EFFECTS OF CALORIC RESTRICTION IN *PODOSPORA ANSERINA*

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NATURAL VARIATION IN THE RESPONSE OF LIFESPAN, PIGMENTATION AND PERITHECIA FORMATION

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ABSTRACT:

Caloric restriction (CR), defined as undernutrition without malnutrition, increases lifespan in a variety of species, including mammals, insects and fungi. *Podospora anserina* is one of the few ageing filamentous fungi and serves as a fungal model of senescence. In *P. anserina*, CR results in faster growth, reduced formation of pigment and perithecia and an increase in lifespan up to more than 10-fold. The response to CR appears to be dose-dependent and differs per fungal strain. In this study, the variation in response to CR was investigated in wild *P. anserina* strains. Strains were grown in tubes containing synthetic media, to test the effects of seven different D-glucose concentrations ranging from 0.02 to 2 % m/v.

Strains varied in the glucose concentration at which the change in lifespan in response to CR started to occur as well as in the acuteness of this response. In addition, for some of the strains a stochastic effect was observed near this transition point. Longevity is known to typically occur together with lack of pigment and perithecia formation. However, after stratification for glucose concentration in the medium, extended lifespan was significantly associated with pigment but not with perithecia formation. *Podospora anserina* could thus be used to distinguish between expression of specific genes involved in senescence and pigment or perithecia formation.

INTRODUCTION:

Caloric restriction (CR) is defined as undernutrition without malnutrition. The term dietary restriction, or DR, is sometimes used instead [1] but this term can also refer to a dietary regimen in which one or more food products or product groups are avoided [2]. CR has been shown to increase lifespan in a variety of species, including rats [3], mice [4], dogs [3], fruit flies [5] and fungi [6]. No consensus has been reached yet as to whether the effects in animals are due to the restriction of caloric intake [4], the restriction of proteins [7] or even specific amino acids [8,9]. Nevertheless, it is the only regimen known to increase not only the average lifespan but also maximum lifespan of a species [4]. CR seems to retard ageing – as it is associated with later onset of age-related diseases – and at the same time decrease fecundity [4].

Under circumstances of food abundance, *Podospora anserina* has a life cycle of approximately two weeks, during which it forms perithecia (fruiting bodies) and black pigment. The fungus shows the following response to caloric restriction: Lack of perithecia formation, lack of pigmentation, increased growth speed and an extended lifespan [10]. The combination of longer lifespan and decreased fertility under CR conditions is as also seen in various animals including mammals [4] and insects [11]. However, some research in fruit flies indicates that the link between these two effects may not be inevitable [12].

The effects of CR on *Podospora anserina* are of particular interest for various reasons. It is a fungus with a short lifespan, as opposed to many fungi that do not senescence at all (and can be regarded as

immortal). This raises the question whether CR could bring *P. anserina* into an immortal state, an effect that can hardly be expected in animal species. Secondly, as opposed to mammals and insects, *P. anserina* can grow on relatively simple growth medium with glucose as a sole energy source, ruling out the possibility that the effects are caused by protein restriction or the ratio between different amino acids. The fact that it can grow on a simple medium also adds to the status of *P. anserina* as an organism that is easy to work with; in addition, spores can be kept in the freezer and the fungus itself requires no husbandry during its life in the incubator. Fourthly, *P. anserina* strains naturally show a lot of variation in at what concentration they respond to CR [13,14,15]. The fact that the variation occurs naturally suggests that the response to CR may be adaptive. Lastly, the response of *P. anserina* to CR can be drastic – up to 10-fold increases in lifespan have been found [6] – compared to the milder responses seen in mammals [3] and fruit flies [8].

Senescence research using *P. anserina* has often focussed on laboratory mutant strains [16,17,18]. Examples of research using wildtype strains include the experiments of Van Diepeningen et al. [13,15] and Rusli [14]. However, Rusli focussed on few fungal strains [14] and Van Diepeningen et al. used only three different glucose concentrations [13,15]. As far as we know, our study is the first to investigate the response to caloric restriction in this over 60 strains very precisely. *Podospora anserina* is known to show inter-strain variation in its response to CR. Van Diepeningen et al. [13,15] have categorized their strains into four categories depending on their response: (1) Strains that show no response to low CR but do to severe CR; (2) Strains that show an average response to low CR and a large response to severe CR; (3) Strains that show a strong effect to low CR; (4) Strains that show no significant response to CR. They tested the strains in two-fold on three different glucose concentrations: 2, 0.2 and 0.02 % (m/v). In their experiment no strains showed immortality on 0.2 % glucose, suggesting that the transition point to the CR phenotype is lower than 0.2 % glucose for all strains tested. However, their data do show a moderate CR effect at 0.2 % glucose, and other data by Rusli [14] show that sensitive and moderately sensitive strains can survive on 1 % glucose for 42 days, which is longer than their life span on control food (2 % glucose). This is suggestive of a dose-response effect of caloric restriction within a certain concentration range.

General information is available on the response of *P. anserina* strains to CR, but data by Rusli [14] suggest that the strains differ in their response in ways not visible when tested using only three different glucose concentrations. In this study we investigated for 63 strains at what glucose concentration they started to exhibit an in increase in lifespan, as well as in what concentration range this increase was established. The main outcome was survival, here defined as the ability to grow for at least 30 centimetres without dying**.** In addition, other phenotypic aspects of the response to CR were recorded, namely pigmentation and perithecia formation. *Podospora anserina* is known to live longer and produces less/no pigment or perithecia if the energy intake is restricted. To find out if these phenotypic aspects were correlated one-on-one, it was investigated to what extent they were correlated to survival.

MATERIAL AND METHODS

Strains and culturing conditions

Fungal samples have been collected between 1991 and 2001 in Wageningen and Arnhem, The Netherlands [13]. New spores formed by the samples have been stored in the freezer at -80 ^{o}C. *Podospora anserina* strains that contained the pAL2-1 senescence plasmid were excluded since this plasmid interferes with the CR effect [10], leaving 63 strains available for testing (See Appendix A for a list of included strains). Spores were plated on *P. anserina* synthetic medium with glucose (2.0 % mass/volume) and acetate (3.9 g/l). Mycelia from these plates were used to inoculate 30 cm long

tubes containing 20 ml PASM with glucose concentrations of 0.02, 0.05, 0.10, 0.20, 0.50, 1.0 and 2.0 grams per 100 millilitre. Each strain was tested in three-fold on all seven glucose concentrations. Tubes were kept in a 28 ^{o}C stove in the dark. Growth was monitored for three months by marking the tubes three times weekly. For composition of the media see Appendix C.

Phenotyping

Lifespan: In the three months the replicas were monitored, they either reached the end of the tube or died. Replicas were said to be 'survivors' if they reached the end of the 30 cm tube, and 'dead' if they had ceased to grow before reaching the end of the tube.

Pigmentation: There were two categories of pigmentation. Yes: Pigmentation was visible to the naked eye; No: No visible pigmentation.

Perithecia formation: There were two categories of perithecia formation. Yes: Perithecia were visible to the naked eye; No: No visible perithecia.

Closer investigation of a selected subset of the strains

A subset of strains (n=10) with different responses were tested in five-fold on a more narrow range of glucose concentrations. The lowest concentration was chosen as one step lower than the lowest concentration at which all replicas in the first experiment had grown 30 cm; the highest concentration as one step higher than the highest concentration at which all replicas had died. Four to five concentrations in between these two extremes were tested. The selected strains and corresponding glucose concentrations can be found in Appendix D.

Classification of strains

The strains were characterised based on their response to CR in the first experiment, using 7 different glucose concentration and 3 replicas per medium. The response was characterised both in terms of acuteness and the strain's sensitivity to CR. Acuteness is defined here as the number of steps along the glucose concentration axis – each step being a 2-fold or 2.5-fold decrease in concentration – needed for this strain to make the difference between no response (i.e. all three replicas dying prematurely) and full response (i.e. all three replicas surviving). Acuteness was scored as 1, 2, 3, >3 or "Not classified", the latter meaning the strain showed no full response at the lowest glucose concentration of 0.02 %. Sensitivity is expressed as the threshold value, that is, the highest tested glucose concentration at which the strain showed at least a partial response to CR (i.e. at least one of the replicas became a survivor). Sensitivity was thus scores as 2.0, 1.0, 0.50, 0.20, 0.10, 0.05, 0.02 or "Not classified", the latter being used if the strain showed no response at any of the tested glucose concentrations.

Statistical analysis

To assess the association between different phenotypic aspects of the CR response, a chi-square tests was performed. Tests were done separately for the association between survival and absence of pigment, and for survival and absence of perithecia formation. Chi-square values were calculated for the entire dataset as well as stratified per glucose concentration. Expected values were the numbers of replicas expected to show each combination of phenotypic aspects if the probabilities for these aspects would be independent. A significance level of 0.05 was used, which means the critical chi-square value was 3.84.

RESULTS

VARIATION IN RESPONSE BETWEEN STRAINS

The strains showed variation in their response to CR both in the sensitivity and the acuteness of the response. The 63 fungal strains were classified according to these two parameters, as shown in Table 1. It was not possible to determine the acuteness for all strains, since not all strains showed a full response at the lowest glucose concentration. Three strains showed no response at any of the seven concentrations tested, meaning both the sensitivity and the acuteness were not classified. Six strains did show partial and/or full responses at some concentrations but no full response at the lowest concentration, meaning the acuteness was still not classified.

Almost one third of the strains (18 out of 63) showed the lowest possible acuteness: One 2.0-fold or 2.5-fold decrease in glucose concentration made the difference between all replicas dying and all replicas surviving. Another third (23 out of 63) showed a moderate acuteness needing two concentration steps. Only three strains were classified as needing more than three concentration steps to achieve this difference. This may be an underestimation since some of the nine strains with not-classified acuteness might have been added to the >3 acuteness group if even lower concentrations had also been tested.

The majority of the strains start to exhibit a (partial or full) CR response at a threshold concentration of 0.20 % (16 strains), 0.10 % (22 strains) or 0.05 % (16 strains). Four strains showed the lowest sensitivity that could be observed in this experiment: A threshold concentration of 0.02 %. One strain showed a partial response already at 1.0 % glucose.

Figures 1 gives a graphic representation of the sensitivity to CR. Figure 2 shows the distribution of the fungal strains across different degrees of acuteness of the CR response. Both figures are based on table 1 and together they depict the same data already given in this table.

TABLE 1: Acuteness of CR responses and sensitivity to CR, characterisation of 63 *Podospora anserina* strains. Threshold glucose concentration: Highest glucose concentration with at least partial CR response occurred. Acuteness: Number of steps needed to make the difference between no response and full response. Solid grey cells indicate impossible acuteness/sensitivity combinations. Totals per row and column are shown in grey.

Threshold glucose concentration (Sensitivity)	Acuteness					
	1	**2**	**3**	**>3**	**Not classified**	**Total**
2.0 %	0	0	0	0	0	0
1.0 %	0	0	0	1	0	1
0.50 %	0	0	0	1	0	1
0.20 %	2	8	5	1	0	16
0.10 %	6	10	5		1	22
0.05 %	8	5			3	16
0.02 %	2				2	4
Not classified					3	3
Total	18	23	10	3	9	63

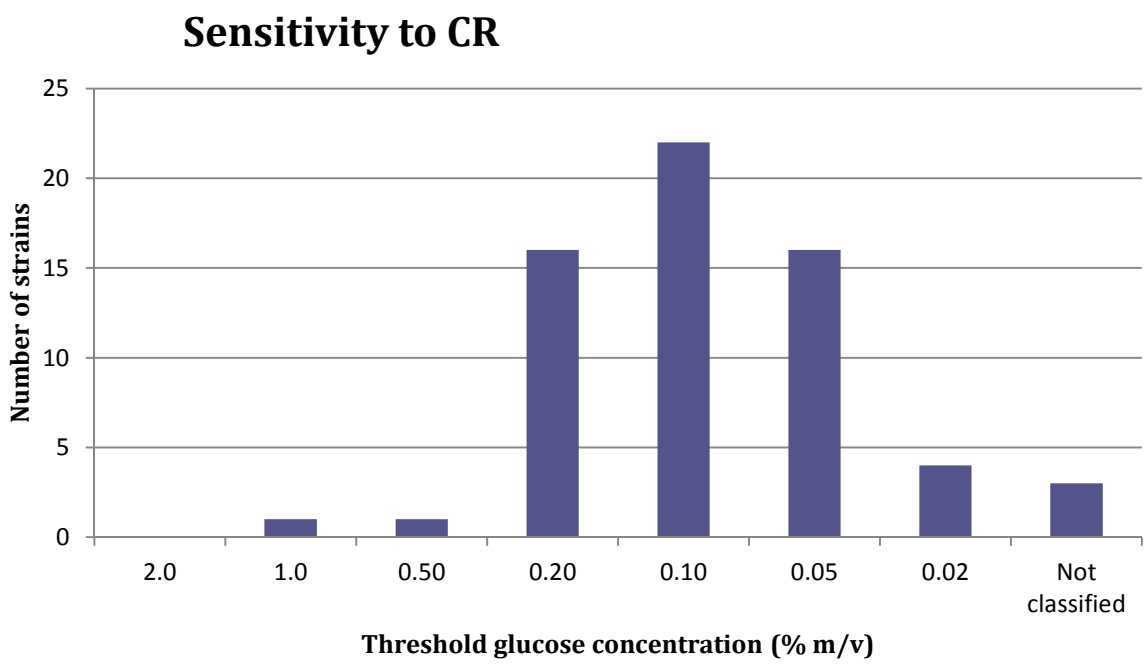

FIGURE 1: Sensitivity of 63 *Podospora anserina* strains to caloric restriction. Number of strains per threshold glucose concentration: The highest concentration at which at least one out of three replicas survived.

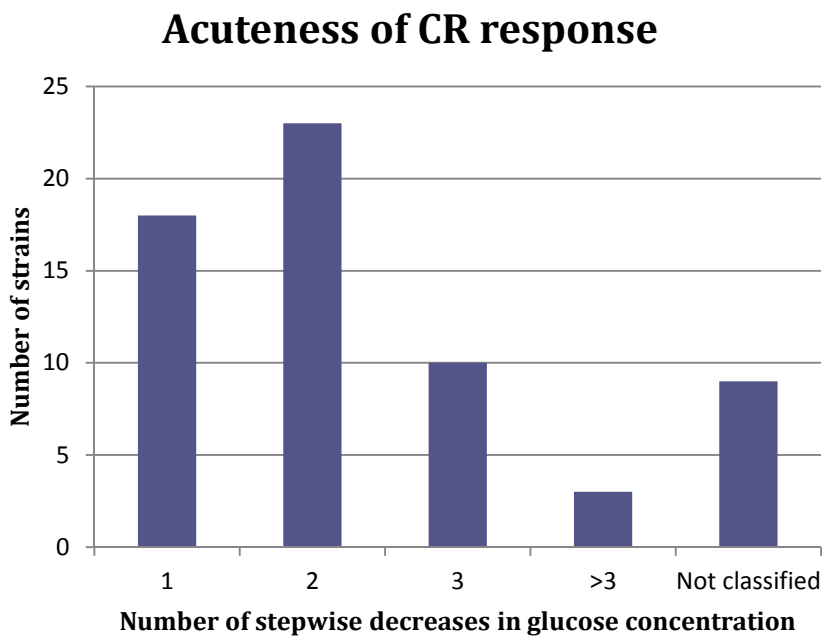

FIGURE 2: Acuteness of the response of 63 *Podospora anserina* strains to caloric restriction. Number of strains per measure of acuteness: The number of stepwise decreases (2.0-fold or 2.5-fold) in glucose concentration required to make the difference between no response (i.e. all three replicas died) and full response (i.e. all three replicas survived).

Although all strains that were classified the same based on this first experiment showed the same response pattern initially, still differences were found when a subset of the strains was re-tested more extensively. Ten strains with different responses were tested in five- to ten-fold per glucose concentration on a strain-specific range of concentrations, which was based on their preliminary results (see Appendix D for details).

Table 2 shows the fractions of survivors, out of five to ten replicas, on various glucose concentrations. Two of the selected strains showed the least acute response measured in the initial experiment (Wa3 and Wa56), five strains responded as acute as possible but differed in their sensitivities to CR (Wa16 and Wa48; Wa13 and Wa14; Wa7), and three strains showed no response or a very late response so the acuteness could not be determined (Wa25, Wa29 and Wa67).

Interestingly, strain Wa29, which did not respond to CR in the initial experiment (all replicas at all concentrations died) had one surviving replica in the second experiment. One of the five replicas on 0.02 % glucose reached the end of the tube. This suggests Wa29 partially responds to severe CR (0.02 % glucose).

TABLE 2: Fractions surviving replicas on a strain-specific glucose concentration range, for 10 *Podospora anserina* strains. Sensitivity and acuteness in the first experiment are given; A question mark indicates sensitivity or acuteness could not be classified in the first experiment. The number of replicas tested per strain per concentration is 5 unless indicated otherwise between brackets. Solid grey cells indicate a strain was not (re-)tested on that glucose concentration.

Strain	**Wa3**	**Wa56**	**Wa16**	**Wa48**	**Wa13**	**Wa14**	**Wa7**	**Wa25**	**Wa29**	**Wa67**
Sensitivity	0.50	0.50	0.20	0.20	0.10	0.10	0.05	0.05	?	0.02
Acuteness	4	4	1	1	1	1	1	?	?	?
[glucose]										
2.0	0.60	0.00								
1.0	0.00	0.00	0.00	0.00						
0.50	0.40	0.00	0.00	0.00	0.00	0.00				
0.40			0.00	0.00						
0.30			0.20	0.40						
0.20	0.40	1.0	0.40	0.40	0.00	0.00	0.00 (10)			
0.17					0.20	0.40				
0.13					0.60	1.0				
0.10	1.0	1.0	1.0	1.0	0.80	1.0	0.11 (9)	0.00	0.00	0.00
0.08							0.70 (10)			
0.07							0.44 (9)	0.00	0.00	
0.05	0.80	1.0			1.0	1.0	1.0 (8)	0.00	0.00	0.20
0.03								0.20	0.00	0.80 (10)
0.02	1.0	1.0					1.0 (8)	0.20	0.20	1.0
0.10								1.0	0.00	1.0

Strains that showed the same response pattern in the initial experiment could still respond differently in the more detailed experiment. For instance, strains Wa13 and Wa14 both had a threshold concentration of 0.10 % and needed only 1 step along the concentration axis to change from no response to full response. In other words, for both strains all replicas died at 0.20 % glucose and higher, and all replicas survived at 0.10 % and glucose and below. Figure 3 shows the results of the subsequent experiment, in which Wa13 and Wa14 were tested on a more narrow range of glucose concentrations. Figure 3 zooms in on the glucose concentrations in between 0.10 % and 0.20 %, and shows that strain Wa14 has a steeper response curve compared to Wa13. The Y axis shows the percentage of surviving replicas. On the X axis the glucose concentration in the growth medium is shown. Similarly, strains Wa16 and Wa48 showed the exact same response to CR in the first experiment (see Appendix A) but differed slightly when tested in more detail (see Table 2). These were the two only pairs of strains included in the second experiment that gave the exact same results in the first experiment.

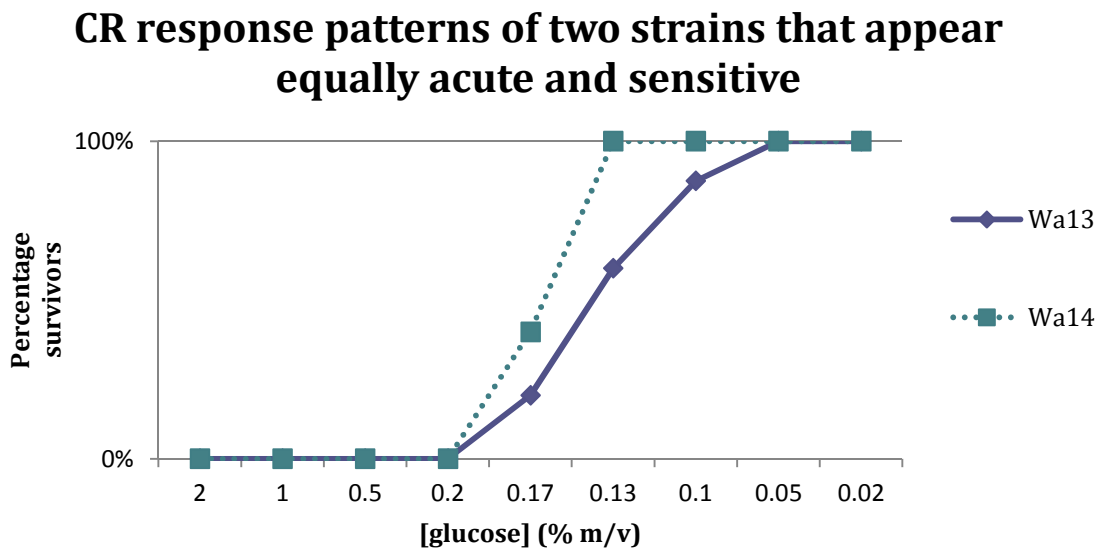

FIGURE 3: CR response patterns of strains Wa13 and Wa14, both with a threshold glucose concentration of 0.10 % and the highest possible acuteness in the initial experiment.

CORRELATION BETWEEN DIFFERENT ASPECTS OF THE RESPONSE: SURVIVAL, PIGMENTATION AND PERITHECIA FORMATION

Table 3 gives chi-square values and p-values for the association between survival and pigmentation. These two phenotypes are related in the pooled data (χ^2 787, p-value <0.001) and in the stratified data (χ^2 53.3-79.3, p-values <0.001). On the two highest glucose concentrations, strains always form pigment and never grow the entire 30 cm. At the lowest concentrations, the opposite is most common: Survival combined with lack of pigmentation. In the middle range (0.05 to 0.5 % glucose), all four possible combinations of death/survival with pigment/no pigment can be found. However, even within one glucose concentration (one column in the table), strains tend to either form pigment and die, or not form pigment and survive.

TABLE 3: Association between survival and pigmentation. Number of observations per phenotype on each glucose level and corresponding chi-square values for the association between the two characteristics.

Survival	Pigment	Concentration glucose (g/100ml)							
		0.02	0.05	0.10	0.20	0.50	1.0	2.0	Pooled
Yes	No	131	115	62	14	0	0	0	322
Yes	Yes	0	1	1	3	2	0	0	7
No	No	11	18	22	7	0	0	0	58
No	Yes	7	15	65	126	148	149	150	660
χ^2 death-pigment p-value		53.5 <0.001	53.3 <0.001	79.3 <0.001	74.4 <0.001	-	-	-	787 <0.001

Table 4 gives chi-square values and p-values for the association between survival and perithecia (fruiting body) formation. Here, too, all four possible combinations of phenotypic aspects are found. The strains tend to either survive and produce no perithecia, or produce perithecia and do not survive. Again, this is especially true for the extreme glucose concentrations. In the middle range (0.10-0.20 % glucose) all possible combinations of phenotypic aspects can be found. In contrast to the relation between pigmentation and survival, the apparent relation between perithecia formation and survival (χ^2 373, p-value <0.001) is lost when the data are stratified for glucose concentration (χ^2 0.0136-0.765, p-values 0.38-0.91). Within one column of the table, there is no correlation between survival and the formation of perithecia. A table in which the data from tables 1 and 2 are combined, can be found in Appendix B.

TABLE 4: Association between survival and perithecia formation. Number of observations per phenotype on each glucose level and corresponding chi-square values for the association between the two characteristics.

Survival	Perithecia	Concentration glucose (g/100ml)							
		0.02	0.05	0.10	0.20	0.50	1.0	2.0	Pooled
Yes	No	131	116	61	15	0	0	0	323
Yes	Yes	0	0	2	2	2	0	0	6
No	No	18	33	86	106	1	1	0	245
No	Yes	0	0	1	27	147	148	150	473
χ^2 death-perithecia p-value		-	-	0.765 0.38	0.704 0.40	0.0136 0.91	-	-	373 <0.001

CONCLUSION AND DISCUSSION

In this study we investigated the lifespan, pigmentation and perithecia formation of 63 naturally occurring strains of *Podospora anserina* on seven different glucose concentrations and showed that strains differ in sensitivity to caloric restriction and in the acuteness of their response. Our results are in line with previous findings [14,15] that most *P. anserina* strains, if grown on medium with a very low glucose concentration (0.02 % m/v), will grow fast, produce no pigment or perithecia and live long, while on a high glucose concentration (2.0 %) they typically show the opposite phenotypes: Pigmentation, perithecia formation, slow growth and death before having grown 30 cm. However, our data also show that pigmentation and perithecia formation are not always accompanied by premature death, and neither does lack of pigment and perithecia always predict survival.

The large numbers of strains and glucose concentrations allowed us to gain more insight into the variation in acuteness of CR response and in the sensitivity to CR that exists among naturally occurring strains of *P. anserina*. This fungus has a life cycle of approximately 2 weeks under non-CR conditions in nature (growing on herbivore dung) as well as in the laboratory (on 2.0 % glucose). The replicas on 2.0 % glucose grew approximately 11 cm (average 11.0 cm, SD 2.8 cm, data not shown). Thus, both the duration of the experiment and the length of the tube were much higher than the normal survival of *P. anserina* in the respective dimensions. Therefore, 'survival' in this experiment clearly indicated a response to CR. Since the behaviour at intermediate concentrations differs per strain, by looking at many different glucose concentrations in a high number of strains we were able to investigate the associations between lifespan extension and other typical CR effects in the transition area. Our data show that perithecia formation is associated with lifespan, but also suggest this is probably due to confounding by glucose concentration. Pigmentation is strongly correlated with lifespan, both across and within glucose concentrations, so pigmentation but not perithecia formation is significantly associated with non-survival when data are stratified for glucose concentration. Furthermore, we found that some strains acutely switch from non-survival to survival when the glucose concentration is decreased by approximately a factor two, whereas other strains need a ten-fold difference in glucose concentration to make this switch. This means there is natural variation in the acuteness of the response to CR. The threshold glucose concentration at which a CR response (in terms of survival) starts to occur also differs per strain, ranging from 1.0 % glucose to 0.02 % or perhaps even lower.

The fact that so many wild-derived strains respond to CR suggests this may be an adaptation. Perhaps the combination of faster growth and higher lifespan allows the fungus to grow from a nutrient-poor area to a more nutrient-rich area. A substantial part of senescence research using *P. anserina* has focussed on laboratory mutant strains [16,17,18]. Research into CR in naturally occurring *P. anserina* strains has often focussed on few fungal strains [14] or few different glucose concentrations [13,15]. As far as we know, this is the first study to investigate the response to caloric restriction in this many strains this precisely. Our data give more detailed insight into the natural variation in acuteness of CR response and sensitivity to CR that exists among *Podospora anserina* strains. The variation in sensitivity to CR and acuteness could be caused by a lack of selection pressure on these characteristics. For instance, if the fungus naturally only encounters extremely nutrient-poor or extremely nutrient-rich environments, there will not be selection for a specific kind of response to intermediate environments. Alternate explanations for these variations include balancing selection and different selection forces acting on different *P. anserina* populations. It should be noted that the strains used in our experiment were collected from different locations, in different years and from different types of dung.

Previous research already showed that *P. anserina* exhibits inter-strain variation in its response to caloric restriction [13,14,15] and our data confirm this. Van Diepeningen et al. [13] investigated the effect of CR in over 100 different strains but only looked at three different glucose concentrations: 2.0 %, 0.20 % and 0.02 %. This raised the question how the strains respond to glucose concentrations in between 2.0 and 0.20 %, or in between 0.20 and 0.02 %. Other research by Rusli [14] focussed on a smaller number of *P. anserina* strains on more different glucose concentrations, and showed strain-specificity on glucose concentrations in between those used by Van Diepeningen et al. In the present study, the advantages of both studies were combined: Over 60 different strains were tested, on at least seven different glucose concentrations, all in three-fold. We found a high amount of variation between naturally occurring *P. anserina* strains in their response to varying glucose concentrations in the medium. This is in agreement with the results found by Van Diepeningen et al. [13,15] and Rusli [14]. There is variation both in the threshold value for the caloric restriction phenotype as well as the acuteness of the response. If the experiments would have been performed in two-fold instead of three-fold and on three glucose concentrations instead of seven, this variation would have been largely disguised. At near-threshold glucose concentrations, there appears to be a stochastic effect for lifespan. This is in line with previous findings by Rusli [14]. The stochastic effect is not visible if the experiments are done in two-fold on three glucose concentrations.

A link between the lifespan extending effect and a decrease in fertility under CR conditions has been observed in many species [4,11]. In humans, severely underweight females are known to cease menstruating, although it is important to note this could also be due to malnutrition. A prevailing hypothesis is that undernutrition (even without malnutrition) leads to a reallocation of nutrients from reproduction to maintenance. This would explain why, at least in fruit flies [11,12], female fertility is more strongly affected by CR. Interestingly, some research in fruit flies indicates that the link between lifespan extension and decreased fecundity may not be inevitable [12]. Our results are in line with this: Perithecia formation is not significantly associated with non-survival when data are stratified for glucose concentration, suggesting the link between lifespan extension and decreased fertility is not inevitable in *P. anserina* either. The lack of pigment under CR conditions is typical for *P. anserina* and to our knowledge has no analogue in the animal kingdom. Nevertheless, it is interesting that although pigmentation is significantly associated with survival, replicas with and without pigment may survive or die. The fact that the different aspects of the CR response in this organism can occur separately from one another, suggests they may be caused by partially different pathways. Therefore, *P. anserina* could be used to distinguish between factors – for instance expression of specific genes – involved in senescence and those involved in pigment and perithecia formation. Similarly, the inter-strain variation in the effect of CR on lifespan could be used to distinguish gene expression specific to ageing outcome from gene expression specific to a certain glucose concentration. This could help elucidate the mechanisms behind the lifespan extending effect of caloric restriction. Separating the factors involved in lifespan extension from those involved in other aspects of the CR response or glucose metabolism could shed light on the mechanisms of senescence. Therefore, *P. anserina* provides us with unique opportunities to increase our understanding of senescence in other organisms.

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APPENDICES

APPENDIX A: STRAINS INCLUDED IN THE EXPERIMENT

TABLE 5: Included strains, acuteness (number of stepwise decreases in glucose concentration needed to switch from no response to full response) and sensitivity (threshold value: Highest concentration at which at least a partial response occurred).

Strain:	Acuteness:	Sensitivity:	Strain:	Acuteness:	Sensitivity:
Wa3	4	0.50	Wa61	Unknown	0.10
Wa7	1	0.05	Wa63	3	0.10
Wa8	2	0.10	Wa64	2	0.10
Wa9	2	0.10	Wa65	2	0.05
Wa13	1	0.10	Wa66	2	0.10
Wa14	1	0.10	Wa67	Unknown	0.02
Wa15	1	0.10	Wa68	2	0.05
Wa16	1	0.20	Wa69	Unknown	0.02
Wa17	2	0.10	Wa72	1	0.05
Wa21	1	0.10	Wa76	2	0.20
Wa23	2	0.20	Wa77	3	0.20
Wa24	2	0.20	Wa78	1	0.05
Wa25	Unknown	0.05	Wa79	1	0.05
Wa27	2	0.20	Wa81	Unknown	Unknown
Wa28	1	0.05	Wa84	2	0.20
Wa29	Unknown	Unknown	Wa86	2	0.05
Wa38	2	0.05	Wa88	2	0.10
Wa39	2	0.05	Wa89	2	0.10
Wa41	3	0.20	Wa93	1	0.10
Wa42	3	0.10	Wa95	Unknown	0.05
Wa43	1	0.05	Wa99	4	0.20
Wa44	2	0.20	Wa100	3	0.20
Wa46	3	0.10	Wa101	3	0.10
Wa47	3	0.10	Wa102	2	0.20
Wa48	1	0.20	Wa103	1	0.05
Wa50	1	0.02	Wa104	3	0.20
Wa54	1	0.02	Wa105	2	0.10
Wa55	2	0.10	Wa109	1	0.10
Wa56	4	0.50	Wa111	3	0.20
Wa58	2	0.20	Wa114	Unknown	Unknown
Wa59	Unknown	0.05	Wa117	2	0.10
Wa60	1	0.05			

APPENDIX B: ASSOCIATIONS BETWEEN SURVIVAL, PERITHECIA FORMATION AND PIGMENTATION

TABLE 6: Association between survival and perithecia formation and pigmentation. Number of observations per phenotype on each glucose level and corresponding chi-square values and p-values for the associations.

Survival	Pigment	Perithecia	Concentration glucose (g/100ml)							
			0.02	0.05	0.1	0.2	0.5	1	2	Pooled
Yes	No	No	131	115	60	12	0	0	0	318
Yes	No	Yes	0	0	2	2	0	0	0	4
Yes	Yes	No	0	1	1	3	0	0	0	5
Yes	Yes	Yes	0	0	0	0	2	0	0	2
No	No	No	11	18	22	7	0	0	0	58
No	No	Yes	0	0	0	0	0	0	0	0
No	Yes	No	7	15	64	99	1	1	0	187
No	Yes	Yes	0	0	1	27	147	148	150	473
χ^2 survival and pigment p-value			53.5 <0.001	53.3 <0.001	79.3 <0.001	74.4 <0.001	-	-	-	787 <0.001
χ^2 survival and perithecia p-value			-	-	0.765 0.38	0.704 0.40	0.0136 0.91	-	-	373 <0.001

APPENDIX C: PODOSPORA ANSERINA SYNTHETIC MEDIUM (PASM)

Preparation of PASM
0.85 gram KH_2PO_4
0.5 gram $MgSO_4.7H_2O$
1 gram ureum
Adjust pH to 5.8
1 litre demineralized water
20 gram agar

Before use: Add to 1 litre medium:
1 ml biotin/thiamine solution
1 ml trace-elements for Podospora solution
Sugar (glucose) solution as needed

PASM with acetate:
PASM with 6.5 ml acetate solution (49 g NH_4 acetate per 100 ml) per 800 ml PASM

Trace-elements for Podospora solution:
500 mg citric acid.H_2O
500 mg $ZnSO_4.7H_2O$
100 mg $(NH_4)_2Fe(SO_4)_2.6H_2O$
25 mg $CuSO_4.5H_2O$
5 mg $MnSO_4$
5 mg H_3BO_3
5 mg $Na_2MoO_4.2H_2O$
100 demineralized water
Filter sterilise

APPENDIX D: STRAINS TESTED IN MORE DETAIL

TABLE 7: Strains re-tested in five-fold to ten-fold, and corresponding glucose concentrations

Strain	**Glucose concentrations (% mass/volume) tested:**						
Wa3	2.0	1.0	0.50	0.20	0.10	0.05	0.02
Wa7	0.20	0.10	0.08	0.07	0.05	0.02	
Wa13	0.50	0.20	0.17	0.13	0.10	0.05	
Wa14	0.50	0.20	0.17	0.13	0.10	0.05	
Wa16	1.0	0.50	0.40	0.30	0.20	0.10	
Wa25	0.10	0.07	0.05	0.03	0.02	0.01	
Wa29	0.10	0.07	0.05	0.03	0.02	0.01	
Wa48	1.0	0.50	0.40	0.30	0.20	0.10	
Wa56	2.0	1.0	0.50	0.20	0.10	0.05	0.02
Wa67	0.10	0.05	0.03	0.02	0.01		