lower (figure 4a). The putative mitophagy-deficient strain is fertile both as female and as male, though development of the fruiting bodies and spore formation are retarded in comparison with the parental strain. By contrast, the autophagy-deficient strains $\Delta PaAtg1$, $\Delta PaAtg8$ and $\Delta PaPspA$ as reported show a reduction in pigmentation and in hyphal density. These strains have lost their female fertility, but do produce spermatia enabling the backcrossing to the parental strain and selection of monokaryotic mutant lines for experiments [16,17].

Normal wild-type hyphae have a smooth shape in which the mitochondria form networks that undergo continuous fusion and fission (figure 4b). However, the $\Delta PaUth1$ mutant shows irregular hyphal morphology (figure 4c) with often bulbous deformations at the hyphal tips and branching at sub-apical tip locations initiating further growth of the mycelial front. Occasionally, bursting hyphal tips were observed,

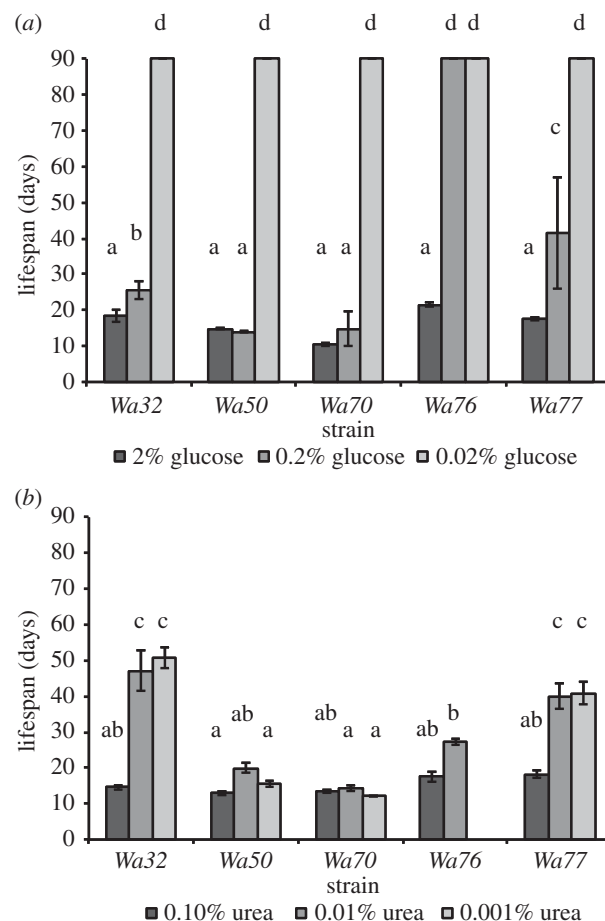


Figure 3. The lifespan of wild-type strains under glucose (a) and urea (b) restriction, respectively. The often small standard deviations are given for all columns, but when all strains lived longer than the cut-off point of 90 days, these are not visible in the graphs. The same letter above columns (a,b,c,d) indicates that these groups do not statistically differ in phenotype, while different letters indicate that these groups do.

even of young cultures (data not shown). This phenomenon could be an indication of early stage death, but this will require additional experimentation. The mitochondria of a $\Delta PaUth1$ lineage crossed with strains expressing GFP in the mitochondria give a diffuse GFP signal throughout the cell without clear mitochondrial networks, but many punctuate units, potentially due to an imbalance between mitochondrial fission and fusion events, and more signal seems to be present within the cell, potentially due to the accumulation of undigested damaged mitochondria (figure 4c).

Explants of the different knock-out mutants derived from 2- to 3-day-old cultures (juvenile samples) were grown on media with normal and either reduced glucose or urea concentrations (figure 5a,b, respectively). The lifespan of mutants grown on glucose-restricted media significantly increased with decreasing glucose concentration (figure 5a; $\chi^2 = 2401.48$, d.f. = 2, $p < 0.001$) showing a lifespan that was similar to their respective wild-type. The exception was $\Delta PaUth1$, which is the only strain in which severe carbon restriction (0.02% (w/v) glucose) does not result in an absolute DR lifespan of well over 90 days, but only of an average of 50 days (post hoc contrast $p < 0.001$).

The effect of nitrogen restriction strongly depended on the gene that was mutated (figure 5b; $\chi^2 = 327.42$, d.f. = 4, $p < 0.001$) and on the concentration of urea (figure 5b; $\chi^2 = 201.52$, d.f. = 2, $p < 0.001$). Interestingly, mutants $\Delta PaAtg1$

Table 1. List of the strains used in this study and their relevant characteristics. Symbol ‘+’ is normal compared with the wild-type.

strain/mutation	parental strain	lifespan versus parent	growth rate versus parent	fertility ♀	fertility ♂	pigmentation	hyphal density	hyphal growth	reference
<i>Wa32</i>	wild-type isolate			+	+	+	+	+	[20]
<i>Wa50</i>	wild-type isolate			+	+	+	+	+	[20]
<i>Wa70</i>	wild-type isolate			+	+	+	+	+	[20]
<i>Wa76</i>	wild-type isolate			+	+	+	+	+	[20]
<i>Wa77</i>	wild-type isolate			+	+	+	+	+	[20]
<i>Cs</i>	laboratory strain			+	+	+	+	+	[16]
$\Delta PaAtg1$	<i>Cs</i>		15% reduced	infertile	+	reduced	+	+	[17]
$\Delta PaAtg8$ (<i>idi-7</i>)	<i>Cs</i>	15% reduced		infertile	+	reduced	+	+	[16]
$\Delta PaPspA$ (<i>idi-6</i>)	<i>Cs</i>			infertile	+	reduced	+	+	[16]
<i>s</i>	laboratory strain			+	+	+	+	+	[18]
$\Delta PaUth1$	<i>s</i>	50% reduced	50% reduced	± delayed fruiting body/ spore formation	+	+	+	abnormal	this paper

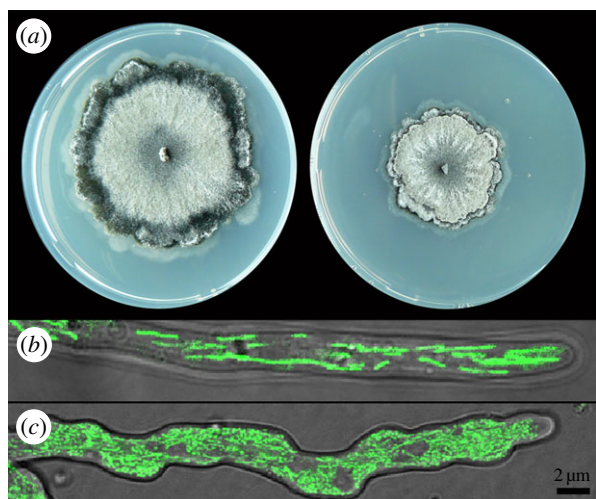


Figure 4. (a) Phenotype of a 5-day-old culture of wild-type collection strain *s* (left) and of the putative mitophagy-deficient $\Delta PaUth1$ mutant (right) on PASM with 2% glucose and 0.1% urea. (b) Confocal laser microscopy picture of a hyphal tip of strain *s* with filamentous mitochondria expressing GFP on PASM with 2% glucose and 0.1% urea. (c) Part of an irregularly formed hyphal tip of $\Delta PaUth1$ mutant with diffuse mitochondria expressing GFP. (Online version in colour.)

and $\Delta PaAtg8$ do not show the same lifespan increase as their ancestor. By contrast, the mutants $\Delta PaUth1$ and $\Delta PaPspA$ behave similar to their respective ancestors with the longest lifespan at intermediate nitrogen restriction (figure 5b). However, the effect of nitrogen restriction in $\Delta PaUth1$ was much more pronounced than in wild-type with a significantly longer lifespan at intermediate nitrogen restriction (0.01% urea) and a significantly shorter one at the standard growth conditions (figure 5b).

(d) The effect of age on the magnitude of the dietary restriction response

Carbon restriction can increase the lifespan of both wild-type and autophagy/mitophagy-deficient mutants in juvenile samples. But how effective is this carbon restriction response, when encountering lean periods later in life? To answer this question both culture collection strain *s* and the four autophagy-deficient mutants $\Delta PaAtg1$, $\Delta PaAtg8$, $\Delta PaPspA$ and $\Delta PaUth1$ were grown to different relative ages on 2% (w/v) glucose and subsequently transferred to severe carbon-restricted conditions (0.02% (w/v) glucose).

The relative response of wild-type strain *s* to a switch to severe carbon-restricted conditions was largest: up to a relative age of 30% of the lifespan all lines could be rescued by carbon restriction and transplants lived at least 90 days. Up to halfway through its lifespan, 50% of the transplants showed the typical DR response and after that the effect of DR diminished (fitted survival curves are given in figure 6; see the electronic supplementary material, figure S1 for the individual replicate data points). However, all three autophagy-deficient mutants, $\Delta PaAtg1$, $\Delta PaAtg8$ and $\Delta PaPspA$, lost their ability to extend lifespan in response to DR at a significantly earlier relative age compared with the wild-type. Mutant $\Delta PaUth1$ shows this effect even stronger as already young strains (relative age 0) have a reduced lifespan extension on severe carbon-restricted medium (see above, figure 5a).

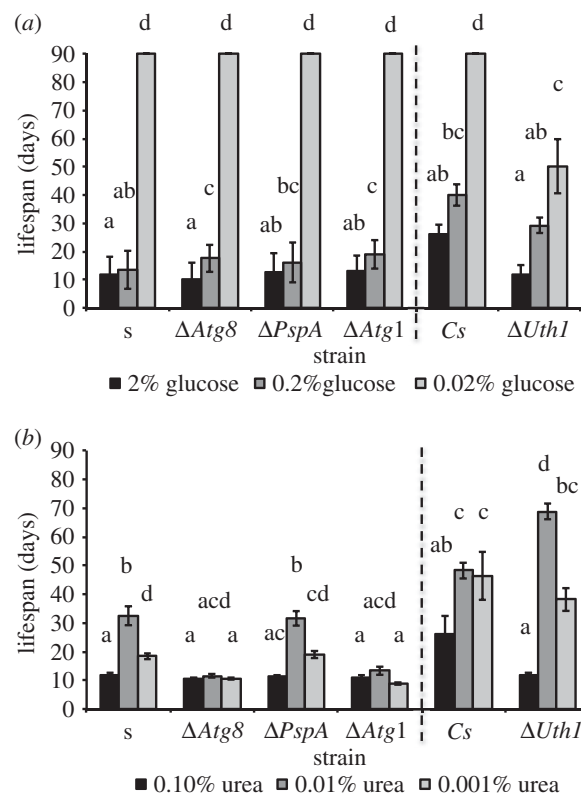


Figure 5. The lifespan of laboratory strains and derived autophagy-deficient mutants $\Delta PaAtg1$, $\Delta PaAtg8$, $\Delta PaPspA$ and $\Delta PaUth1$ under glucose (a) and urea (b) restriction, respectively. All autophagy mutants are given to the right of the laboratory strain they originated from. The error bars indicate the standard deviation per experimental condition. Under experimental conditions in which all samples lived beyond 90 days there are no error bars, because the experiment was terminated at this point. The same letter above columns indicates that these groups do not statistically differ in phenotype.

4. Discussion

Extension of lifespan by DR appears to be evolutionarily conserved as it is found in a large range of organisms, from yeasts to potentially primates and humans [1–4,6]. However, the mechanisms through which this occurs in the various organisms are largely unknown. Starvation and other stress conditions induce autophagy, a large-scale lysosomal degradation of (damaged) cytoplasmic content including organelles such as mitochondria. Therefore, a more efficient recycling of damaged mitochondria could be a mechanism to extend lifespan during DR. Here, we show that in the filamentous fungus *P. anserina*, for which mitochondria have been unambiguously implicated in ageing, DR (both C- and N-restriction) early in life has a profound effect on lifespan, for which autophagy is not required. By contrast, however, for later-onset DR autophagy appears to be essential.

(a) Dietary restriction

Restriction in the amount of available carbon or nitrogen can both lead to lifespan extension—or reduced death rates—in the filamentous fungus *P. anserina*. There is a maximum increase in lifespan at an optimum amount of urea in the medium that ranges between 0.001 and 0.01% (w/v); further reduction of nitrogen source decreases lifespan by starvation. However, reducing the carbon source results in maximum lifespan at the lowest measured concentration; strains growing on 0.02% (w/v) glucose have been shown to grow continuously

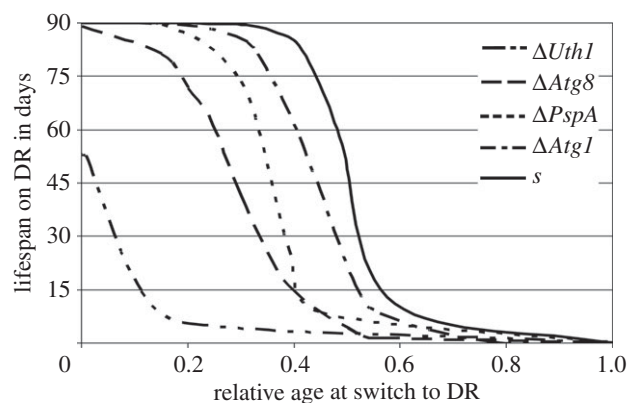


Figure 6. Fitted curves of the DR response at relative ages at onset of DR (PASM with 0.02% w/v glucose) for the autophagy-deficient mutants ($\Delta PaUth1$, $\Delta PaAtg8$, $\Delta PaPspA$ and $\Delta PaAtg1$), and the wild-type parental strain *s* (in order of appearance from left to right; growth experiments were stopped after 90 days, *p*-values fitted curves of less than 0.001). Refer to the electronic supplementary material, figure S1 for the individual replicate data points.

for over 3.5 years, more than 50 times the life expectancy of two to three weeks under standard nutrient-rich conditions [31,32]. The typical mitochondrial genome rearrangements correlated with senescence in *P. anserina* on normal media are also observed in carbon- and nitrogen-restricted conditions. However, for the severe carbon-restricted conditions where in the long-lived strains the mitochondrial stability is enhanced, the prominent rearrangements due to senDNA α are absent and only occasional rearrangements due to other known senDNA regions are observed (for *Wa32*, [31]; for mutants, AD van Diepeningen 2009, personal observation).

DR extends healthy lifespan in diverse organisms and reduces fecundity under those conditions. In *P. anserina*, carbon-restricted conditions may reduce reproduction up until effective sterility, but once conditions are favourable again the fertility is often restored and thus reproduction happens well beyond the period in which *Podospora* reproduces under normal nutritional conditions [31,32]. Grandison *et al.* [38] showed in *D. melanogaster* that specific amino acids may restore fertility even under DR conditions, indicating that a somatic lifespan extension through DR may well cause malnutrition with respect to fertility, and that lifespan and reproduction thus do not rely fully on the same specific resources. DR in *P. anserina* does not only result in reduction in fertility, but also in less dense mycelium with increased (carbon restriction) or decreased (nitrogen restriction) growth rates. Pigmentation under nitrogen restriction is normal or even increased, whereas under carbon restriction it is absent, indicating different responses within the cell to reduction in different food components. These differences are likely related to the changes in mycelial growth and growth rates ('thinning' and 'condensing' effects).

(b) Autophagy

Autophagy is one of the mechanisms proposed to be involved in the degradation and recycling of (damaged) cell components and thus may have a role in the DR responses. Autophagy was found to be involved in, but neither necessary nor sufficient to cause lifespan extension in *C. elegans* [13]. Under normal culture conditions, several *P. anserina* autophagy mutants have no or little adverse effects on lifespan or growth rate although they are infertile [16,17]. However, the $\Delta PaUth1$ mutant shows

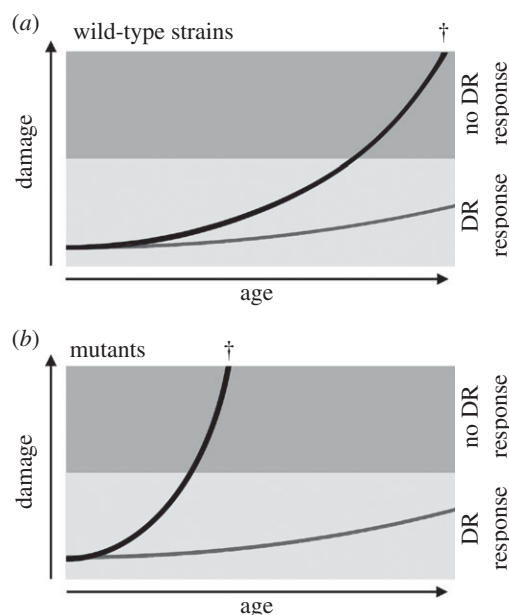


Figure 7. Models of damage accumulation over time for wild-type (a) and autophagy mutant (b) strains growing under nutrient-rich (black line) and calorie-restricted (grey line) conditions. The shaded areas depict the damage levels in which a lifespan extending DR effect is (light shade) or is no longer (darker shade) possible. Normal accumulation of damage under nutrient-rich conditions will lead to a threshold level of damage after which cell death (cross symbol) becomes inevitable. Below such damage levels DR may postpone senescence phenomena and cell death for a long time. Mutants impaired in their autophagy pathway will show faster ageing processes under nutrient-rich conditions, resulting in a lack of response to DR conditions earlier in the life time of the mutants.

severe effects on cell and mitochondrion morphology, growth rate and lifespan, while this mutant is still able to reproduce (though sexual spore formation is delayed). In yeast, it was found that UTH1 acts on various cellular pathways beside mitophagy such as the response to oxidative stress [39], mitochondrial biogenesis [40] and apoptosis [41]. The phenotypes of the mutant described in our paper may be due to pleiotropic effects of these or other processes. Moreover, it is not the only gene involved in mitophagy [42].

The mutants deficient at different positions in the main-stream autophagy pathway all show a lifespan-extending effect of carbon restriction, but two— $\Delta PaAtg1$ and $\Delta PaAtg8$ —fail to do so under nitrogen-restricted conditions, and thus seem to mediate the effect of nitrogen restriction on lifespan extension. The $\Delta PaUth1$ mutant gave a smaller response to carbon-restricted conditions than its parental strain, but a larger response on nitrogen restriction suggesting that under these conditions UTH1 may actually be involved in processes with an adverse effect on lifespan. Our results indicate that the tested set of different genes from the complex autophagy pathway can be involved in different ways in the response to carbon and/or to nitrogen restriction in *P. anserina*.

The *P. anserina* mutant $\Delta PaUth1$ has other characteristics than the 'youthful' *Uth1* mitophagy mutant of *S. cerevisiae*: *Uth1* has been identified in yeast based on its enhanced stress resistance and its longer lifespan [33,34], but notably our $\Delta PaUth1$ strain has a shorter lifespan. Microscopically, *Uth1*-deficient yeasts show no (clear) abnormalities in either cell or mitochondrial morphology [34]. However, the $\Delta PaUth1$ *P. anserina* strain has a very irregular growth and shows many malformed hyphal tips, while its mitochondrial

networks are severely reduced, and many punctuate mitochondria are present. These punctuate mitochondria may be the result of a lack of autophagic degradation as they may be the units normally tagged for the autophagy pathway and are reminiscent of aged cells [43].

Clearly, autophagy is not the only mechanism involved in DR responses, nor is the involvement the same when different resources are limited. Proteolytic enzymes might well provide complementary and partly redundant mechanisms. In *P. anserina*, autophagy has been linked not only to DR, but also to reactions that resemble programmed cell death when two different strains undergo hyphal fusions (a.k.a. heterokaryon or vegetative incompatibility reactions; [15–17]). Also, proteolytic activity has been shown to increase during such incompatibility reactions [14]. In turn, programmed cell death in yeast has been linked to regulating mitochondrial fission proteins [44], which lead us back to the observed punctuated mitochondria in our $\Delta PaUth1$ strain. Thus, $\Delta PaUth1$ may interfere with the mitochondrial fusion and fission processes.

(c) Timing of dietary restriction

DR leads to lifespan extension through a still to be unravelled mechanism and generally has the consequence of severe but often reversible reductions in fecundity. But when should one start with DR to have an optimal lifespan? In *D. melanogaster*, DR has been shown to be effective already after 48 h and reduced the short-time risk of death [3]. Note though, that in this study the effect was measured until the age of 28 days, which is well before the mortality rate starts to increase under the standard culture conditions for *Drosophila*. Here, for *P. anserina* however, we investigated the DR response over the whole lifespan, and show that only when a wild-type strain is switched to DR in the first half of its life when it is also still fully fertile, will it acquire the near ‘immortal’ lifespan typical for growth under DR. Later in life, a switch to DR results only in a slight increase in life expectancy of a mere few days.

Our data thus show that the DR response is age-dependent in *P. anserina*. It is not clear why *Podospora* is only showing such a DR response early in life. Maybe its growth on herbivore dung, a short-lived substrate, has dictated strong selection for early reproduction and an early potential DR response. Under such a scenario, selection for an effective late-life DR response would have been weak as at that point in life the fungus has normally already reproduced. Such weak selection would allow accumulation of mutations and/or damage to the cellular machinery essential for the response with age, both within the lifespan of the fungus as well as over evolutionary time. Combining this reasoning with the age-dependent responses of the wild-

type and mutant strains results in the model depicted in figure 7.

In this model, in a wild-type strain grown under dietary-rich conditions damage will be accumulating with age, finally resulting in cell death (figure 7a, black line). Switching to dietary-restricted conditions will result in lifespan extension up to a certain point in life, a threshold, after which there will be too much accumulated damage and switching will only result in a very limited lifespan-extending response. The same strain grown under DR conditions will not accumulate as much damage and will reach the critical accumulated damage for DR response and cell death at a much later time point, if at all (figure 7a, grey line).

The fact that autophagy-deficient strains of *P. anserina* show a reduced lifespan-extending effect of a switch to DR suggests that during early onset DR in the fungus *P. anserina* autophagy forestalls ageing by preventing the critical accumulation of highly suppressive damaged mitochondria leading to a senescent state that is culminating in cell death (figure 7b, grey line). A switch to DR late in life apparently cannot revert this ageing process by autophagic removal of accumulated damage in *P. anserina*. In the model, an autophagy-deficient mutant will accumulate damaged cell components faster and thus also get to the threshold point earlier after which DR will no longer be leading to a strong lifespan-extending response (figure 7b, black line). Critically, grown under DR conditions from the start there will be little difference between mutant and wild-type strains (figure 7a,b).

Our results on the involvement of autophagy genes in the reproduction and lifespan response to DR thus indicate that there is both a direct, for nitrogen restriction, and indirect, for the late-onset response, connection. We hypothesize that the DR response depends on an intact (mitochondrial) cellular machinery as the DR effects are limited to the first half of the lifespan and are truncated in all the autophagy mutants. As a consequence, our results are the first to indicate that the normal ageing process and the response to DR conditions are linked, at least in *P. anserina*.

Acknowledgements. We thank Dr Bérangère Pinan-Lucarre and Dr Corinne Clavé from the Centre de Génétique Moléculaire of the Université de Bordeaux 2, in France for kindly providing the three *P. anserina* autophagy-deficient strains.

Funding statement. This research was part of the MiMage project on the role of mitochondria in conserved mechanisms of ageing and supported by funding from the Community's Sixth Framework Programme (LSHM-CT-2004-512020). This work was further supported by the European Union's FP7 Programme (IDEAL FP7/2007-2011/259679, <http://www.ideal-ageing.eu/>, to B.J.Z.).

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