Coffee extract and caffeine enhance the heat shock response and promote proteostasis in an HSF-1-dependent manner in *Caenorhabditis elegans*

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Abstract As the population ages, there is a critical need to uncover strategies to combat diseases of aging. Studies in the soil-dwelling nematode *Caenorhabditis elegans* have demonstrated the protective effects of coffee extract and caffeine in promoting the induction of conserved longevity pathways including the insulin-like signaling pathway and the oxidative stress response. We were interested in determining the effects of coffee and caffeine treatment on the regulation of the heat shock response. The heat shock response is a highly conserved cellular response that functions as a cytoprotective mechanism during stress, mediated by the heat shock transcription factor HSF-1. In the worm, HSF-1 not only promotes protection against stress but is also essential for development and longevity. Induction of the heat shock response has been suggested to be beneficial for diseases of protein conformation by preventing protein misfolding and aggregation, and as such has been proposed as a therapeutic target for age-associated neurodegenerative disorders. In this study, we demonstrate that coffee is a potent, dose-dependent, inducer of the heat shock response. Treatment with a moderate dose of pure caffeine was also able to induce the heat shock response, indicating caffeine as an important component within coffee for producing this response. The effects that

we observe with both coffee and pure caffeine on the heat shock response are both dependent on HSF-1. In a *C. elegans* Huntington's disease model, worms treated with caffeine were protected from polyglutamine aggregates and toxicity, an effect that was also HSF-1-dependent. In conclusion, these results demonstrate caffeinated coffee, and pure caffeine, as protective substances that promote proteostasis through induction of the heat shock response.

Keywords Coffee · Caffeine · *C. elegans* · Heat shock response · Huntington's disease · HSF-1 · HSP70

Introduction

The average life expectancy of the population increases each year, resulting in a need to uncover interventions to delay the onset of aging-related diseases. Caffeine is a bioavailable compound that is consumed in large quantities worldwide with implications in promoting human healthspan and aging-associated neuropathologies (Cunha and Agostinho 2010). Moderate caffeine consumption has been suggested to promote protection against numerous neurodegenerative disorders including Alzheimer's disease, dementia, and Parkinson's disease (Ascherio et al. 2001; Eskelinen and Kivipelto 2010). Additionally, epidemiological studies have correlated moderate caffeine consumption with improved memory (Hameleers et al. 2000) and reduced cognitive decline and mortality (Paganini-Hill et al. 2007; Santos et al. 2010). Thus, moderate caffeine consumption has been suggested to promote healthy aging and longevity in humans.

Studies in the multi-cellular nematode *Caenorhabditis elegans* have demonstrated the positive impact of caffeine on longevity. Caffeine was uncovered as a longevity-

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promoting substance in a screen aimed at uncovering FDA-approved compounds that could extend the *C. elegans* lifespan (Lublin et al. 2011). The lifespan extension observed in response to caffeine treatment was found to be temperature- and dose-dependent and mediated in part through the insulin-like signaling pathway (Bridi et al. 2015; Lublin et al. 2011; Sutphin et al. 2012). Additionally, a worm Alzheimer's model treated with caffeinated coffee extract was protected against β -amyloid-induced paralysis, an effect that was found to be dependent on the oxidative stress response factor SKN-1 (Dostal et al. 2010). Taken together, these results suggest that caffeine and coffee treatment may act through conserved longevity pathways to promote healthy aging in *C. elegans*.

The cytoprotective heat shock response (HSR) is a highly conserved response employed by cells exposed to protein denaturing stressors, such as heat, which functions to maintain proteostasis (Gidalevitz et al. 2011). Vertebrates express for major heat shock transcription factors (HSF1–4), with HSF1 being the critical factor for the response to heat (Dayalan Naidu and Dinkova-Kostova 2017). During stressful insults, HSF1 enhances the expression of heat shock protein (*hsp*) genes which encode chaperone proteins. Chaperones serve multiple cytoprotective functions including the prevention of aggregate formation, promotion of protein folding, and mediation of protein degradation (Frydman 2001; Hartl et al. 2011). Regulation of the HSR by HSF1 is thus an important cytoprotective mechanism utilized during heat stress to promote survival.

C. elegans contains one HSF transcription factor, HSF-1 (Garigan et al. 2002). HSF-1 is an essential protein whose activity is required for heat shock and proteotoxicity responses, larval development, innate immunity, and regulation of adult lifespan (Barna et al. 2012; Hsu et al. 2003; Morley and Morimoto 2004; Silva et al. 2011; Singh and Aballay 2006). *C. elegans* HSF-1 is structurally similar to vertebrate HSF1, containing an N-terminal DNA binding and trimerization domains and a putative transactivation domain at the C-terminus (Hajdu-Cronin et al. 2004). The same activity steps required for mammalian HSF1 activation, including trimerization, hyperphosphorylation, induction of DNA binding, and stress granule formation, are also required for worm HSF-1 activation by heat shock (Chiang et al. 2012; Morton and Lamitina 2013). Knockdown of *hsf-1* in *C. elegans* decreases lifespan and induces rapid aging, while its overexpression increases lifespan (Hsu et al. 2003; Morley and Morimoto 2004; Morton and Lamitina 2013). Also, increased expression of *hsp-70* in a *C. elegans* Huntington's disease model protects against protein aggregate formation and its associated toxicity (Satyal et al. 2000). Thus, enhancing HSF-1 activity may promote longevity and proteostasis.

Many diseases of protein quality control, such as neurodegenerative disorders, are associated with the misfolding, aggregation, and accumulation of disease-associated

proteins. Neurodegenerative diseases have been associated with a decline of the HSR, and decreased proteome maintenance, during the process of aging (Muchowski 2002; Muchowski and Wacker 2005; Westerheide and Morimoto 2005). In order to combat diseases of protein quality control, the identification of compounds that can be harnessed therapeutically to activate the HSR has been an active area of research over the past decade. However, many of the compounds currently known to modulate HSF1 activity are not therapeutically feasible due to cytotoxicity and bioavailability issues (West et al. 2012). Therefore, uncovering alternative HSR activators may assist in the design of new potential therapies for diseases of protein conformation, and aging, such as neurodegenerative disorders.

An interesting characteristic of HSR activators is that they often function synergistically with heat shock (HS) to enhance induction of the HSR. For example, the anti-inflammatory drug indomethacin synergizes with a mild heat stress to increase *hsp* induction (Lee et al. 1995). Similarly, the triterpenoid celastrol (Westerheide et al. 2004), the inflammatory pathway intermediate arachidonic acid (Jurivich et al. 1994), the hydroxylamine derivative bimoclomol (Torok et al. 2003), the caloric restriction (Raynes et al. 2012), and the pyrimidine analog fluorodeoxyuridine (Brunquell et al. 2014) can also function synergistically with HS to enhance *hsp* expression compared to treatment with each compound alone. Thus, many currently known HSR activators have synergistic effects on *hsp* expression when combined with a heat stress.

In this study, we demonstrate caffeine as a component in coffee that mediates induction of the HSR in *C. elegans*. Using *hsp-70* as a marker for induction of the HSR, we show a dose-dependent role for caffeine in inducing the HSR alone and together with HS, greater than that of decaffeinated or caffeinated coffee extracts. Furthermore, a *C. elegans* Huntington's disease model was protected from polyglutamine aggregation and toxicity in response to treatment with caffeinated coffee extract, and even more so in response to treatment with a moderate dose of pure caffeine, an effect that is dependent on HSF-1. We therefore conclude that caffeinated coffee and, to a greater extent, caffeine are protective compounds that can induce the HSR and suppress age-associated protein aggregation in *C. elegans*.

Materials and methods

C. elegans strains and maintenance

The wild-type N2, p*C12C8.1*::GFP (Morley and Morimoto 2004), and Q35::YFP (Morley et al. 2002) strains were used in this study. Worms were maintained at 23 °C on standard nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50. A synchronous population of

nematodes was obtained by standard 20% hypochlorite treatment and a 24-h rotation at 220 rpm in M9 buffer without food.

RNAi feeding

Synchronous L1 nematodes were plated onto standard NGM plates supplemented with 25 µg/mL ampicillin and 1 mM isopropyl-beta-D-thiogalactopyranoside seeded with sequence-verified empty vector control RNAi (control, L4440) or *hsf-1* RNAi isolated from the Ahringer RNAi library (Kamath et al. 2003).

Heat shock conditions

Synchronous nematodes were grown on plates, wrapped in parafilm, and submerged in a 33 °C water bath for either 15 or 30 min as indicated. For qRT-PCR, worms were allowed to recover for 15 min prior to RNA extraction.

Coffee extraction and cafestol and caffeine medium preparation

We performed an aqueous extraction protocol to obtain decaffeinated and caffeinated coffee extract that was developed in the Pallanck laboratory (Trinh et al. 2010). This extraction method was previously determined to contain 0.032 mM caffeine in the decaffeinated coffee extract and 3.6 mM caffeine in the caffeinated coffee extract (Trinh et al. 2010). Briefly, 18.48 g of caffeinated and decaffeinated Starbucks House Blend whole bean coffee was ground for 3 min, placed into boiling water for 30 min, filtered with a French press, and sterilized with a 0.2-µm filter. Coffee extract was then added at a final volume of 10% to plates. The indicated concentrations of pure caffeine (Fisher Scientific, cat# S93153) were added to NGM prior to autoclaving, similarly to Sutphin et al. (Sutphin et al. 2012). The indicated concentration of cafestol (Sigma, cat# C1305) was added to plates after autoclaving. Worms were exposed to each treatment condition from the L1 larval stage until the L4 larval stage (~40 h) for gene expression analyses, until day 3 of adulthood for polyglutamine aggregate analyses, and until day 5 for the paralysis assay.

RNA preparation and cDNA synthesis

Total RNA was extracted using TRIzol® reagent (Ambion®, cat# 15596-026) by standard protocol and purified using RNeasy columns (QIAGEN, cat# 74104). RNA was reverse-transcribed using a High Capacity complementary DNA (cDNA) Reverse Transcription Kit (Applied Biosystems, cat# 4368814) according to manufacturer's instructions.

cDNA was diluted to 50 ng/µL prior to being used as a template for qRT-PCR.

Quantitative RT-PCR

qRT-PCR was performed with the StepOne Plus Real-time PCR system (Applied Biosystems, cat# 4376600) using iTaq™ Universal SYBR® Green Supermix (BioRad, cat# 1725121) according to the manufacturer's instructions. Data analysis was performed according to standard calculations using the comparative Ct method (Bookout and Mangelsdorf 2003). Relative messenger RNA (mRNA) levels were normalized to *gapdh* and calculated from independent biological triplicates and technical duplicates. Primer sequences are available in Supplementary File 1, Table S1.

Fluorescence microscopy and quantification

Worms were collected in 1 mL of M9 buffer, placed onto a glass slide, and anesthetized with 10 mM levamisole prior to imaging. An EVOS fluorescence microscope was used for phase contrast, GFP, and DAPI imaging. Fluorescence intensity was quantified using ImageJ (ImageJ Software, Bethesda, MD, USA, <http://imagej.nih.gov/ij/>) for 50 worms per treatment condition in independent biological triplicates (Schneider et al. 2012).

Polyglutamine aggregation assay

Synchronous Q35::YFP animals were cultured on control or treatment plates as indicated. Worms were picked daily to new plates until day 3 of adulthood to avoid progeny contamination. At day 3 of adulthood, five worms were lined up side by side on a NGM plate spotted with 10 µL of 10 mM levamisole to induce paralysis. Worms were photographed as described above, and ImageJ (ImageJ Software, Bethesda, MD, USA, <http://imagej.nih.gov/ij/>) was used to apply the triangle method as a means to set an automatic color threshold. Particle analysis was then used to count the number of aggregates of 50 worms per condition in independent biological triplicates (Brunquell et al. 2014; Schneider et al. 2012; Zack et al. 1977).

Paralysis assay

Synchronous Q35::YFP animals were cultured on control or treatment plates as indicated. Worms were picked to new plates daily until day 5 of adulthood to avoid progeny contamination. Paralysis was determined by transferring 100 live worms per condition, in biological duplicates, to a corresponding fresh plate and observing movement within 1 min. Worms that did not move within that time frame were scored as paralyzed.

Statistical analyses

Statistical analyses were carried out with GraphPad Software (GraphPad Software, La Jolla, CA, USA, <http://www.graphpad.com>) using ANOVA followed by the Bonferroni post hoc test. Error bars are representative of standard deviation between independent biological replicates as indicated.

Results

Treatment with caffeinated coffee extract, decaffeinated coffee extract, and cafestol enhances HS-induced *hsp-70* promoter activity

To determine the effects of coffee on the HSR, we grew worms on plates supplemented with caffeinated or decaffeinated coffee extracts, or with the coffee component cafestol, and then assessed *hsp-70* promoter activity by visualizing GFP expression under the control of the HS-inducible *C12C8.1* (*hsp-70*) promoter (*phsp-70::GFP*) (Fig. 1 and Supplementary File 1, Fig. S1). Synchronous *phsp-70::GFP* nematodes were left untreated (control) or subjected to treatment with decaffeinated or caffeinated coffee extract added at a 10% vol/vol to NGM plates, or the indicated doses of cafestol added to the plates, from the L1 larval stage to the L4 larval stage prior to treatment with or without a 15-min HS. As expected, this moderate HS treatment of the control showed a low level of induction of HS-induced GFP expression as compared to the untreated control (Fig. 1a). Treatment

with caffeinated (3.6 mM caffeine) and decaffeinated (0.032 mM caffeine) coffee extracts both enhanced HS-induced GFP expression as compared to the HS-treated control, with the caffeinated coffee extract having a stronger effect on *hsp-70* promoter activity (Fig. 1a). To quantify the GFP expression of the fluorescent images in Fig. 1a, fluorescence intensity was measured for each treatment condition using ImageJ (Fig. 1b). Treatment with decaffeinated and caffeinated coffee extract increased HS-induced fluorescence intensity 10- and 20-fold as compared to the HS-treated control, respectively.

As our data indicates that there is a component present in both caffeinated and decaffeinated coffee that enhances the HSR, we also tested the effect of cafestol, a compound present in both types of coffee extracts, on the HSR. Treatment with cafestol was able to enhance HS induction of *hsp-70* promoter activity in a dose-dependent fashion (Supplementary File 1, Fig. S1). Overall, we conclude that components present in decaffeinated coffee, including cafestol, can enhance the HSR, while caffeinated coffee further increases this effect, suggesting a role for coffee and caffeine in modulating the HSR.

We were next interested in more closely examining tissue-specific induction of the *hsp-70* promoter in response to treatment with caffeinated coffee extract alone (Supplementary File 1, Fig. S2). We observed that GFP protein expression driven by the *hsp-70* promoter::GFP construct accumulates in intestinal gut granules. *C. elegans* gut granules are acidic lysosome-like organelles that serve as sites for fat storage and nutrient metabolism (Coburn and Gems 2013). These granules fluoresce under ultraviolet light due to the accumulation of glycosylated anthranilic acid and can be visualized with a

Fig. 1 Treatment with coffee extract enhances HS-induced *hsp-70* promoter activity. **a** Fluorescent images are shown of synchronous *phsp-70::GFP* nematodes left untreated (control) or subjected to treatment with decaffeinated and caffeinated coffee extract (added at a 10% vol/vol to NGM plates), from the L1 larval stage to the L4 larval

stage prior to treatment with or without a 33 °C 15-min heat shock (HS) followed by a 12-h recovery. **b** The GFP intensity of 50 worms given the same treatment conditions in **a** was quantified using ImageJ, and significance was determined using the Bonferroni post hoc test compared to the control and between treatment conditions. * $p < 0.05$; *** $p < 0.001$

DAPI filter (Coburn and Gems 2013). Corresponding DAPI images are therefore shown to visualize the location of gut granules. Overlay of the GFP/DAPI images confirmed the localization of GFP expression to these intestinal granules in response to treatment with caffeinated coffee extract. Thus, treatment with caffeinated coffee extract results in tissue-specific activation of the *hsp-70* promoter in gut granules in the absence of stress, and enhancement of *hsp-70* promoter activity throughout the worm during HS.

Treatment with caffeinated coffee extract induces *hsp-70* mRNA expression with and without HS, and treatment with decaffeinated coffee extract enhances HS-induced *hsp-70* mRNA

We next used qRT-PCR to measure the expression of the endogenous *hsp-70* family members *C12C8.1*, *F44E5.4*, and *F44E5.5* in response to treatment with caffeinated and decaffeinated coffee extracts (Fig. 2). Synchronous wild-type worms were left untreated (control) or subjected to treatment with decaffeinated or caffeinated coffee extract (added at a 10% vol/vol to NGM plates) from the L1 larval stage to the L4 larval stage prior to treatment with or without a 15-min HS. As expected, HS treatment of the control increased the expression of each *hsp-70* family member as compared to the untreated control. Consistent with the results in Fig. 1, decaffeinated coffee extract did not induce *C12C8.1* mRNA expression upon treatment alone, but was able to enhance HS-induced *C12C8.1* mRNA expression 6-fold as compared to the HS-treated control. Additionally, treatment with caffeinated coffee extract alone induced *C12C8.1* mRNA expression 3-fold and enhanced HS-induced *C12C8.1* mRNA gene expression 35-fold. We also observed that treatment with caffeinated coffee extract had a stronger effect (13-fold) on *C12C8.1* mRNA

expression compared to treatment with decaffeinated coffee extract (Fig. 2a). A similar trend is also observed for the *hsp-70* family members *F44E5.4* and *F44E5.5* (Fig. 2b–c). Treatment with caffeinated coffee extract therefore has a stronger effect on inducing *hsp-70* mRNA expression and enhancing *hsp-70* mRNA expression upon HS, as compared to treatment with decaffeinated coffee extract. Thus, while these data thus further suggest a role for caffeine as a component in coffee that can activate the HSR, there must be a component in decaffeinated coffee that can synergize with HS to activate the HSR.

Treatment with pure caffeine robustly enhances *hsp-70* mRNA expression in a dose-dependent manner

We next determined the effects of pure caffeine on the HSR. Before initiating these studies, we first wanted to determine whether caffeine treatment affects worm development. Synchronous wild-type worms were left untreated (control), treated with caffeinated coffee, or subjected to treatment with low (0.5 and 1 mM), moderate (3.6 mM), and high (5 and 10 mM) doses of pure caffeine, from the L1 larval stage to the L4 larval stage prior to a 30-min HS treatment. We observed that low and moderate doses of caffeine did not affect the rate of development of the worm, whereas high doses stunted development (Supplementary File 1, Fig. S3). We therefore focused our studies on low and moderate doses of caffeine to avoid changes in the developmental stages of the worms from affecting our data. qRT-PCR was then performed for the *hsp-70* family members *C12C8.1*, *F44E5.4*, and *F44E5.5* in response to treatment with 0.5, 1, and 3.6 mM of caffeine (Fig. 3). As expected, HS treatment increased the induction of each HS-inducible *hsp-70* family member (80–200-fold) as compared to the untreated control. Similar to the trend observed in Fig. 2, treatment with caffeinated coffee

Fig. 2 Treatment with caffeinated coffee extract enhances *hsp-70* mRNA expression greater than decaffeinated coffee. **a–c** mRNA expression for the *hsp-70* genes *C12C8.1*, *F44E5.4*, and *F44E5.5* was determined with qRT-PCR using synchronous wild-type worms left untreated (control) or subjected to treatment with decaffeinated and caffeinated coffee extract (added at a 10% vol/vol to NGM plates), from the L1 larval stage to the

L4 larval stage prior to treatment with or without a 33 °C 15-min heat shock (HS) followed by a 15-min recovery. Results are representative of technical duplicates from biological triplicates, and significance was determined using the Bonferroni post hoc test compared to the control and between treatment conditions. * $p < .05$; *** $p < 0.001$

extract alone induced the expression of each *hsp-70* family member (5–20-fold) and enhanced the expression of each *hsp-70* family member when combined with HS (150–300-fold), compared to the respective controls. Interestingly, pure caffeine treatment has a robust, and dose-dependent, effect on *hsp-70* mRNA expression compared to treatment with caffeinated coffee extract. In fact, treatment with 3.6 mM pure caffeine alone induced *C12C8.1* mRNA expression (50-fold) similarly to that of HS (80-fold), indicating caffeine as a strong inducer of the HSR (Fig. 3a). Additionally, treatment with 3.6 mM pure caffeine (the amount of caffeine resulting from our caffeinated coffee extract protocol) resulted in the largest enhancement of HS-induced *C12C8.1* mRNA expression, compared to treatment with caffeinated coffee extract and low-dose caffeine treatment. A similar trend is also observed for the *hsp-70* family members *F44E5.4* and *F44E5.5* (Fig. 3b, c). These data thus demonstrate caffeine as a component in coffee that induces and enhances the HSR.

Induction of *hsp-70* mRNA expression in response to treatment with caffeinated coffee extract and moderate caffeine is dependent on HSF-1

We next used qRT-PCR to assess the role of HSF-1 in regulating *hsp-70* mRNA expression in response to treatment with caffeinated coffee extract and pure caffeine. Wild-type worms were either left untreated or subjected to treatment with caffeinated coffee extract, or 3.6 mM pure caffeine, from the L1 larval stage to the L4 larval stage prior to treatment with or

without a 30-min HS in the presence of control or *hsf-1* RNAi (Fig. 4). As expected, HS treatment of the control increased the expression of each *hsp-70* family member as compared to the untreated control in an HSF-1-dependent manner. Consistent with the results in Fig. 3, treatment with caffeinated coffee extract and 3.6 mM caffeine induced *C12C8.1*, *F44E5.4*, and *F44E5.5* mRNA expression upon treatment alone, and also collectively with HS (Fig. 4, black bars). Additionally, the ability of worms to enhance *hsp-70* mRNA expression in response to caffeinated coffee extract and pure caffeine treatment is dependent on HSF-1. Worms fed *hsf-1* RNAi showed a 500–1500-fold decrease in their ability to enhance *hsp-70* mRNA expression upon treatment with caffeinated coffee extract or 3.6 mM caffeine during HS (Fig. 4, blue bars). Thus, induction of the HSR in response to caffeinated coffee extract and pure caffeine treatment is dependent on HSF-1.

Caffeinated coffee extract and pure caffeine treatment protect a *C. elegans* Huntington's disease model against polyglutamine aggregation and toxicity in an HSF-1-dependent manner

We were next interested in testing the effects of caffeinated coffee extract and 3.6 mM pure caffeine alone, in the absence of HS, on proteostasis by observing protein aggregate formation in a *C. elegans* Huntington's disease model (Fig. 5). The Huntington's disease model we used harbors 35 polyglutamine repeats fused to YFP under the control of a muscle promoter (Q35::YFP) and develops insoluble protein aggregates in the

Fig. 3 Treatment with pure caffeine enhances *hsp-70* mRNA expression greater than caffeinated coffee extract in a dose-dependent manner. **a–c** mRNA expression for the *hsp-70* genes *C12C8.1*, *F44E5.4*, and *F44E5.5* was determined with qRT-PCR using synchronous wild-type worms grown on standard plates left untreated (control), and plates supplemented with caffeinated coffee extract (for comparison) or various doses of pure caffeine, as indicated, from the L1 larval stage to

the L4 larval stage before treatment with or without a 33 °C 30-min heat shock (HS) followed by a 15-min recovery. The amount of caffeine previously found to be present in the protocol we used to obtain our caffeinated coffee extract is 3.6 mM (Trinh et al. 2010). Results are representative of technical duplicates from biological triplicates, and significance was determined using the Bonferroni post hoc test compared to the control and between treatment conditions. * $p < 0.05$; *** $p < 0.001$

Fig. 4 Induction of *hsp-70* mRNA expression in response to treatment with caffeinated coffee extract and caffeine is dependent on HSF-1. mRNA expression for the *hsp-70* genes *C12C8.1*, *F44E5.4*, and *F44E5.5* was determined with qRT-PCR using synchronous wild-type worms grown on RNAi plates fed empty vector RNAi (control) or *hsf-1* RNAi to knockdown gene expression. Worms were then left untreated (control) or grown on plates supplemented with caffeinated coffee extract

(added at a 10% vol/vol to NGM plates) or 3.6 mM pure caffeine, as indicated, from the L1 larval stage to the L4 larval stage before treatment with or without a 33 °C 30-min HS followed by a 15-min recovery. Results are representative of technical duplicates from biological triplicates, and significance was determined using the Bonferroni post hoc test compared to the control and between treatment conditions. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

body wall muscle in an age-dependent manner (Morley et al. 2002). Synchronous Q35::YFP worms were left untreated (control) or subjected to treatment with caffeinated coffee extract or 3.6 mM pure caffeine from the L1 larval stage until day 3 of adulthood in the presence of control or *hsf-1* RNAi. We observed a decrease in punctate-YFP aggregation upon treatment with caffeinated coffee extract and 3.6 mM pure caffeine, an effect that is dependent on HSF-1 (Fig. 5a). ImageJ was used on threshold-adjusted images to allow quantification of the number of aggregates per worm for each treatment condition (Fig. 5b). Treatment with caffeinated coffee extract and 3.6 mM pure caffeine suppressed aggregate formation by a magnitude of 10 and 15 less aggregates per worm, respectively, compared to the control (Fig. 5b, black bars). The decrease in aggregate formation observed in response to treatment with caffeinated coffee and 3.6 mM pure caffeine was dependent on HSF-1, as worms cultured in the presence of *hsf-1* RNAi did not exhibit a decrease in aggregate formation in response to either treatment condition (Fig. 5b, blue bars). These data suggest that treatment with caffeinated coffee extract and pure caffeine protects against polyglutamine aggregate formation in a *C. elegans* Huntington's disease model in an HSF-1-dependent manner.

We next assessed the toxicity associated with polyglutamine expansions by observing paralysis in the Huntington's disease model in response to treatment with caffeinated coffee extract or pure caffeine (Fig. 5c). Synchronous Q35::YFP worms were left untreated (control) or subjected to treatment with

caffeinated coffee extract or 3.6 mM pure caffeine from the L1 larval stage until day 5 of adulthood in the presence and absence of *hsf-1* RNAi. Treatment with caffeinated coffee extract reduced paralysis by 25%, while 3.6 mM pure caffeine reduced paralysis by 39%, compared to the control (Fig. 5c, black bars). The ability of caffeinated coffee extract and 3.6 mM caffeine to reduce paralysis is abolished in the presence of *hsf-1* RNAi, showing dependence on HSF-1 (Fig. 5c, blue bars). This data correlates to Fig. 5b, suggesting that the number of aggregates present in the worm may be associated with paralysis. Thus, treatment with caffeinated coffee extract and 3.6 mM pure caffeine decreases polyglutamine aggregate formation and paralysis in an HSF-1-dependent manner in a *C. elegans* Huntington's disease model.

Discussion

In this study, we demonstrate caffeine as a component in coffee that activates the HSR and promotes proteostasis in *C. elegans* (for a summary of the results, see Fig. 6). We observe that treatment with caffeinated coffee extract results in greater activation of the HSR as compared to decaffeinated coffee extract, and find a dose-dependent role for pure caffeine in activating the HSR. Furthermore, we show a central role for HSF-1 in regulating the response to caffeinated coffee extract and pure caffeine treatment on *hsp-70* mRNA induction. Additionally, treatment with caffeinated coffee extract and a moderate dose of pure

Fig. 5 Treatment with caffeinated coffee extract and 3.6 mM pure caffeine promotes proteostasis in a *C. elegans* Huntington's disease model in an HSF-1-dependent manner in the absence of HS. **a** Polyglutamine aggregates in a *C. elegans* Huntington's disease model are suppressed upon treatment with caffeinated coffee extract and caffeine. Fluorescent images and threshold-adjusted images (black and white) are shown of synchronous worms expressing 35 polyglutamine tracts fused to YFP (Q35::YFP) under the control of a muscle promoter that were grown on RNAi plates with empty vector RNAi (control) or *hsf-1* RNAi. Worms were left untreated (control) or subjected to treatment with caffeinated coffee extract (added at a 10% vol/vol to NGM plates) or

3.6 mM pure caffeine from the L1 larval stage until day 3 of adulthood. **b** Quantification of polyglutamine aggregates. Aggregates were scored with ImageJ particle analysis using the threshold-adjusted images from **a** for 50 worms per condition in biological triplicates. **c** Treatment with caffeinated coffee extract and 3.6 mM caffeine prevents paralysis in a *C. elegans* Huntington's disease model. Worms given the same treatment conditions in **a** were grown until day 5 of adulthood and a worm was considered paralyzed if no movement was observed in 1 min for 100 worms per condition in biological duplicates. Significance was determined using the Bonferroni post hoc test compared to the control and between treatment conditions. * $p < 0.05$; ** $p < 0.01$

caffeine protects against polyglutamine aggregation and toxicity in a *C. elegans* Huntington's disease model in an HSF-1-dependent manner. Overall, this work suggests that caffeinated coffee extract and moderate caffeine consumption may protect against age-associated neurodegeneration by promoting proteostasis through activation of the HSR.

Caffeine is well known for its bioavailability in natural products including coffee, tea, and chocolate (Oba et al. 2010). The degree of caffeine consumption varies worldwide. The average amount of caffeine consumed in the USA is approximately 168 mg per day, the equivalent of two cups of coffee (Fredholm et al. 1999). Our data suggests that the effects of caffeine are highly dose-dependent and that an optimum dose of caffeine can maximize health

benefits while decreasing health risks. Our treatment conditions consisted of low (0.5 and 1 mM), moderate (3.6 mM), and high-dose (5 and 10 mM) chronic caffeine exposure from the first larval stage to the last larval stage of development. Moderate caffeine treatment enhanced the HSR with no developmental delays, whereas high-dose caffeine treatment severely stunted development. We therefore conclude that moderate chronic caffeine consumption may be beneficial in mammalian systems by promoting proteostasis while avoiding off-target phenotypic effects.

Interestingly, we note that while our caffeinated coffee extract contains ~100× the caffeine present in our decaffeinated coffee extract, caffeinated coffee only increases HS-induced *hsp-70* promoter activity by one third over decaffeinated

Fig. 6 Summary of the effects of caffeinated coffee extract and pure caffeine on the heat shock response and proteostasis in *C. elegans*. In this study, we have found that *C. elegans* treated with caffeinated coffee extract and pure caffeine induce *hsp-70* mRNA expression upon treatment alone, and in combination with HS, in an HSF-1-dependent manner. The level of induction of HSF-1-dependent *hsp-70* mRNA expression observed in response to treatment with caffeinated coffee extract and pure caffeine treatment corresponds to the extent of polyglutamine aggregate suppression and paralysis observed in a *C. elegans* Huntington's disease model. We therefore conclude that moderate caffeine consumption may promote proteostasis through induction of the HSR

coffee. Coffee extract contains many dissolved solutes other than caffeine, including chlorogenic and phenolic acids, aromatic compounds, and diterpene molecules, among others (Farah and Donangelo 2006; Houessou et al. 2005; Moeenfarid et al. 2016). Therefore, caffeine-independent induction mechanisms must exist. We have demonstrated cafestol as a component in decaffeinated and caffeinated coffee extract that can enhance the HSR. We also note that, when assaying effects on *hsp-70* mRNA induction, the effect of 3.6 mM caffeine is higher than the effect of coffee extract, which also contains 3.6 mM caffeine. Therefore, there must be an inhibitor of the HSR present in caffeinated coffee that is not present in pure caffeine. Altogether, these observations suggest that other compounds found in decaffeinated and caffeinated coffee may impact the HSR or the effectiveness of caffeine.

In mammals, the pharmacological effects of caffeine are mediated by stimulation of the central nervous system through non-selective inhibition of neuronal adenosine receptors (Elmenhorst et al. 2012; Nehlig et al. 1992). In *C. elegans*, caffeine-mediated lifespan extension was found to be dependent on adenosine signaling (Bridi et al. 2015), suggesting a partially conserved mechanism. Interestingly, the density of serotonergic receptors, and serotonin levels, are increased in response to caffeine intake in mammals (Shi et al. 1993), while the metazoan HSR is induced through stimulation of serotonergic neurons (Tatum et al. 2015). Therefore, it would be interesting to determine the dependency of adenosine receptors and serotonin signaling in caffeine-mediated induction of the HSR in *C. elegans*.

The metazoan HSR is a highly complex system that employs cell-nonautonomous signaling to maintain proteostasis

between tissues (Pralhad et al. 2008; van Oosten-Hawle et al. 2013). The release of neuropeptides from neurons, such as serotonin, can act as a signaling mechanism to activate the HSR in distal tissues (Tatum et al. 2015; van Oosten-Hawle and Morimoto 2014). Treatment with caffeinated coffee extract results in unique tissue-specific activation of the *hsp-70* promoter in gut granules, a process that may be elicited through coffee-mediated neurostimulation. Gut granules are located predominately in the intestine and are major sites for fat storage and nutrient metabolism (Ashrafi et al. 2003; Roh et al. 2012; Schroeder et al. 2007). The HSR has been linked to the metabolic state of an organism. For example, caloric restriction can synergize with heat to enhance the HSR (Raynes et al. 2012). Although caffeinated coffee extract treatment does not affect *C. elegans* feeding or initiate a secondary caloric restrictive response (Dostal et al. 2010), our data suggests that gut granular metabolic processes may influence the HSR. Further investigation into coffee-induced neuronal stimulation, and the effects on tissue-specific *hsp-70* promoter induction, may uncover novel neuronal receptors that mediate cell-nonautonomous signaling.

C. elegans is a powerful model that can be utilized to uncover therapeutic compounds for neurodegenerative diseases. Many of the compounds found to elicit positive responses in *C. elegans* neurodegenerative disease models have a translational impact in mammalian systems (Chen et al. 2015). Small molecule activators of the HSR have been suggested as possible therapeutic strategies to prevent aggregate-associated neurodegenerative disorders (Balch et al. 2008; Calamini and Morimoto 2012; Neef et al. 2010; Westerheide and Morimoto 2005). HSR inducers often function synergistically with one another to enhance induction of the HSR (Brunquell et al. 2014; Raynes et al. 2012; Westerheide et al. 2004). We can now add coffee extract and caffeine to the list of compounds that induce the HSR alone and that function synergistically with heat to promote induction of the HSR. Ultimately, this finding may aid in the design of potential therapies for diseases of protein quality control.

Overall, this study demonstrates caffeinated coffee extract and a moderate dose of caffeine as activators of the HSR, both alone, and in combination with HS. Our studies suggest that the genetic mechanisms behind the neuroprotective benefits associated with coffee and caffeine supplementation depend, at least in part, on activation of the HSR and HSF-1. Caffeinated coffee extract and moderate caffeine treatments are promising therapeutic options for age-associated neurodegenerative diseases due to bioavailability and low toxicity. Additionally, caffeine readily crosses the blood-brain barrier, making it an ideal therapeutic candidate for neurodegenerative disorders (McCall et al. 1982). Caffeinated coffee extract and caffeine may elicit their protective benefits, in part, through induction of the HSR, thus warranting further studies in mammalian systems.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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