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The ageing lysosome of *Caenorhabditis elegans*

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Summary – Autophagy represents a unique process of self-eating and has been intensively studied in the past decade due to its importance in various biological processes. However, many of the previous studies in this field revealed contradictory results regarding its biological role, molecular mechanism and genetic regulations. In *Caenorhabditis elegans* the autophagic pathway was reported to play a key role in lifespan extension of *daf-2* insulin/insulin-like growth factor 1 (insulin/IGF-1) receptor-defective mutants. It was shown that when an important autophagy gene *beclin 1* (T19E7.3) was knocked-down using RNA interference (RNAi), the lifespan extension in the *daf-2* mutant was no more seen. But whether autophagy is responsible for prolonging life in *C. elegans* still remains a topic of many debates. To contribute towards understanding whether up-regulation of autophagy is linked to accelerated or decelerated ageing, two different mutants considerably differing in their lifespan were compared (*glp-4 daf-16; daf-2* vs. *glp-4 daf-2*). Comparisons of activity of major lysosome-associated enzymes relevant for the autophagic process (acid phosphatases, cathepsins and lipases) were done. In addition, specific genes responsible for the above mentioned enzymes were down-regulated by means of the RNAi technique to find important genes which determine global enzymatic activities and lifespan. Findings of the current study indicate much lower acid phosphatase and negligible cathepsin activity levels in long-lived *glp-4 daf-2* worms compared to normal-lived *glp-4 daf-16; daf-2* mutants. The low activity of lysosome-associated enzymes in lifespan-extended *glp-4 daf-2* worms suggests low levels of autophagy and hence protein turnover which is contradictory to what was proposed in previous studies. Whether autophagy shortens lifespan in *C. elegans* needs to be studied. The *pho-14* (T13B5.3) gene was found to be responsible for the global acid phosphatase levels in both strains. However, down-regulation of this gene's expression did not affect their lifespan. RNAi treatment of another acid phosphatase gene *pho-11* (C05C10.4) reduced lifespan of both the strains suggesting its involvement either in development or ageing independent from the DAF-16/FOXO pathway. The current study provides hints to the differences between *glp-4 daf-16; daf-2* and *glp-4 daf-2* mutants' protein turnover rates which have to be still studied in depth.

Keywords – autophagy, acid phosphatase, cathepsin, lipase, *C. elegans*.

Autophagy represents a catabolic process, by which damaged or unnecessary cellular macromolecules undergo degradation by the help of the lysosomal machinery. This unique process was first discovered by an American anatomist Sam Clark (1957), while the term "autophagy" and the discovery of the specific organelle involved in it are contributions made by a Belgian cytologist Christian de Duve (1963). Since then, the structure and functions of the lysosome have been extensively studied. Lysosomes are considered the cell's disposal system as they act in breakdown of "cellular waste". This process is accomplished by the action of different lysosomal hydrolytic enzymes, the variety of which exceeds 50.

It was proposed that the autophagic pathway and lysosomes are essential in lifespan extension in a wide range of organisms, from yeasts (Dwivedi & Ahnn, 2009; Yen & Klionsky, 2008) to mammals (Sandoval et al., 2008). Another study shows that the increased fragility of lysosomal membrane due to age may lead to leakage of "enzymatic soup" into the cytoplasm (Chondrogianni et al., 2002) resulting in cell death (Samara & Tavernarakis, 2003). On the other hand, it was proposed that lysosomal enzymes remain active only in optimal acidic pH (Rauch-Lüllmann, 2005) and slightly alkaline pH of cytosol immediately deactivates/reduces activity of most of them (Turk & Turk, 2009). However, it is an indisputable fact that cathepsins, essential lysosomal proteases, play an important role in apoptosis and necrosis (Turk & Turk, 2009). Thus, it still remains unclear whether autophagy is important for lifespan extension or, vice versa, promotes cellular death.

The process of ageing leads to various alterations in organisms at different levels and the lysosome is not an exception. Senescence of the lysosome results in morphological and biochemical changes, which are common in most organisms (Cuervo & Dice, 2010). But the importance of lysosomal hydrolytic enzymes and the age-related alterations have yet to be studied in more details. For this purpose, the nematode *Caenorhabditis elegans* (*C. elegans*) was chosen as a model due to its diminutiveness, short lifespan and ease of handling under laboratory conditions. The objective of the current study was: to characterize 3 groups of hydrolytic enzymes (acid phosphatases, cathepsins and lipases) and reveal their importance in lifespan extension of the organism. To meet this objective the difference in enzymatic activity between lifespan-extended *glp-4 daf-2* and normal-lived *glp-4 daf-16; daf-2* mutants was studied: (1) along the age series; and (2) by specifically knocking-down 23 lysosome-associated genes with the help of the RNA interference (RNAi) technique. Moreover, RNAi method was applied to check the influence of knock-down of 15 selected genes of acid phosphatases and cathepsins on lifespan. This study contributes towards the understanding of the importance of autophagy in *C. elegans*.

Material and methods

Strains, growth condition and synchronisation

The following mutants were used for the current study: *glp-4 daf-2(e1370)*, and *glp-4 daf-16*; *daf-2(e1370)* (hereafter referred as *daf-2* and *daf-16* respectively). Both of the strains were obtained from the *Caenorhabditis* Genetics Center. For culture maintenance worms were grown on nutrient agar (NA) plates with *Escherichia coli* (*E. coli*) strain K-12 at 17°C.

A synchronised nematode culture was obtained by treating gravid hermaphrodites with a hypochlorite solution (Sulston & Hodgkin, 1988). Eggs, recovered from dissolved bodies, were incubated in S-buffer overnight on a rotary shaker (120 rpm at 17°C). The normal-lived triple mutant was considered as control to long-lived *daf-2* mutant. All experiments were replicated by time trice.

Sampling along age series

L1's of both strains of *C. elegans* were grown on NA plates with additional agar №1 (0,0125g/ml) with an *E. coli* K-12 lawn. Worms were maintained at 17°C during ~2 days until they reached L3 stage. Then, plates were shifted to 24°C for the remaining part of the experiment in order to activate *glp-4* mutation and inhibit gonadal development. When nematodes reached L4 stage fluorodeoxyuridine (FUdR) was added to a final concentration of 0.1 mg/ml. The first day of adulthood was considered as day 0. Sampling of *daf-16* mutants was performed every second day (age series 0-2-4-6-8-10-12-14) and for *daf-2* worms every third day (age series 0-3-6-9-12-15-18-21-24-27-30). Besides, dauers of *daf-2* mutants were obtained by incubating worms at 24°C from L1 stage. In order to make comparisons between and within strains possible, sampling of dauer *C. elegans* was performed on the same day 0 as for other two strains. All samples were immediately frozen (-80°C).

Sampling after RNAi exposure

Animals were specifically exposed to 24 different RNAi bacteria including control L4440-containing bacteria (Table 1). Bacterial cultures were obtained as described before (Timmons et al., 2001). The difference was only in growing bacteria until OD_{595}=0.2 when diluted twice with distilled water. A large population of worms were grown on bacteria, freshly seeded on NGM plates containing antibiotics and isopropyl β-D-1-thiogalactopyranoside (IPTG) with additional agar №1 as described previously. The transfer of animals from 17 °C to 24°C was performed at L3 stage. Both strains were treated with FUdR at L4 stage to ensure no progeny. Sampling was performed at day 3 to achieve high RNAi exposure dose.

Table 1. Target genes used for knock-down in RNAi treatments.

	Gene name	Gene ID
Acid phosphatases	*pho-1*	EGAP2.3*
	pho-4	T16D1.2
	pho-5	B0361.7
	pho-7	T21B6.2*
	pho-8	R13H4.3*
	pho-10	C05C10.1
	pho-11	C05C10.4*
	pho-12	C27A2.4
	pho-13	F07H5.9*
	pho-14	T13B5.3*
	acp-1	2K563.6
	acp-2	F14E5.4*
	acp-3	F14E5.3*
	acp-5	F13D11.1

	Gene name	Gene ID
Cathepsins	*cpr-1*	C52E4.1*
	cpl-1	T03E6.7*
	cpz-1	F32B5.8*
	asp-6	F21F8.7*
	asp-3	H22K11.1*
		T28H10.3*
		F57F5.1*

	Gene name	Gene ID
Lipases	*lipl-4*	K04A8.5
		ZK856.5

All the above mentioned genes were selected for RNAi knock-down followed by enzyme assay. *Genes selected for knock-down and lifespan assay.

Different bacterial treatment

To check the effect of different bacteria on both *C. elegans* mutants, the *E. coli* strains K-12, OP50 and HT115 were used, establishing 2 conditions: fully fed and dietary restricted. The overnight grown culture of K-12 and OP50 in lysogeny broth (LB) medium, and 5x concentrated L4440 vector containing HT115 were used for maintaining fully fed condition. Dietary restricted condition was established by diluting the mentioned bacteria 10-times with S-buffer. Feeding was performed on a daily basis.

Enzyme assays

All samples were homogenized by bead beating method in 50 mM Na/K phosphate buffer, pH 6.8. Bead beating was performed in a Precellys24 homogenizer, for 30 s at 6000 rpm. To release lysosomal enzymes into the homogenate, CHAPS detergent was added at a final concentration of 1% and kept on ice for 15 min. The bead beating step was repeated. Insoluble debris was removed by centrifugation at 14000 rpm for 8 min at 4°C. A part of the obtained supernatant was subjected to determination of total protein concentration using a bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). The rest of homogenate was used for acid phosphatase, cathepsin and lipase enzyme assays.

Acid phosphatase assay was performed using SpectraMax microplate reader. 10 µl of sample was mixed with 90 µl of reaction mixture, containing 4-methylumbelliferyl phosphate (4-MUP) at a final concentration of 97.5 µM in 50 mM citrate buffer, pH 4.6. The excitation and emission wavelengths, optimal for unsubstituted 4-MUP substrate, were set at 360 nm and 450 nm respectively.

Cathepsin activity was measured using a Magic Red^{TM} (MR) Cathepsin detection kit (Immunochemistry Technologies, LLC) based on fluorometry. The reaction mixture consisted of 10 µl of homogenate, 190 µl of sodium acetate buffer (50 mM, pH 4.6) and 8 µl of MR 26x solution.

The measurement of lipase activity was performed using a QuantiChrom^{TM} Lipase assay kit (BioAssay Systems). The pH of the working reagent was changed from 8.5 to 4.2.

Lifespan assay using RNAi

For lifespan analysis, L1's were treated with 16 different RNAi bacteria including HT115 with an empty vector as a control independently (Table 1). Bacterial cultures and culture conditions were similar to those in 3-day treatment with RNAi. 15 worms were grown on each 10x concentrated bacteria, seeded dropwise on 6 cm nematode growth medium (NGM) plates with previously mentioned additives. Lifespan of populations was measured every second day for *daf-16* mutants and every third day for *daf-2* mutants until the last worm had died. Death was evaluated as no response to several slight pokes with a platinum wire.

Data analysis

The enzymatic activity of all samples is calculated according to initial 30 min slope for acid phosphatase assays and 90 min slope for cathepsin assays. An average of first 30 min is used to estimate lipase activity due to precipitations occurring during this assay. All data is normalized to 1mg/ml protein. Statistical analysis for enzyme assays is performed using t-test. Lifespan assay data is checked for statistical significance applying Kaplan-Meier analysis. The survival rate of each RNAi-treated population is compared with the population fed on control RNAi bacteria using the log rank test. A p-value of ≤ 0.05 was considered as significantly different from control.

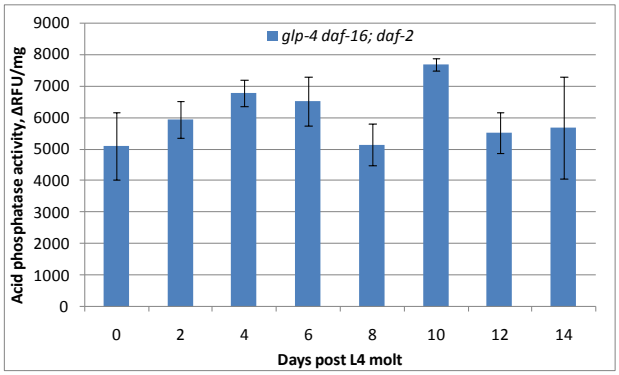
Results

Enzyme assays along age series

The lifespan-extended *daf-2* and normal-lived *daf-16 C. elegans* strains were subjected to acid phosphatase, cathepsin and lipase enzyme assays throughout their life. In all samples, the acid

phosphatase activity in *daf-16* worms was found to be higher than in the long-lived *daf-2* mutants. *daf-16* worms revealed gradual increase in acid phosphatase levels till day 4, followed by a decline (Fig.1, A & C). In comparison to control (5085 ΔRFU/mg protein), the activity of acid phosphatase was almost twice lower in *daf-2* mutants on day 0 (2827 ΔRFU/mg protein) (Fig.1, B & C). The activity was the highest on day 3, revealing gradually dropping trend afterwards.

A



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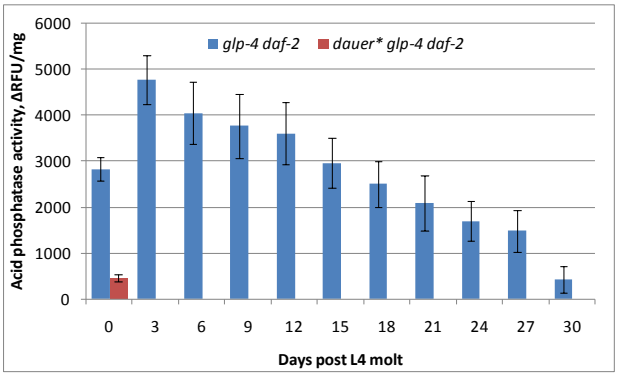

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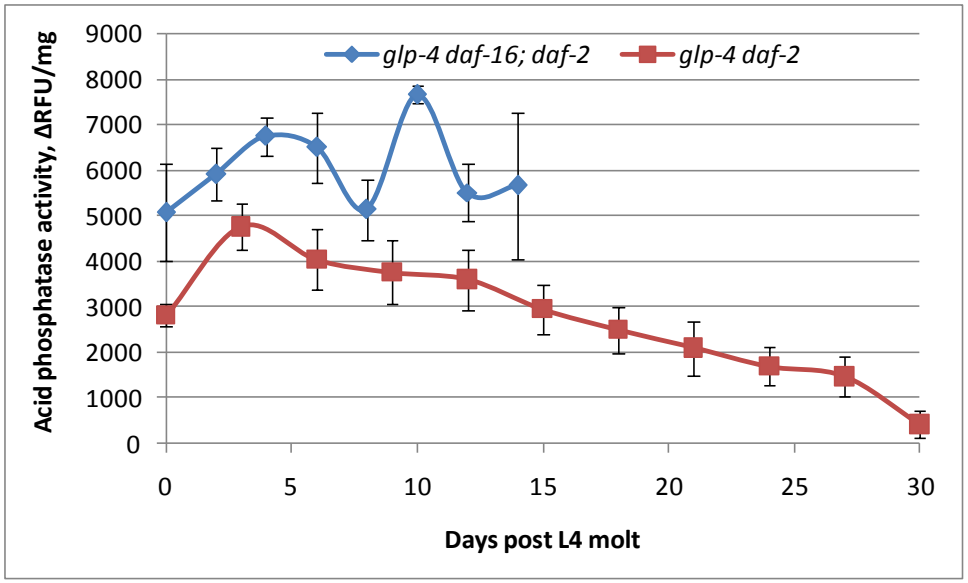
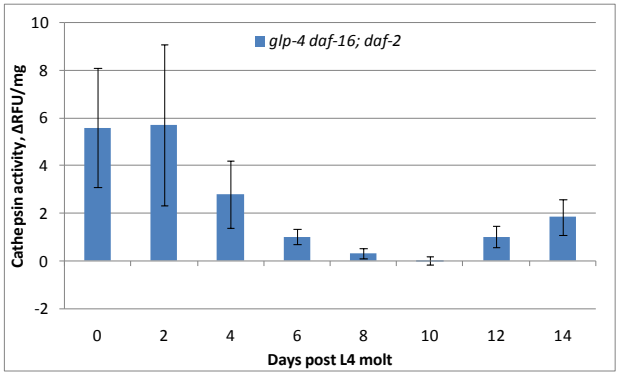


Fig.1. Acid phosphatase activity in *daf-16* (A) and *daf-2* (B) mutants, and their comparison (C) along age series. Enzymatic activity was measured as relative fluorescence units (ΔRFU) normalized to protein (mg). Activity was detected using a slope on a time course (0 min to 30 min) on the Time vs. RFU graph. *Sampling of dauers was performed on the day 0 of *daf-2* mutants. Error bars represent standard errors.

The activity of cathepsin in *daf-16* worms are the highest during the initial 2 days, revealing steep decreasing trend thereupon till day 10 and a slight raise during the final days of their life (Fig.2, A & C). Comparative to control, the activity of cathepsin is negligible or absent in *daf-2*

worms throughout their life (Fig.2, B & C). The last 2 days induced cathepsin levels in lifespan-extended worms to increase similar to that seen in control worms.

A



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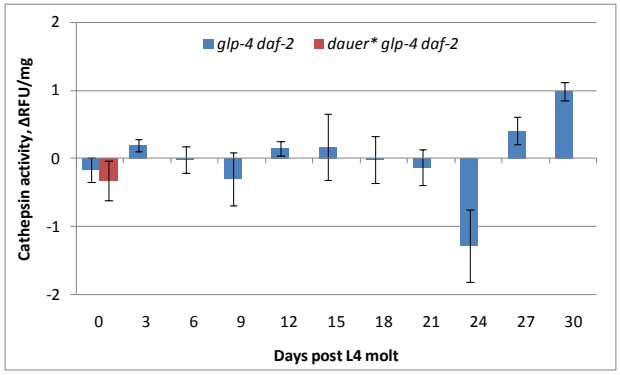

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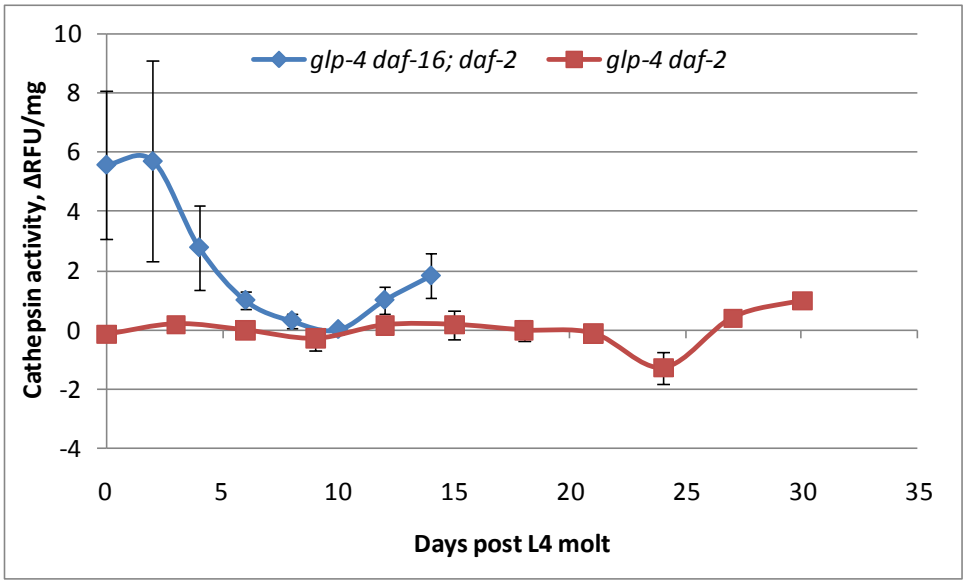


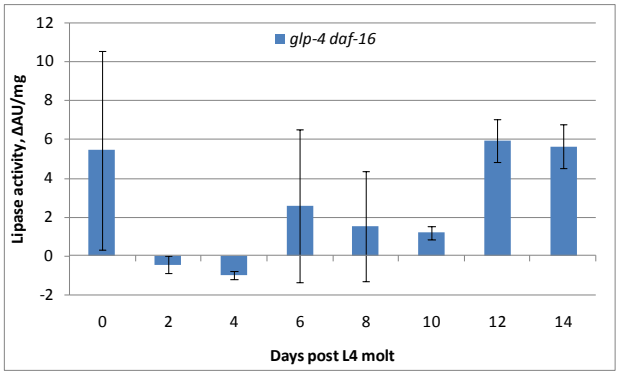
Fig.2. Cathepsin activity in *daf-16* (A) and *daf-2* (B) mutants, and their comparison (C). Enzymatic activity was measured as ΔRFU normalized to protein (mg). Activity was detected using a slope on a time course (0 min to 90 min) on the Time vs. RFU graph. *Sampling of dauers was performed on the day 0 of *daf-2* mutants. Error bars represent standard errors.

The results of lipase activity assay showed no significant difference between *daf-16* and *daf-2* animals due to large fluctuations (Fig.3; A, B & C).

In addition to comparisons between *daf-16* and *daf-2* mutants, dauer stage of *daf-2* worms was also examined. Dauer larvae are known to survive harsh conditions for extended periods of time. Dauer formation is controlled by insulin/IGF-1 pathway, which also regulates adult ageing. This suggests that dauers share some common mechanisms with long-lived *daf-2* adults in terms of lifespan extension, which might be reflected by common changes (e.g. enzymatic activity). Thus, it seemed reasonable to check activity of lysosome-associated enzymes also in dauer

larvae. The acid phosphatase activity in dauer larvae was 6-fold lower compared to *daf-2* adults, sampled on day 0 (Fig.1, B). The cathepsin (Fig.2, B) and lipase (Fig.3, B) activity profiles were similar to those found in *daf-2* adults. Caution is needed in the interpretation of activity levels in dauers, which represent a special juvenile phase of *C. elegans* and not an adult stage.

A



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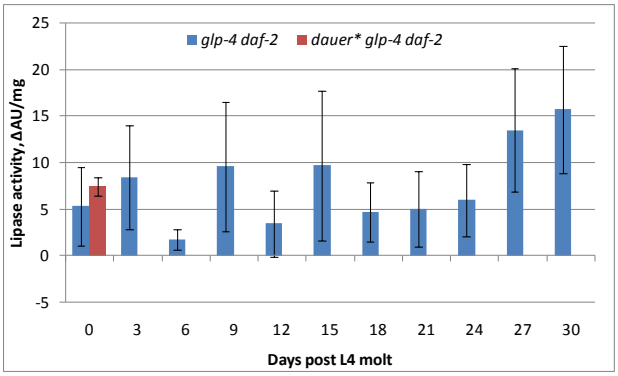

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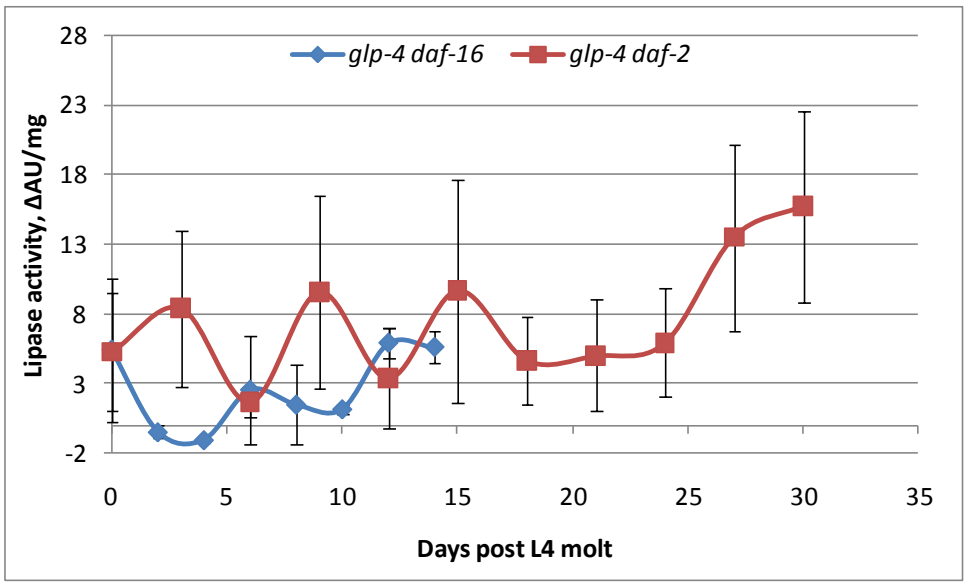

Fig.3. Lipase activity in *daf-16* (A) and *daf-2* (B) mutants, and their comparison (C) along age series. Enzymatic activity was measured as absorbance unit (ΔAU) normalized to protein (mg). Activity was detected using an average value of absorbance on a time course (0 min to 30 min) on the Time vs. AU graph. *Sampling of dauers was performed on the day 0 of adult *daf-2* mutants. Error bars represent standard errors.

Enzyme assays after RNAi exposure

The expression of specific acid phosphatase, cathepsin and lipase genes were knocked-down by using RNAi technique. According to the results, there was a clear difference between the acid phosphatase activity profiles between the two strains (Fig.4). The long-lived *daf-2* worms exhibited 4-times lower activity compared to *daf-16* mutants. Besides, the acid phosphatase gene

pho-14 was found to be contributing greatly for the global acid phosphatase activity in *daf-16* (*p*-value<0.001) as well as *daf-2* (*p*-value=0.05) worms. Knocking-down of this gene resulted in 7.5- and 8-fold decrease of the overall acid phosphatase level in *daf-2* and *daf-16* mutants respectively. Down-regulation of other acid phosphatase genes did not reveal any significant differences in comparison to the control L4440 in both the strains.

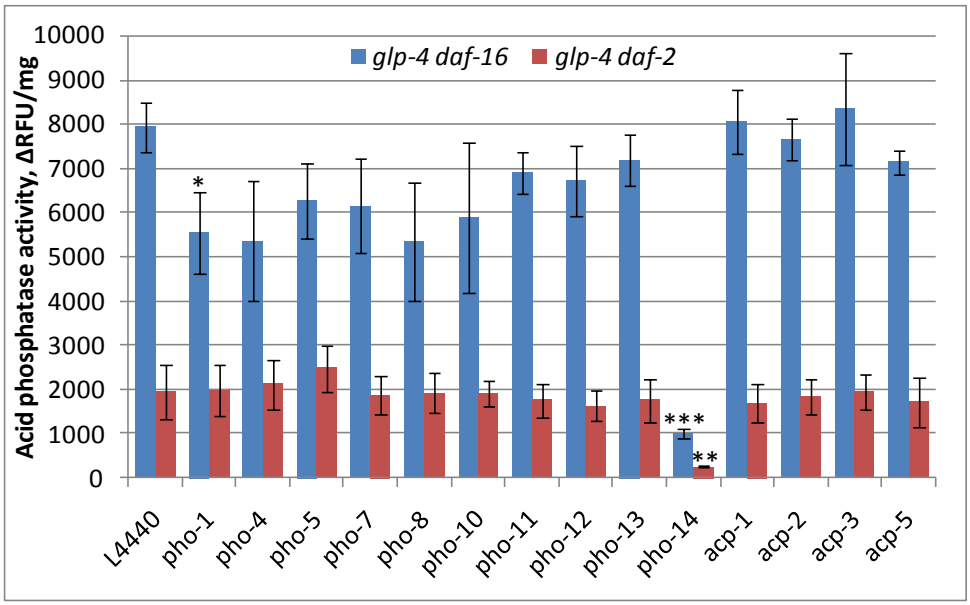

Fig.4. Acid phosphatase activity levels after specific knock-down of 14 lysosome-associated acid phosphatase genes by applying RNAi technique in *daf-16* and *daf-2* mutants. L4440 is an empty vector control. Error bars represent standard errors. ****p*-value≤0.01, ***p*-value≤0.05, **p*-value≤0.1.

RNAi treatment against specific cathepsin genes revealed differences in enzyme activity of *daf-16* worms (Fig.5). No differences were found when cathepsin genes were knocked-down in *daf-2* mutants. However, the interpretation of results of RNAi treatment on *daf-16* worms is complicated due to negligible cathepsin activity in the control treatment. The cathepsin level in *daf-16* control population was as low as the cathepsin activity found in the long-lived *daf-2* control mutants. As was seen in the experiment along age series, *daf-2* worms show negligible cathepsin activity on day 3 of adulthood, whereas *daf-16* worms are expected to exhibit cathepsin activity of ≥2.5 ΔRFU/mg protein. But this was not the case in the current experiment. Therefore, before making any conclusions on the obtained result, attempts were done to clarify this issue. It was hypothesised that the RNAi bacteria with L4440 insert might affected the global cathepsin levels for unknown reasons. To check this idea an additional experiment was conducted using three different *E. coli* strains for making comparisons (see "Different bacterial treatment" in the "Results" section). The suggested above hypothesis would be true if the results of the follow-up test reveal the great difference in cathepsin activities between *daf-16*

populations fed on HT115 bacteria with an empty L4440 vector and other two *E. coli* strains (K-12 & OP50). In addition, it was decided to test whether the bacterial quantity has an influence on the global cathepsin activity to check the diet condition of the current experiment.

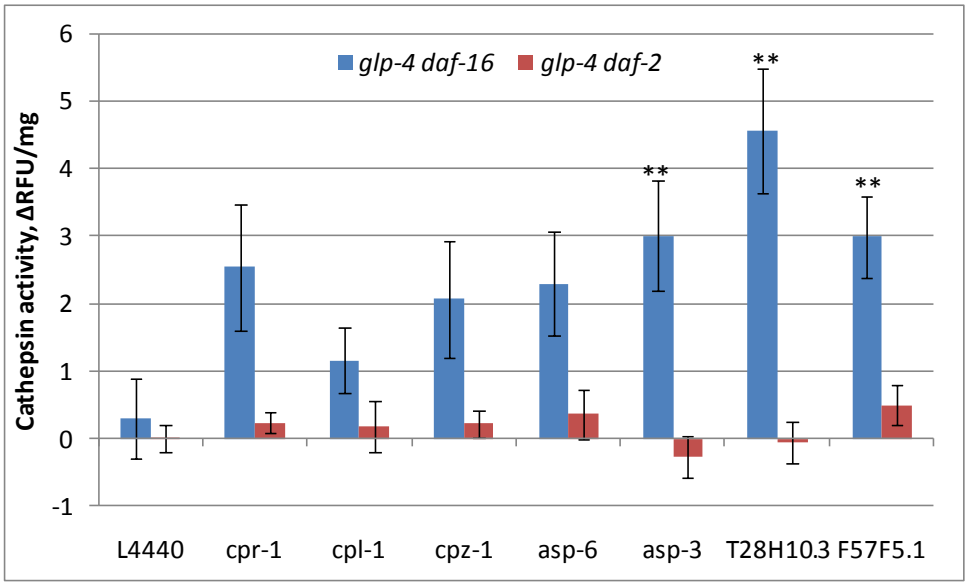

Fig.5. Cathepsin activity levels after specific knock-down of 7 lysosome-associated cathepsin genes by applying RNAi technique in *daf-16* and *daf-2* mutants. L4440 is an empty vector control. Error bars represent standard errors. **0.01<*p*-value≤0.05.

The knock-down of the lipase K04A8.5 gene did not show any significant difference in both the strains. The knock-down of ZK856.5 gene leads to a 3-fold increase in the total lipolytic activity in *daf-16* mutants, whereas it shows a 6-fold decrease in *daf-2* mutants. However, the results of RNAi treatment of lipase genes were not significant due to large fluctuations.

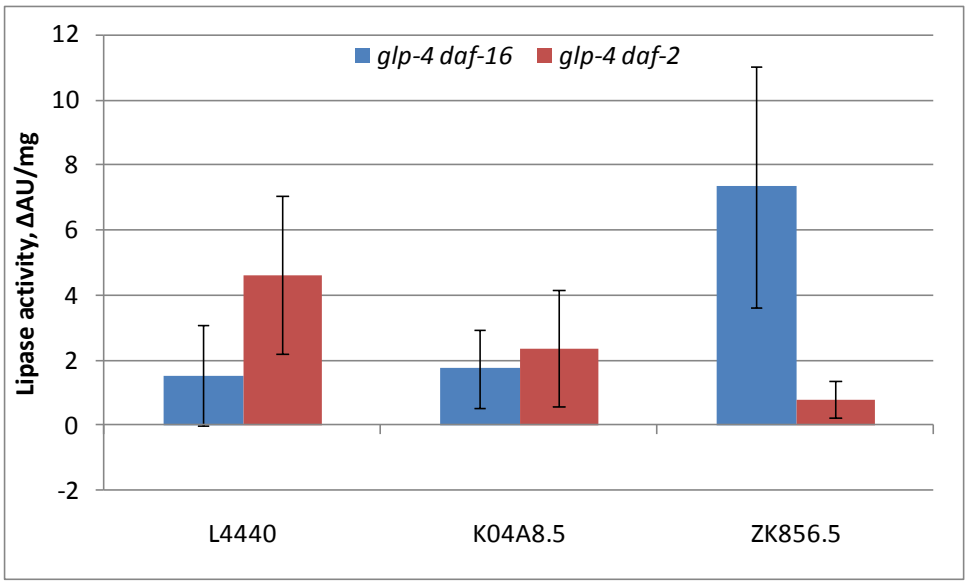

Fig.6. Lipase activity levels after specific knock-down of 2 lysosome-associated lipase genes by applying RNAi technique in *daf-16* and *daf-2* mutants. L4440 is an empty vector control. Error bars represent standard errors.

Influence of bacterial quality and quantity on cathepsin activity

In order to check whether the control HT115 bacteria with an empty vector might be involved in down-regulation of cathepsin activity, a follow-up experiment was conducted using *E. coli* strains K-12, OP50 and HT115. Moreover, worms were exposed to dietary restriction (DR) in addition to normal fed (FF) worms to determine whether feeding conditions influence the enzyme activity levels.

Well-fed *daf-16* mutants did not reveal any differences in cathepsin activity while subjecting to three different strains of *E. coli* (Fig.7). The difference between bacterial treatments becomes pronounced in the dietary restricted case (DR K-12 vs. DR HT115, p-value<0.05). Moreover, dietary restriction seems to promote cathepsin activity in *E. coli* strain HT115 (p-value<0.01). No significant difference was found for *daf-2* animals in neither conditions nor bacterial strain.

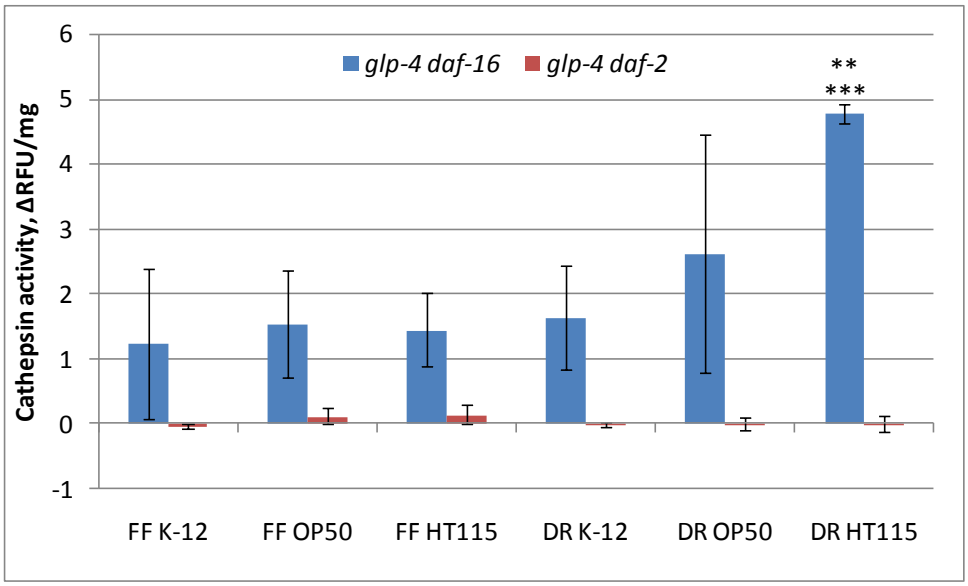

Fig.7. The cathepsin activity levels in *daf-16* and *daf-2* strains after specific feeding on three different types of *E. coli* bacteria in fully fed (FF) and dietary restricted (DR) conditions. The *E. coli* strains K-12 and OP50 are commonly used for maintenance of *C. elegans* cultures and experiments. HT115 containing an empty vector L4440, is widely used as control in RNAi experiments. Error bars represent standard errors. ***p-value≤0.01 (FF vs. DR), **p-value≤0.05 (K-12 vs. HT115).

Lifespan assay using RNAi

Specific lysosome-associated genes were knocked-down in *daf-16* and *daf-2* mutants using RNAi bacteria to identify any changes in lifespan in comparison to control-L4440 treated populations (Table 2). No significant reduction or extension in lifespan was detected in both the strains when cathepsin genes were knocked-down (Fig.8, A & C). The result indicates that when the expression of an acid phosphatase gene *pho-11* was down-regulated in *daf-16* mutants, a significant decrease of lifespan (p-value≤0.01) was identified (Fig.8, B). *daf-16* mutants also show reduced lifespan when subjected to *pho-11* RNAi (p-value=0.06) (Fig.8, D). The knock-down of *pho-11* resulted in significant lifespan reduction in both the mutants compared to their L4440-fed controls (Table 2). The mean and maximum lifespan (data not shown) of *daf-16* worms was reduced from 12.2±1.3 days to 8.5±0.3 days and from 18.3±0.3 days to 9.3±1.3 days respectively. The long-lived *daf-2* mutants revealed more pronounced mean and maximum lifespan (data not shown) reduction: from 42.1±6.1 days to 28.1±2.3 days and from 83±3.6 days to 54±12.1 days respectively. The knocking-down of other acid phosphatase genes did not show any significant results.

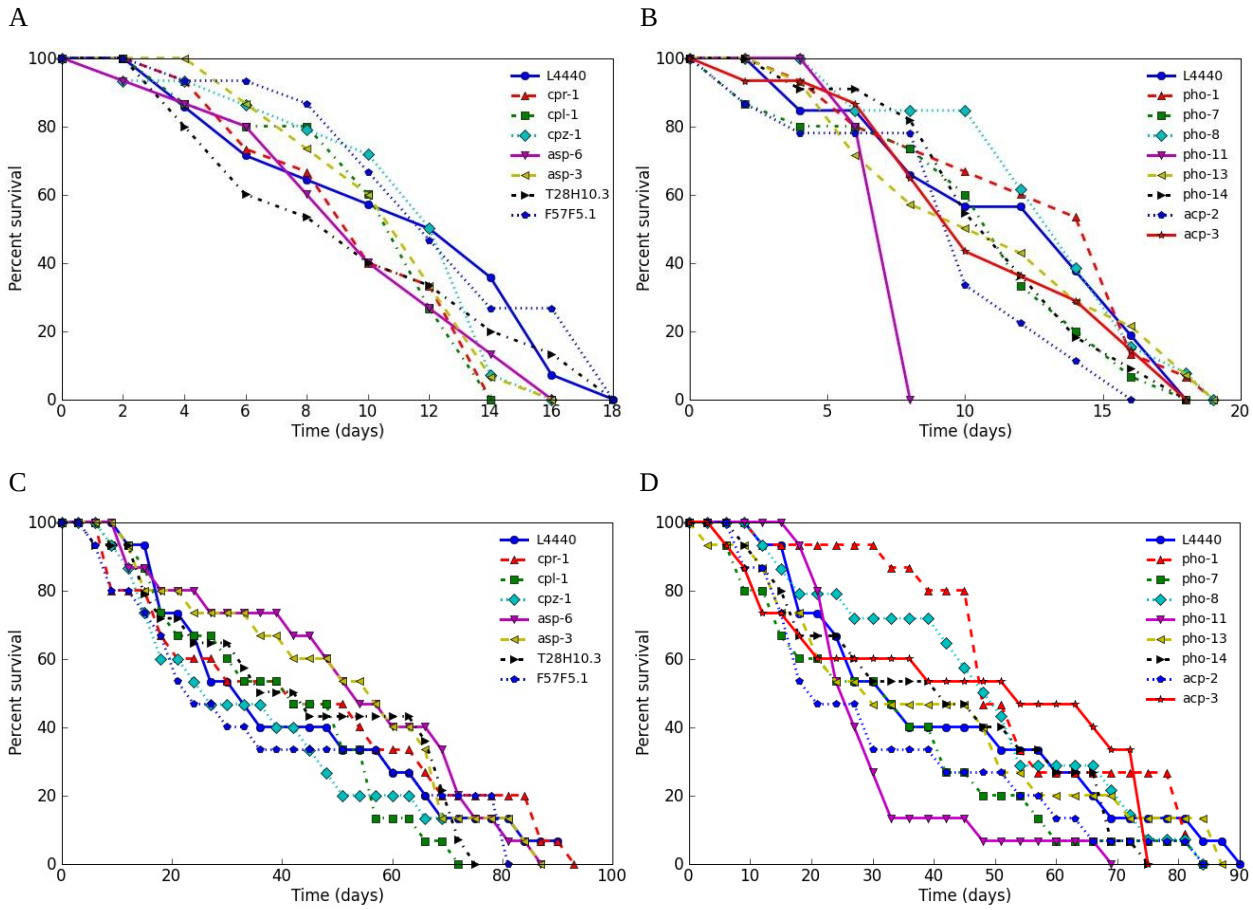

Fig.8. Kaplan-Meier survival curves after RNAi exposure of *daf-16* (A & B) and *daf-2* (C & D) mutants to down-regulate a set of cathepsin (A & C) and acid phosphatase (B & D) genes at 24°C as described.

	glp-4 daf-16; daf-2	*glp-4 daf-2*
Gene/RNAi	**Mean lifespan**	**Mean lifespan**
L4440	12.2±1.3	42.1±6.1
cpr-1	11.8±1.0	44.4±6.8
cpl-1	11.8±1.1	43.1±6.2
cpz-1	12.0±1.0	37.8±5.6
asp-6	11.3±1.2	43.4±6.1
asp-3	11.4±1.0	45.5±6.1
T28H10.3	12.6±1.1	42.2±5.8
F57F5.1	11.5±1.0	42.3±6.1
pho-1	12.4±1.0	51.2±6.1
pho-7	11.2±1.0	38.9±5.1
pho-8	12.1±0.9	45.3±5.6
pho-11	8.5±0.3	28.1±2.3
pho-13	12.0±1.2	34.7±5.1
pho-14	12.0±1.0	37.6±5.7
acp-2	12.1±1.1	40.0±6.1
acp-3	11.6±1.1	43.9±6.2

Table 2. Mean lifespan of *daf-16* and *daf-2* worms grown on RNAi bacterial strains (for knocking-down specific cathepsin & acid phosphatase genes). Mean lifespan ± standard error at 24°C as described.

Discussion

Enzyme assays along age series

The results of the present study show a difference in lysosomal enzyme activity between the normal-lived and long-lived *C. elegans* strains. Moreover, the activity of "lysozymes" is changing in both mutants in age-dependent manner pointing on their interrelation with ageing. However, it is not still clear whether these changes are the cause or result of physiological senescence. The ageing process may be postponed by lifespan extension as it was shown in *daf-2* worms (Kenyon et al., 1993). And autophagy, which involves the lysosomal machinery, hence lysosomal enzymes, plays a key role in prolongation of life granting extended youth to the organism. The *daf-2* insulin/IGF-1 pathway mutants were proposed to have a lifespan extension due to the efficient "waste" disposal system (Meléndez et al., 2003). It was speculated that enhanced turnover of cellular material may rejuvenate cells by removal of old and damaged structures (Vicencio et al., 2008). In addition, previous studies demonstrate the importance of

the autophagic process in *daf-2* mutants by RNAi of several autophagy genes (e.g. *bec-1*), resulting in significantly shortening of their lifespan (Meléndez et al., 2003). These facts suggest that the rate of autophagy should be higher in *daf-2* strains than in *daf-16* ones. Controversially, findings of the present study indicate that the activity of lysosomal enzymes such as acid phosphatases and cathepsins are several fold less in long-lived worms in comparison to *daf-16* animals during the entire life. Moreover, it was already reported that the "garbage" recycling system alone is not sufficient in lifespan extension of insulin/IGF-1 mutants (Hansen et al., 2008). It could also hint that recycling or protein turn-over is higher in the *daf-16* mutants compared to the *daf-2* mutants.

The physiological activity of lifespan-extended *C. elegans,* having mutation in the *daf-2* gene, is known to be decelerated at adulthood. The pharyngeal pumping rate and hence the feeding rate, and motility in long-lived *daf-2* mutants were twice as low compared to the normal-lived *daf-16* worms (personal communication – Shanmugam N.). Because of low nutrient uptake and with a possible low protein turnover, *daf-2* mutants may not need to produce and store the lysosome-associated enzymes in a large amount. This may serve as a possible explanation for low enzymatic activity, detected in *daf-2* worms in the current study.

An interesting phenomenon of increased cathepsin and lipase activity in the final stages of life was detected in both the strains. One reason for that may be accumulation of enzymes due to low demand in the already old organism. Although, this hypothesis is not consistent with the acid phosphatase levels, which reveal decreasing trend in age-dependent manner. It seems that cathepsins may be intentionally activated or up-regulated during the final days of life of worms in order to initiate necrosis, result in death of the organism and consequently degrade the dead body. The involvement of cathepsins in cell death was discussed by Turk & Turk (2009). But apoptosis is not in scope of this study.

In the nematode *C.elegans*, the lifespan extension and dauer formation are regulated by the common insulin/IGF-1 signalling pathway. Hence, regulations of specific genes, the expression of which are similar in both dauers and *daf-2* mutants in comparison to *daf-16; daf-2* worms illuminates potential longevity determinants (McElwee et al., 2004). The results of the current work indicate the low activity of acid phosphatase in both stages of *daf-2* populations compared to *daf-16* mutants. Although, the direct comparison of dauers with *daf-2* and *daf-16* adults is not possible in the current study as the former represents an alternative juvenile stage. Thus, it can be logical that dauer larvae did not have sufficient time to generate a comparable amount of acid phosphatases as in adults. On the other hand, the expression of lipase was the same between all three examined populations and the cathepsin activity of dauers was similar to those found in 0-day *daf-2* adults. Recent studies also support the down-regulation of histidine acid phosphatase

and up-regulation of lipolytic enzymes in both *daf-2* dauers and adult worms (McElwee et al., 2004). However, the interpretation of lipase activity in all the examined animals in the current study should be done with caution due to some reasons which are discussed in "Knock-down of lipases" in the "Discussion" section.

Knock-down of acid phosphatases

Three main groups of lysosome-associated enzymes were analysed via RNAi technique in this study. The first group represents acid phosphatases, which catalyze liberation of phosphate groups during the hydrolytic breakdown of phosphomonoesters (Bull et al., 2002; Fukushige et al., 2005). Among 20 acid phosphatase genes in *C. elegans*, the *pho-1* gene is the most studied one. The expression of PHO-1 is limited to the intestinal brush border, which was proposed as a convenient biochemical marker for intestinal development in *C. elegans* (Beh et al., 1991). Not much is known about other acid phosphatase family members.

The present work revealed significant suppression of the global acid phosphatase activity in both *daf-2* and *daf-16* worms while subjected to the *pho-14* knock-down. Hence it can be concluded that this gene contributes to the greatest part of acid phosphatase activity residing in lysosomes of *C. elegans*. On the other hand, *daf-16* worms treated with *pho-14* RNAi lived as long as control ones indicating no effect of this gene on lifespan extension. However, enzymatic assay after RNAi exposure supported the experiment along age series by indicating several fold lower acid phosphatase activity in *daf-2* populations in comparison to *daf-16* ones (in the control treatment).

Recently it was reported that there are differences in expression levels of another acid phosphatase PHO-11 between *daf-2* vs. *daf-16* (McElwee et al., 2003). However, there was no significant down-regulation of acid phosphatase activity in RNAi treatment for this gene in the current study. PHO-11 seems interesting as its knock-down reduced the life duration of *daf-16* and *daf-2* mutants in lifespan analysis, while worms treated on other RNAi bacteria remained unaffected.

Knock-down of cathepsins

The second class of enzymes, used in this study, are cathepsins. Cathepsins represent an essential group of proteases, which act in the breakdown of other proteins. The knock-down of 7 specific cathepsin genes using RNAi did not display any changes in lifespan of both *daf-16* and *daf-2* animals. The measured cathepsin activities in the RNAi treatment were compared to the cathepsin levels of control populations. The cathepsin expression profile was not affected in long-lived worms after RNAi treatment. Similarly, no significant influence of *cpr-1, cpl-1, cpz-*

1 and *asp-6* down-regulations was observed on cathepsin activity in *daf-16* populations (*p*-values of 0.11, 0.32, 0.17 and 0.11 respectively).

The *cpr-1* gene encodes a cysteine protease of the cathepsin B-like cysteine protease family, which is important in embryogenesis (Britton et al., 1996). Unfortunately nothing is known about the role of *cpr-1* after hatching. The cathepsin L family member gene *cpl-1* is responsible for embryonic viability and development of *C. elegans* (Hashmi et al., 2002). It was shown that microinjection with *cpl-1* dsRNA resulted in about 98% of L1 arrested embryos. It would be worth mentioning that during the lifespan assay of the present work few juveniles were detected on plates, when gene expression of *cpl-1* was down-regulated. This may be due to the fact that microinjection into embryos demonstrates stronger effect than feeding on RNAi bacteria from L1 stage. It is also a well known fact that certain RNAi work better in the second or third generation. This gene has to be studied further growing worms on RNAi bacteria for second or third generation. The cathepsin Z family representative *cpz-1* was reported to be localized in hypodermal cells, the expressed enzyme of which functions in the molting pathway by its involvement in the cuticular degradation (Hashmi et al., 2004). Hashmi et al. (2004) reported severe morphological defects and larval arrest, when soak-treated with RNAi bacteria containing *cpz-1* dsRNA. However, the feeding methodology reduced the molting phenotype up to 20%. No observation of this abnormality was found in the present study possibly due to differences in the dsRNA introducing method. Another cathepsin gene the knock-down of which had no effect on nematodes was aspartic protease 6 (*asp-6*). The expression of *asp-6* was detected in the intestine and to a lesser extent in muscles and epithelium (Lochnit et al., 2006). Kamath & Ahringer (2003) revealed no phenotypic changes in *C. elegans* when ASP-6 was knocked-down using RNAi, which is consistent with the findings of the present study. Unfortunately not much is known about the biological function of this gene.

Significant differences were detected in cathepsin activity levels for *asp-3*, T28H10.3 and F57F5.1 knock-down treatments in *daf-16* worms. Aspartic protease 3 (*asp-3*) was shown together with other three proteases to act in neurodegeneration in *C. elegans* (Syntichaki et al., 2002). No information is available about the hemoglobinase-type cysteine proteinase gene T28H10.3 and F57F5.1. Interestingly, the RNAi treatment of the above mentioned genes stimulates cathepsin activity in comparison to the control. On average the induction was as high as 10-fold for both *asp-3* and F57F5.1, and 15-fold for T28H10.3 in comparison to control. These genes seem interesting in terms of cathepsin regulation and further studies are needed to provide an insight in this issue. In *daf-2* mutants knock-down of cathepsin genes did not reveal any differences in activity compared to control (possibly due to very low or negligible activity in *daf-2* worms and hence the difference could not have been detectable). This suggests that the

regulation of cathepsin is different in *daf-16* and *daf-2* mutants. However, the down-regulation of cathepsin genes had no effect on lifespan of neither *daf-2* nor *daf-16* worms. Previous studies propose that there is a lot of redundancy among cathepsins, as down-regulation of a single cathepsin did not affect intracellular protein turnover due to up-regulation of some other cathepsins (Brix et al., 2008; Turk & Turk, 2009; Vasiljeva et al., 2007) which is consistent to our findings.

Because of the leak in expression of the *glp-4* mutation in *daf-16; daf-2* strains in the present study, progeny was observed in the lifespan RNAi treated plates. Surprisingly, no progeny was detected when the expression of *pho-1* was knocked-down even-though eggs were noticed in the worms. This could be due to embryo arrest due to maternal incapability to provide essential nutrients to developing progeny (Fukushige et al., 2005). The absence of progeny was also noticed when the cathepsin gene F57F5.1 and acid phosphatase *pho-11* were knocked-down. Studies need to be done to check if they act in the same way as *pho-1*. However, detection of larval arrest was not in scope of this study, therefore no emphasis or interest was laid on it.

Knock-down of lipases

Lipases represent the third group of enzymes, on which the current work is orientated. RNAi treatment of two lysosome-associated lipases, namely *lipl-4* (K04A8.5) and ZK856.5, was performed. The *lipl-4* gene encodes a triglyceride lipase and seemed as an interesting candidate due to its narrow link with autophagy and lifespan extension (Lapierre et al., 2011; Wang et al., 2008). Recently Wang et al. (2008) demonstrated that prolonged life of germline-less *glp-1* worms and *daf-2* mutants is related with increased expression of the LIPL-4 enzyme. The authors propose that overexpression of this protein is sufficient to promote lifespan extension in germline-deficient animals. Lapierre et al. (2011) reported that there is a link between autophagy and LIPL-4, as autophagy plays an important role in maintaining high lipase activity in germline-less worms. The authors suggest that it is precisely this link that is relevant to longevity in germline-deficient animals. Unfortunately nothing is known about the role and functions of ZK856.5 lipase. The RNAi treatment of both lipase genes in the present study did not give any statistically significant results due to variations. The minor differences in treatment conditions between replicates might have caused such variations, although measures were taken to standardize the whole experimental procedure. Besides, the pH of the working reagent (QuantiChrom^{TM} Lipase assay kit), required for determination of lipase activity, was changed from alkaline to acidic. This was done to measure the activity of only lysosome-associated lipases which have an optimal pH of 4-5 (Zechner et al., 2012). But unfortunately there were some precipitations at the bottom, which were formed due to altered pH. This would have

blocked passage of the signal and may explain why the lipolytic activity did not reveal an increasing trend during the whole assay. A better optimization of the method is required in order to determine the real activity of lysosome-associated lipases.

Influence of bacterial quality and quantity on cathepsin activity

The RNAi treatment of specific cathepsin genes in *daf-16* mutants revealed unexpected results. The control population fed on an empty vector containing bacteria HT115 showed low cathepsin activity in comparison to other populations treated with RNAi bacteria. As this phenomenon was considered suspicious, it was decided to check whether bacteria might influence the global cathepsin levels in *daf-16* worms. For this purpose, three different *E. coli* strains, K-12, OP50 and HT115, were selected as bacterial diet. In addition, two different conditions: fully fed (FF) and dietary restriction (DR) were established in order to test not only the influence of bacterial quality, but also the quantity. In this experiment, *daf-2* populations were considered as a control.

As expected, *daf-2* mutants had negligible or absent cathepsin activity in contrast to *daf-16* worms. The long-lived worms were affected neither by bacterial strains nor by treatment conditions. A comparison of FF HT115 vs. DR HT115 indicates that the cathepsin activity in the control *daf-16* mutants in the RNAi enzyme assay experiment lies in the same range as of FF HT115. This implies that previously obtained data is trustable. There were no differences in cathepsin activity in *daf-16* animals while exposed to different *E. coli* strains in fully fed condition. However, the differences become pronounced in low food condition between K-12 and HT115 treatments, indicating significant increase in global cathepsins while fed with HT115 (p-value <0.02). Moreover, significant differences were detected in DR HT115 vs. FF HT115 (p-value<0.01) indicating that bacterial amount also plays a role in determination of cathepsin levels in *C. elegans daf-16* strain. On the other hand, treatments on K-12 and OP50 did not reveal differences between FF and DR conditions.

There are much more differences between K-12 and OP50 than K-12 and HT115. OP50 is derived from an *E. coli* strain B (Brenner, 1974), while HT115 from K-12 (Takiff et al., 1989). But the current study did not detect any differences in cathepsin levels when worms were fed on OP50 compared to worms fed on other two closely related bacteria. Despite close relationships between K-12 and HT115, the latter one was changed genetically via genetic engineering methods for making it suitable in RNAi assay applications. No differences in cathepsin levels, detected in the current experiment between three *E. coli* strains in FF condition, suggest that bacterial strain does not affect enzymatic activity. On the other hand, the DR condition indicates the reverse (K-12 vs. HT115). DR is a special dietary regimen which restricts the uptake of

calories by an organism and leads to lifespan prolongation in many organisms. Lifespan extension via caloric restriction is an evolutionary conserved pathway from yeasts, flies, *C. elegans* to mammals (reviewed in Meléndez & Levine, 2009). Several earlier studies indicate that DR-mediated longevity correlates with the increased autophagic process (Hansen et al., 2008; Jia & Levine, 2007). The results of the current study also indicate higher cathepsin activity in DR condition for *daf-16* mutants exposed to HT115 compared to FF condition. This increase in cathepsin activity is possibly due to the activation of autophagy in caloric restricted regimen. On the other hand, DR did not reveal any changes in *daf-16* populations fed on K-12 or OP50 in comparison to FF condition. Generally, in *C. elegans* DR can be achieved using mutants defective in their genes, which regulate the rate of pharyngeal pumping (e.g. *eat-2*, *eat-3*, *pha-3*) or in liquid cultures. In the present study, DR was established on plates with live bacteria. HT115 is known to grow and propagate much slower than K-12 and OP50. It seems that the current experiment was successful in establishing and maintaining DR condition for HT115, while it could provide only a mild DR for K-15 and OP50, thus explaining the differences between FF vs. DR conditions for three *E. coli* strains. A possible improvement of the DR-establishing method used for the present experiment can be application of dead but intact bacterial cells to prevent their reproduction.

Moreover, the treatment conditions (NA for K-12 vs. NGM, antibiotics, IPTG for HT115) were not the same which also might have played a role in the cathepsin activity determination. Further studies are required to understand the real reasons underlying this issue.

Conclusion

Several studies indicate that the autophagic process, which enhances protein turnover in cells, as the main secret of lifespan extension in *glp-4 daf-2* worms. However, the present study demonstrates that *glp-4 daf-16; daf-2* animals that lack lifespan extension possess much higher enzymatic activity (acid phosphatases and cathepsins) compared to long-lived *daf-2* worms. In both the strains *pho-14* was found to be the major contributor to the global acid phosphatase level and a knock-down of which leads to lower global acid phosphatase levels. On the other hand, knock-down of cathepsin genes *asp-3*, T28H10.3 and F57F5.1 induce higher cathepsin levels in *daf-16* mutants, while cause no alterations in *daf-2* worms. In addition, cathepsin activity in *daf-16* animals seems to be diet-dependent in terms of quality and quantity. It still needs to be established whether these enzyme activities really contribute to the protein turnover and lifespan extension, and also the role of *pho-11* in prolonging life needs to be analysed in depth.

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