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RESEARCH ARTICLE

Feeding a grape seed extract extends the survival of the red flour beetle *Tribolium castaneum* under heat-stress depending on *nrf-2*, *jnk-1*, and *foxo-1* homologous genes but independent of catechin monomers

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Abstract

Besides caloric restriction, a diet rich in fruits and vegetables is believed to delay the ageing process thus providing a powerfull tool in preventive medicine. To investigate underlying interactions between food ingredients and genes simple models, such as the red flour beetle *Tribolium castaneum*, appear especially constructive. Here we show that 1 % of a grape seed extract containing 30 % of procyanidins, significantly increases the survival of *T. castaneum* at 42 °C when added to flour as a dietary source. The beneficial effects of grape seed extract could not be reproduced by supplementing flour with single catechins of which the oligomeric procyanidins consist. We identified previously stress resistance genes responsible for a survival extension by dietary ingredients and show here by the use of RNA-interference that a knockdown of transcripts encoding homologues of Nrf-2 or Jnk-1 blocks the effects of grape seed extract on survival. Interestingly, grape seed extract under knockdown of Foxo-1 caused a significant survival reduction, stressing the hormetic response as underlying the survival extension by the dietary interventions.

In conclusion, our studies provide evidence that a procyanidin-rich extract is able to extend the survival of the model organism T. *castaneum*. Catechin monomers, however, appear not to mediate the effects. The active ingredients, moreover, need the presence of stress resistance factors, and here especially of Foxo-1, in order to promote their preventive activities with regard to degenerations.

Keywords:

Longevity, stress resistance, *Tribolium castaneum*, food-gene interactions, catechins

INTRODUCTION

Epidemiological studies show that one of the most promising strategies to reduce the burden of non-communicable diseases, such as cancers, cardiovascular diseases, and diabetes, is to choose a diet rich in fruits and vegetables and low in calories [1, 2]. A number of studies have demonstrated in recent years that a reduction in caloric intake is the main

factor involved in reduction of pathology risk [2]. In spite of that knowledge, non-communicable diseases are responsible for about two-thirds of deaths worldwide and it therefore appears that this situation is hardly to change by dietary recommendations [1]. A more constructive strategy could be to simulate molecular mechanisms responsible for the extension of lifespan and healthspan provided by caloric restriction [3]. In this regard, it has been demonstrated that activation of proteins involved in stress response, such as the sirtuins, is crucial for mediating the effects of caloric restriction on hormesis and longevity [4, 5]. Hormesis is defined here as the adaptive response to mild stress that may overcompensate for the stress, thereby protecting the organism from cellular damage that would accumulate in non-stressed individuals and contributes to the ageing process [6]. As a matter of fact a number of phytochemicals appear to promote longevity by inducing the expression of genes encoding cytoprotective proteins, such as chaperones or antioxidant enzymes [3]. Moreover, in humans it was shown that hormetic adaptations induced by exercise could be efficiently blocked by scavenging of reactive oxygen species [7], suggesting that health promoting phytochemicals might be regarded as xenobiotics that similarly to stressful radicals trigger defense mechanisms of the organism.

Using the red flour beetle *Tribolium castaneum* as a model organism, we have recently shown that adding freeze-dried broccoli to the flour they are usually fed, on increases their lifespan at temperatures of 32 °C but also under heat-stress at 42 °C [8]. This diet-induced prolongation of survival was dependent on the functional presence of genes with homologies to human stress resistance genes, such as *jnk-1* or *foxo-1*, but interestingly also of a gene which is homologous to *nrf-2*, encoding a transcription factor necessary for the induction of enzymes involved in phase-II xenobiotic metabolism [9, 10]. In the present study we used *T. castaneum* to assess the effects of feeding an extract containing 30 % oligomeric procyanidins on survival at increased temperatures. In humans, an increase of energy levels which represents a surrogate parameter of a health promoting hormetic effect was shown in response to the consumption of oligomeric procyanidins [11]. Here we asked whether the dietary application of a grape seed extract affects the ultimate parameter of ageing, i.e. the death of the organism. Moreover, we also investigated whether influences on the survival were dependent on distinct genes involved in stress response and detoxification. Therefore, RNA-interference (RNAi) was accomplished. Based on the fully sequenced *T. castaneum* genome [12] we were able to construct PCR-amplified cDNAs with T7 extensions to allow the synthesis of dsRNA for microinjection by *in vitro* transcription.

MATERIALS AND METHODS

Tribolium castaneum maintenance

Wild-type *Tribolium castaneum* strain San Bernardino were reared in darkness on a whole-grain flour diet supplemented with 5 % yeast powder (basal medium) on glass Petri dishes (20 cm diameter) at 32 °C and 70 % relative humidity, as described elsewhere [13].

Grape seed extract

Grape seed extract was from Plantextrakt GmbH & Co. KG, Vestenbergsreuth, Germany, and contained 44.1 % procyanidins as determined by VIS spectroscopy (determination of procyanidins calculated as cyanidine chloride according to Ph. Eur. 7.0). Total phenolics were measured with a modified version of the Folin-Ciocalteu method according to DIN ISO/DIS 14502-1 using gallic acid as a reference. The sample or standard solutions were combined with Folin-Ciocalteu reagent and a saturated sodium carbonate solution before absorbance at 725 nm was assessed after a 60 min incubation time. Total polyphenolics were determined as 45.9 % in the extract. For detection of the monomers HPLC with UV-

detection was used (according to ISO/WD CD 14502-2 mod.). Therefore, the extracts were dissolved and analyzed against an external standard. Catechins are separated by an RP-phenylhexyl column using an acetonitrile-water gradient and detected by UV-detector. Contents of (+)-catechin, (-)-epicatechin, and (-)-epicatechin-gallate were 2.95 %, 1.85 % and 0.18 %, respectively.

Preparation of media

Extracts were ground to fine powder using a mortar and pestle. We mixed 1–100 mg of extract powder with enough basal medium to make the total weight up to 1 g, resulting in final concentrations of 0.5–5 % (w/w). The medium was mixed thoroughly to ensure an even distribution of the vegetable powder. The same procedure was used to spike the feed with isolated catechins (Plantextract GmbH & Co. KG, Vestenbergsreuth, Germany) except that the fine chemicals were first dissolved in 70 % ethanol to make 0.2–20 µg/ml stocks, 5 ml of which was pipetted onto 6-cm Petri dishes loaded with 1 g basal medium to achieve relevant final concentrations. The liquid was allowed to evaporate overnight under a laminar flow hood and then ground to fine powder using a mortar and pestle. Control plates were treated with 70 % ethanol only.

Measurements of food intake

Beetles were starved for 6 h before 10 beetles per group were randomly assigned to feed on one of the grape seed extract concentrations during 2 h, 4 h, or 6 h, after which individuals were frozen at -18 °C. Food uptake was determined by measuring the wet weight of the 10 pooled individuals on a microbalance with 8 replicates per concentration per species.

Determination of survival

Synchronized adult beetles were obtained by distributing at least 50 pupae per condition on Petri dishes containing the basal (control) or supplemented media. Pupae were hatched completely after 4 days and beetles were fed thereafter for 14 days. After 7 days at 32 °C and 70 % humidity, beetles were transferred to new plates containing freshly prepared media. After another 7 days of feeding, beetles were transferred to freshly prepared media and to an incubator at 42 °C and 70 % humidity for the thermotolerance assay. Dishes were checked every 12 h for dead beetles to estimate the survival.

RNA-interference

Gene-specific DNA templates cloned into the pCR-2.1 vector were amplified by PCR using 10 ng of gene-specific RNAi plasmid and corresponding primer pairs containing a flanking T7 promoter sequence as described [8]. A fragment of the *Galleria mellonella* insect *metalloproteinase inhibitor* (*impi*) gene was amplified as a control [8]. Gene-specific primers containing a flanking T7 promoter sequence (5'- TAA TAC GAC TCA CTA TAG GG-3' represented in the following sequences as 'T7') were supplied by Eurofins MWG Operon (Ebersberg, Germany) and had the following sequences: *impi*-fw-5'-T7-AGA CGG TGG AGC CTG CGA TAA TG-3', *impi*-rev-5'-T7- AGA CGA CGG TGG AGG GGA GTC AA-3'; *nrf-2*-fw-5'-T7-TCG CCG TAC CAA TACAGT C-3', *nrf-2*-rev-5'-T7-GTC AGT CAC TCG CAT TCA TC-3'; *jnk-1*-fw-5'-T7- ATG TGA CGC ACG CTA AAA G-3', *jnk-1*-rev-5'-T7-AGG GAA ACA GCA CAT CGG G-3'); *foxo-1*-fw-5'-T7-CAC CAC TCC TAG TCC TAG TTC-3', *foxo-1*-rev-5'-T7-ATG CGG ATA CGA AGG CGA C-3'.

1 µg of purified template DNA was used to produce dsRNA by *in vitro* transcription using the MEGAscript®RNAi kit (Ambion, Austin, USA) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometry using the Nanodrop ND-1000 (peqlab, Erlangen, Germany) and RNA integrity was monitored by 1.4 % denaturing agarose gel electrophoresis.

For RNAi experiments, approximately 0.1 µg of the indicated dsRNA (1 µg/µl) was injected laterally between abdominal segments 3 and 4 into pupae by using a nanoliter

2000 microinjector (WPI, Sarasota, USA). After injection, pupae were transferred to 6-cm Petri dishes containing basal medium with or without supplements.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 80 beetles per group using Trizol reagent (Invitrogen). One-step real-time RT-PCR was carried out in triplicate using 1 µl of RNA template, the Brilliant II SYBR Green 1-step Q-RT-PCR-Kit and appropriate primers in a CFX 96 Real-Time PCR Detection System (BioRad, München, Germany). Changes in the target gene expression were calculated according to Pfaffl [14] using equation $2^{-\Delta\Delta CT}$. For each sample, the fold change in the target gene was normalized to rpS18 RNA and was expressed in relation to control gene expression. To determine RNAi efficiency, unique primer pairs recognizing only cDNA derived from endogenous mRNA were designed to avoid cross-reaction between genomic DNA and the recombinant dsRNA. The primers for rpS18 were fw-5'-ATG GTT GCA AAG CTG AAA CT-3' and rev-TCC CGT GTT GAG TCA AAT TA-3', those for nrf-2 were fw-5'-CAG CAC AAC CAT ACG TAC CAC C-3' and rev-5'-CAA ATG CTC CTC GTC ACC CTT C-3', those for jnk-1 were fw-5'-ACC GTT GAG GTG GGC GAC AC-3' and rev-5'- CGC CCT TTT AGC GTG CGT CA-3', and those for foxo-1 were fw-5'-CCC AAC GAA GAG GGC AAC AAG TGC-3' and rev-5'-GGT TGC CCC AGG CGT TCC GT-3'. Relative mRNA levels compared to the control (imagos injected with impi dsRNA) were estimated in dsRNA-injected imagos after feeding with the control medium over 14 days at 32 °C, and were 0.25 ± 0.04 ($p < 0.05$) for nrf-2, 0.34 ± 0.05 ($p < 0.05$) for jnk-1, and 0.21 ± 0.03 ($p < 0.01$) for foxo-1 RNAi, respectively.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (v5.01, GraphPad, La Jolla, CA, USA) to present the longevity data as Kaplan-Meier survival curves. Survival curves were compared using the Log rank test with a significance threshold of $p < 0.05$. For group comparisons, analysis of variance (ANOVA) was performed. Differences between groups were determined by Bonferroni-Holm multiple comparison test. Results are presented as means ± SD with significance levels of $p < 0.001$ (***). All experiments have been repeated at least twice with similar results.

RESULTS

Grape seed extract increases the survival of **T. castaneum** *under heat stress.*

Beetles were fed on a standard flour diet supplemented with 0.5 %, 1 %, 2 %, and 5 % of a grape seed extract for 2 weeks, and were tested against controls fed on a non-supplemented diet in a humidified chamber at 42 °C. The inclusion of grape seed extract at a concentration of 0.5 % increased the survival of the beetles under heat-stress conditions significantly with an increase of average survival time of 20.3 % from 91.8 ± 20.0 h to 110.5 ± 25.1 h (Fig. 1A). Higher concentrations of 1 % (Fig. 1B) and 2 % (Fig. 1C) did not influence the survival of beetles. Concentrations of 5 % grape seed extract displayed adverse effects in as far as survival of *T. castaneum* at 42 °C was reduced by 15.2 % resulting in reductions of average survival from 99.2 ± 15.9 h to 84.2 ± 16.7 h (Fig. 1D).

Food intake was not affected by the supplementation of 0.5 % or 5 % grape seed extract to the diet (Fig. 2), demonstrating that neither survival extending nor reducing effects of the extract were caused by alterations of caloric intake (Fig. 2).

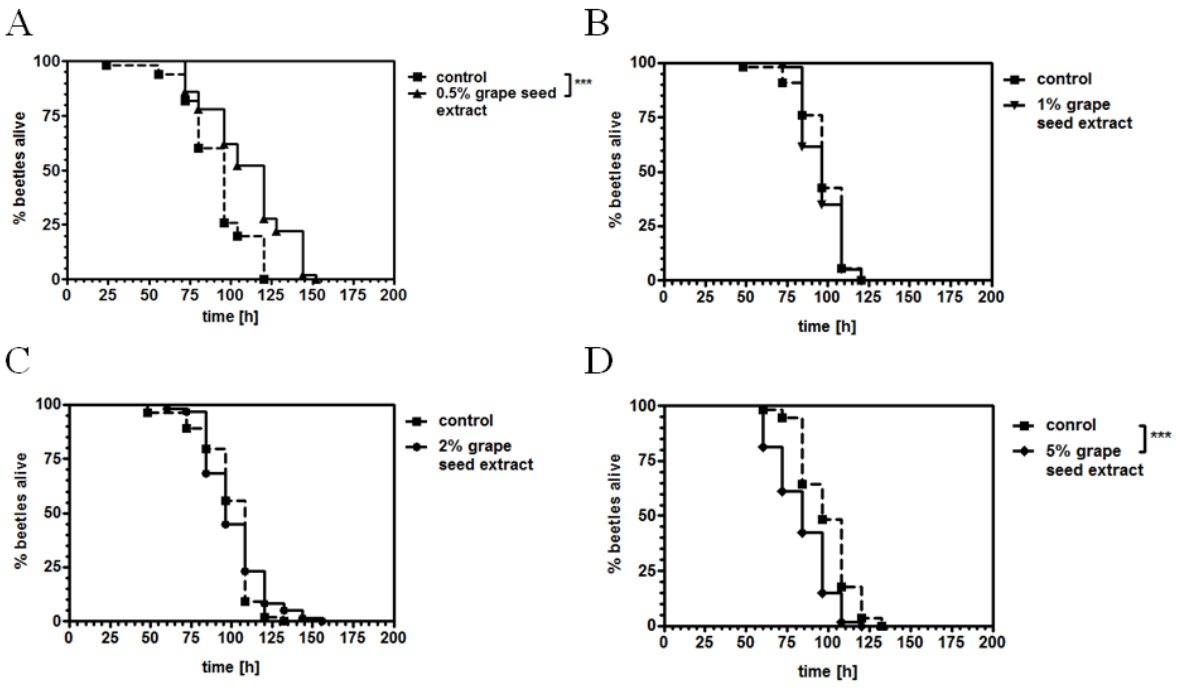

Figure 1: Grape seed extract exerts a dose-dependent effect on survival. Kaplan-Meier survival curves demonstrate the increased survival of beetles fed on a diet supplemented with 0.5 % grape seed extract (n = 50) and then exposed to heat stress (42 °C) compared to beetles fed on a control diet (n = 50) (**A**). Beetles fed for 2 weeks on flour containing 1 % (n = 54 for control, n = 60 for grape seed extract) (**B**), or 2 % grape seed extract (n = 54 for control, n = 60 for extract) (**C**), lived as long at 42 °C as the control. 5 % grape seed extract fed to *T. castaneum* caused a significant reduction of thermotolerance (n = 56 for control, n = 54 for extract) (**D**). A Log-rank (Mantel-Cox) test was used to determine the significance of differences between the survival curves. *** $p < 0.001$ versus the control.

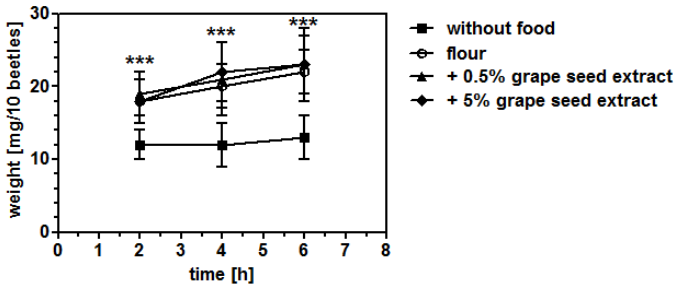

Figure 2: Food intake is not affected by feeding 0.5 % or 5 % grape seed extract. Beetles were starved for 6 h before they were fed on flour alone or supplemented with 0.5 % or 5 % grape seed extract, or in the absence of food (control). After 2 h, 4 h, or 6 h individuals were frozen at -18 °C and food intake was determined by measuring the wet weight of 10 pooled individuals per setting on a microbalance.

Monomeric catechins are not responsible for the extension of survival by grape seed extract

The health-promoting characteristics of grape seed have been attributed to the presence of oligomeric procyanidins, which are oligomeric compounds formed from catechin and epicatechin molecules. In order to test also the building blocks of the oligomeric procyanidins and given that the grape seed extract consisted of 44 % procyanidins we tested the effects of 0.15 % of its constituents (Fig. 3). Those concentrations thus simulated the composition of the applied procyanidins of a single monomer and almost complete hydrolysis by the beetles when the grape seed extract was included at 0.5 %. As shown in Fig. 3, none of the monomeric catechins was able to influence the survival of the beetles when fed over two weeks at 32 °C before temperatures were increased to 42 °C.

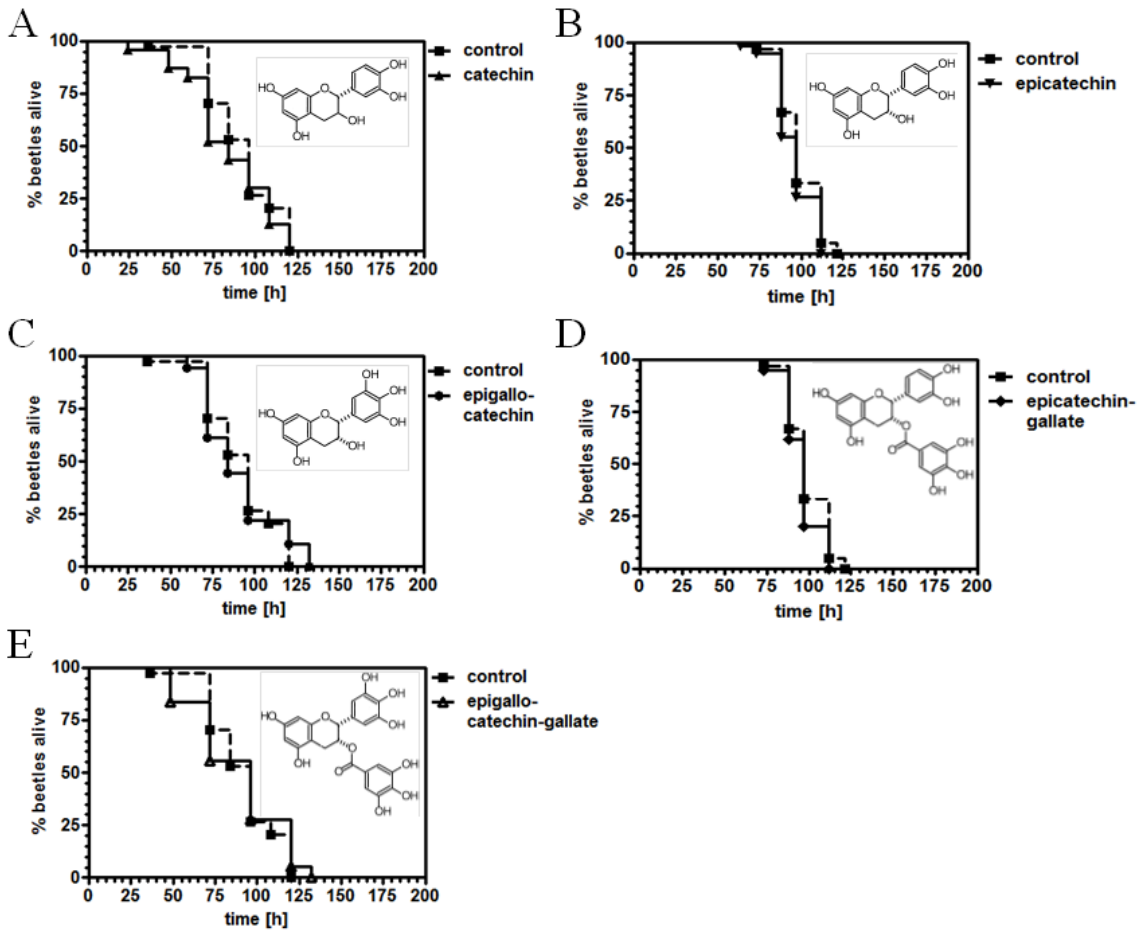

Figure 3: The effect of selected monomeric catechins on the survival of *T. castaneum* at 42 °C, based on their maximal concentration in a flour diet supplemented with 0.5 % grape seed extract. The concentrations were set to 0.15 % catechin (n = 56 for control, n = 54 for catechin) (**A**), epicatechin (n = 60 for control, n = 60 for apicatechin) (**B**), epigallocatechin (n = 56 for control, n = 52 for epigallocatechin) (**C**), epicatechin-gallate (n = 60 for control, n = 60 for epicatechin-gallate) (**D**), or epigallocatechin-gallate (n = 56 for control, n = 54 for epigallocatechin-gallate) (**E**), respectively. Structures of the monomeric catechins have been included into the graphs as insets.

Hormetic adaptations by the grape seed extract are mediated by homologues of nrf-2, jnk-1*, and especially* foxo-1*.*

To search for possible candidates which mediate the survival extending effects of grape seed extract, we focused on Nrf-2, a key transcription factor required for the induction of phase II metabolism in mammalian cells, Jnk-1, a stress-activated protein kinase, and Foxo-1, a transcription factor which increases the expression of stress response and longevity genes and whose nuclear translocation is increased by the phosphorylation through Jnk-1. The importance of the corresponding genes for stress response is demonstrated by the reductions of survival at 42 °C in response to their knockdown by RNAi versus the control, which has been injected with dsRNA from the *Galleria mellonella* gene *impi* for which there is no orthologue in *T. castaneum* (Fig. 4). The reductions of average survival time were 50.4 % (37.3 ± 14.8 h versus 75.1 ± 25.0 h in the *impi* control) due to *nrf-2* RNAi and 35.2 % for *jnk-1* RNAi (48.7 ± 15.4 h). Interestingly, both the knockdown of *nrf-2* and also *jnk-1* prevented an increase of survival of 30.0 % due to feeding 0.5 % of the grape seed extract that was observed in the *impi* dsRNA injected control (Fig. 4A and 4B).

RNAi for *foxo-1* not only prevented the increase of survival by grape seed extract but made the beetles especially vulnerable resulting in a further survival reduction (Fig. 4C). The reduction of survival versus the impi injected control was 39.9 % due to *foxo-1* RNAi (45.2 ± 14.4 h average survival) in the absence of grape seed extract and 59.6 % (30.4 ± 15.4 h average survival) in the presence of 0.5 % grape seed extract.

This result stresses the importance of the *foxo-1* homologous gene and the encoded protein for hormetic adaptations as induced by grape seed extract.

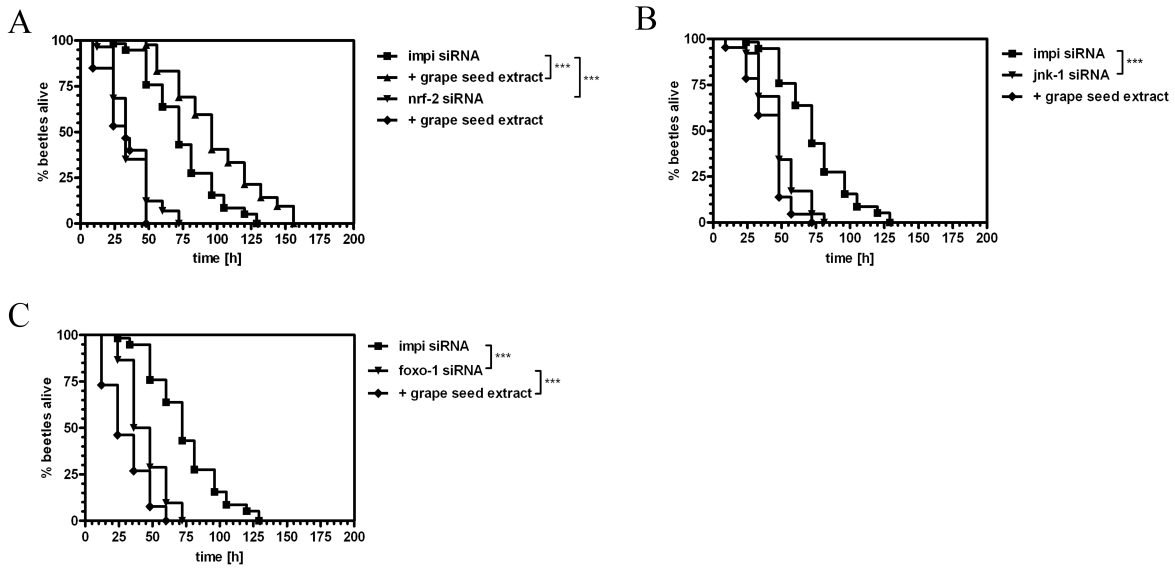

Figure 4: The knockdown of foxo-1 turns the survival extension due to grape seed feeding into a reduction. Transcripts encoding Nrf-2 (n = 58) (**A**), Jnk-1 (n = 65) (**B**), or Foxo-1 (n = 53) (**C**) were knocked down in *T. castaneum* by RNAi, achieved by injecting dsRNA. *Galleria mellonella impi* was used as a control (n = 58) because there is no ortholog in *T. castaneum*. Young adult beetles were fed for two weeks at 32 °C before they were transferred to 42 °C and their survival was recorded. Grape seed extract was added to the flour to reach a final concentration of 0.5 % (n = 52 for the *impi* control, n = 60 for *nrf-2* RNAi, n = 65 for *jnk-1* RNAi, n = 52 for *foxo-1* RNAi) + grape seed extract always refers to the condition mentioned in the line above.

DISCUSSION

Dietary habits can affect health dramatically as exemplified by western-style diets which are associated with increased rates of obesity and the occurence of type- II diabetes, coronary heart disease, and cancer [15, 16]. Most of obesity-resistant inbred mouse strains are also resistant to atherosclerosis, suggesting the existence of common pathways that play an important role in the regulation of energy balance and organismal health [17]. However, besides depending upon interactions between multiple genes and the environment, a high quality diet including functional ingredients is one of the most promising factors in primary and secondary prevention of non-communicable diseases [18]. The prevention of obesity or regain of normal weight must be the major aim of such intervention strategies in humans, based on the convincing outcome that caloric restriction, commonly defined as reducing the energy intake from food by approximately 30 % without limiting the supply of micronutrients, drastically reduces the appearance of age-related diseases in primates [19-21]. Interestingly, those interventions prevent simultaneously the development of many if not all age-related diseases, suggesting that suppression of ageing itself is more rational than to treat each disease separately and often only symptomatically [22]. Besides a necessary change of dietary patterns in order to achieve the beneficial effects of caloric restriction, the identification of compounds that activate the signalling pathways mediating the effects of caloric restriction to increase longevity could enable to reach the goal without dietary restrictions [23]. The red wine polyphenol resveratrol was one of the most potent caloric restriction mimetics identified [24] and has already been shown to induce metabolic changes in obese individuals by mimicking the effects of caloric restriction [25]. It has been shown consistently, that caloric restriction exerts its effects on longevity by the induction of hormesis [26, 27], which refers to the beneficial effects of a treatment that at a higher intensity is harmful [27]. The beneficial effects are manifested at the level of expression of so called vitagenes, which are involved in preserving cellular homoeostasis during stressful conditions [28-30].

To assess health benefits of dietary interventions directly in humans, however, seems complicated due to experimental and ethical conditions. These challenges can be addressed by developing simple experimental models that allow the impact of gene–food interactions on the ageing process to be investigated within a convenient time frame. We here used the red flour beetle *T. castaneum* as a model organism for the investigation of gene–food interactions and their relevance in terms of stress resistance and ageing. The dietary supplement of interest was a grape seed extract that consisted of 30 % oligomeric procyanidins. Oligomeric procyanidins have been identified as the principal vasoactive polyphenols in red wine and to be associated with increased longevity in the population [31, 32]. Also in simple invertebrate models, such as the nematode *Caenorhabditis elegans*, procyanidins were shown to activate vitagenes and to trigger longevity [33]. Grape seeds, moreover, contain also monomeric catechins [32] and non-gallated monomeric forms of them were shown to expand lifespan, presumably through the activation of stress response and repair systems [34].

In the present study we show that the grape seed extract containing 44 % oligomeric procyanidins that was provided at 0.5 % by addition to the flour as a dietary source, increased the survival of *T. castaneum* at 42 °C. Higher concentrations when tested under heat stress displayed either no effect on survival, as observed when added at 1 % or 2 %, respectively, or reduced survival significantly, as observed after feeding 5 % of grape seed extract. These results point to the perception of oligomeric procyanidins as a nutritional threat which triggers a hormetic response. This response, however, appears saturable, in as far as it is not adequate to

compensate for high concentrations of ingested oligomeric procyanidins. That the hormetic response is indeed caused by oligomeric forms of procyanidins is suggested by the lack of survival extending effects when monomeric catechins were fed to the beetles.

The hormetic response of *T. castaneum* to grape seed extract is further stressed by the observation that knockdown of a Foxo-1 homologue not only prevented the extension of survival due to the feeding of 0.5 % grape seed extract but caused a further reduction of survival in comparison to beetles with knocked down Foxo-1 that were fed on flour only. In so far, the importance of Foxo-1 in *T. castaneum* for stress adaptation and longevity reflects the data obtained in Chinese women [35], whereas the role of Foxo-genotypes for longevity in other places is discussed controversial [36]. It is nevertheless noteworthy, that Foxo was made responsible for Hydra's immortality and that an evolutionarily conserved role has been ascribed to the transcription factor in controlling longevity from Hydra to humans [37].

Although the feeding of 0.5 % grape seed extract to *T. castaneum* did not result in reduced survival when Nrf-2 or Jnk-1 was knocked down, RNAi for each factor prevented the survival increasing activity of the plant extract. Therefore, both factors are needed for an adequate stress response as was previously also described for survival extending effects of feeding broccoli in the same species [8] but were demonstrated to cause adaptive cellular stress responses also in other species [3, 38].

In conclusion, our studies demonstrate that feeding a moderate concentration of grape seed extract to the red flour beetle *T. castaneum* induces a hormetic stress response which enables the beetles to tolerate better heat-stress and which increases their survival. Genes encoding for factors necessary for an adequate stress response, such as *foxo-1*, *nrf-2*, and *jnk-1* homologues, were identified to mediate those hormetic effects of grape seed extract. However, the demonstration of survival reducing activities when higher amounts of grape seed extract were ingested shows that hormetic adaptations are saturable and that optimal doses have to be defined.

COMPETING INTERESTS

The authors have declared no conflict of interest.

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