RESEARCH ARTICLE

Phytoestrogens genistein and daidzein affect immunity in the nematode *Caenorhabditis elegans* via alterations of vitellogenin expression

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Scope: Phytoestrogens, such as the soy isoflavones genistein and daidzein, are suggested to beneficially affect lipid metabolism in humans and thereby contribute to healthy ageing. New evidences show that phytoestrogens might slow ageing processes also by affecting immune processes.

Methods and results: We tested in the nematode *Caenorhabditis elegans* the effects of 17β-estradiol, genistein, and daidzein on resistance versus the nematode pathogen *Photorhabdus luminescens* with focus on vitellogenins, which are invertebrate estrogen-responsive genes that encode homologues to ApoB100 with impact on immune functions. Here, we show that the estrogen 17β-estradiol increases the resistance of *C. elegans* versus *P. luminescens* by enhancing vitellogenin-expression at the mRNA and protein level. Knockdown of single out of five functional vits by RNA-interference blunted the life-extending effects under heat-stress of 17β-estradiol, demonstrating a lack of redundancy for the vitellogenins. RNAi for nhr-14, a suggested nuclear hormone receptor for estrogens, displayed no influence on 17β-estradiol effects. The soy isoflavone genistein reduced vitellogenin-expression and also resistance versus *P. luminescens* whereas daidzein increased resistance versus the pathogen in a vitellogenin-dependent manner.

Conclusion: Our studies show that induction of estrogen-responsive vitellogenin(s) by the phytoestrogen daidzein potently increases resistance of *C. elegans* versus pathogenic bacteria and heat whereas genistein acts in an antiestrogenic manner.

Keywords:
C. elegans / Daidzein / Genistein / Innate immunity / Vitellogenin

1 Introduction

Phytoestrogens are secondary plant compounds with similar structure to endogenous 17β-estradiol, suggested to provide atherosclerosis-preventing and cardioprotective activities [1, 2]. One major dietary source of phytoestrogens is soy with the isoflavones genistein and daidzein, as derived by the fermentation process or intestinal processing from the glycosides genistin and daidzin, being predominant phytoestrogens therein [3, 4]. Lowering of low-density lipoproteins (LDL) is a factor discussed to underly the beneficial effects of phytoestrogens that, however, on the other hand has been questioned [5–7]. On a molecular basis, lipid-lowering effects could be accomplished by increased expression of the LDL-receptor [8]. Of course, a diminished atherosclerosis would inevitably influence longevity, but in this regard other mechanisms have been demonstrated as well to be exerted by isoflavones that could delay ageing processes, such as the enhanced expression of longevity-associated genes including Mn-superoxide dismutase [9, 10]. Risk assessments, however, provided evidence that dietary exposure to phytoestrogens, such as daidzein and genistein, poses a relatively

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Abbreviations: ALS, average lifespan; **ER**, estrogen receptor; **ERE**, estrogen-responsive elements; **HRP**, horseradish-peroxidase; **NHR**, nuclear hormone receptor; **PVDF**, polyvinylidene fluoride; **RME**, receptor-mediated endocytosis; **RNAi**, RNA-interference; **VIT**, vitellogenin

higher health risk for humans than synthetic endocrine disrupting compounds [11], indicating that potential health risks of phytoestrogens should not be neglected per se.

Since phytoestrogens also displayed impact on immunity [12] and moreover immunity affects a number of processes which might have an influence on the lifespan of organisms [13, 14], it appeared reasonable to investigate the effects of endogenous estrogen and phytoestrogens genistein and daidzein on immunity and stress-resistance in the nematode *Caenorhabditis elegans*. The relevance of immunity for stress-resistance was depicted from the lifespan of *C. elegans* measured at 37°C subsequently to an exposure versus the nematode pathogenic bacterium *Photorhabdus luminescens* [15]. *Photorhabdus luminescens* uses *C. elegans* as a vector to infect its original host, i.e. insects [16]. In *C. elegans*, the bacteria infect the intestinal tract, where they produce nematicides encoded by toxin-complex genes, such as *tcd* [15]

In the present study, special emphasis was put on exploring the role of estrogen-responsive *vitellogenin*(*vit*)-genes [17], that have been recently shown to act as acute-phase-proteins and possess bacterial binding and inhibiting activities [18]. VITs are a class of yolk proteins that occur in egg-laying vertebrates and in invertebrates [19] that display the closest homologies to human apolipoprotein B-100 [20]. In the invertebrate *C. elegans* five genes, *vit-2—vit-6*, encode members of the VIT family [21]. VITs are necessary for the endocytotic uptake of cholesterol in the intestine and oocytes [22] and their internalization takes place via RME-2 (receptor-mediated endocytosis-2) [23], demonstrating a functional similarity between cholesterol transport in invertebrates and in vertebrates. Finally, we investigated the importance for the nuclear hormone receptor NHR-14 for 17β-estradiol mediated effects since it was shown that expression of VITs is significantly reduced in a strain mutant for NHR-14 [17].

The effects of treatments with 17β-estradiol, genistein, or daidzein on the resistance of *C. elegans* versus *P. luminescens* were investigated by measuring the lifespan of nematodes at 37°C subsequent to infection. Expression effects of the treatments were characterized at the mRNA levels by quantitative PCR (qPCR) and at the protein level by Western blotting. Finally, the influence of single VITs on the immune response and lifespan under heat-stress was assessed by *knockdown* of vit-mRNAs using RNA-interference (RNAi).

2 Materials and methods

2.1 Materials

NucleoSpin Extract II was obtained from Macherey Nagel (Düren, Germany), Trizol RNA-isolation reagent, Topo TA cloning Kit, and SYTOX green nucleic acid stain were from Invitrogen (Karlsruhe, Germany), and T4-ligase from Roche (Grenzach, Germany). Brilliant II SYBR Green QRT-PCR Mastermix-Kit was ordered from Stratagene Products (Waldbronn, Germany). Ninety-six- and 384-well plates were purchased from Greiner Bio-One (Frickenhausen, Germany). Polyclonal antibodies purchased for Western blotting were rabbit-antiactin (Sigma-Aldrich, Hamburg, Germany), and as horseraddish-peroxidase (HRP)-conjugates goat antirat IgG polyclonal (Dianova, Hamburg, Germany), and goat-antirabbit-IgG (Santa Cruz, Heidelberg, Germany). Polyvinylidene fluoride (PVDF) membranes for protein transfer were from Millipore (Schwalbach, Germany).

2.2 *Caenorhabditis elegans* and bacterial strains

Caenorhabditis elegans strains were grown on nematode growth medium (NGM) agar plates carrying a lawn of *Escherichia coli* strain OP50 at 20°C as previously described [24]. Strains wild-type N2, variation Bristol, and the deletion mutant RB2365 *ok3211* [*vit-2*] were obtained from the *C. elegans* Genetics Center, CGC (University of Minnesota, MN). Methods such as freezing nematodes and obtaining synchronous populations using a bleaching method with hypochlorite treatment of egg-laying adults were performed according to standard protocols [25]. RNAi clones were obtained from MRC Gene Service Ltd. (Cambridge, UK) and included a negative control (L4440), vit-3 (F59D8.1), vit-5 (C04F6.1), and nhr-14 (T01B10.4). RNAi clones of vit-2 (C42D8.2) and vit-6 (K07H8.6) were constructed using standard cloning techniques.

In brief, they were generated by isolation the total RNA of *C. elegans* N2 strain with Trizol and a subsequent reverse transcription to cDNA, followed by amplifying the cDNA of *vit-2* and *vit-6*, respectively. Primers used were as follows: vit-2: 5′-CTCAAGAACGAGGAGTGCGAA-3′ (fw), 5′-AAGTGCCG GTCTAGCTTAA-3′ (rev); vit-6: 5′-ACCCCATGCTACTC CGTTCTC-3′ (fw), 5′-GATGGGAGGCAGTAGACGGAG-3′ (rev). The resulting PCR amplification products were cloned by the use of the Topo TA cloning Kit. After amplification, the vit-2 and vit-6 sequences and the PL4440-vector were digested with *HindIII* and *XhoI*, respectively. The digested products were separated by agarose gel electrophoresis and were purified by the NucleoSpin Extract II, before the vit-2 or vit-6 inserts were ligated by T4-ligase in the digested L4440-vector. The vectors were transferred into bacterial HT115(DE3) strains using standard heat shock protocols and plated on antibiotic-containing 2xYT-agar plates. The sequence was verified by custom sequencing with M13 and T7 sequencing primers (JLU, Microbiology Institute, Gießen, Germany).

2.3 RNAi experiments, application of compounds, and infection

RNAi experiments were performed in liquid cultures as previously described [26, 27]. In brief, expression of gene-specific dsRNA in the corresponding RNAi-feeding strain was induced with 1 mM isopropyl-β-d-thiogalactopyranoside for 1

h at 37°C to trigger interference. Subsequently, bacterial cells were washed and resuspended in NGM. A volume of 40 μL equivalent to 5 × 10^7 bacteria of this suspension was dispensed in each well of a 96-well plate, to which 10–15 synchronized L1 larvae were added. In general, L1 larvae reached the adult stage within 3 days of incubation with agitation at 20°C.

17β-estradiol [10 μM], genistein [100 μM], and daidzein [100 μM] were applied as a single dose in liquid culture from L1 stage up to adult stage. Compounds were prepared as stock solutions in ethanol and diluted in the final medium to a maximal ethanol concentration of 1%. Controls were treated with identical amounts of ethanol. For infection, synchronously staged adult egg-laying hermaphrodites were challenged with *P. luminescence* subsp. *Laumondii* strain DSM 15139 [28] that was obtained from DSMZ GmbH (Braunschweig, Germany). In each well, 5 × 10^6 *P. luminescence* bacteria were mixed with 10–15 nematodes in NGM for 24 h and survival rates were determined subsequently within 16 h at 37°C. To assess dead worms, the microplate thermotolerance assay as described below was used.

2.4 Thermotolerance assay

Nematode lifespan at 37°C was determined using a microplate thermotolerance assay as described [29]. In brief, nematodes were washed off the wells with M9-buffer into 15-mL tubes followed by additional three washing steps. In each well of a black 384-well low-volume microtitre plate, 6.5 μL M9-buffer/Tween20 (1% v/v) solution was added. Subsequently, one nematode was dispensed in 1 μL M9 buffer under a stereo-microscope (Breukhoven Microscope Systems, Garching, Germany) into each well and mixed with 7.5 μL SYTOX green (2 μM). To prevent water evaporation, the plates were sealed with Rotilabo sealing film and covered with a lid (Greiner Bio-One). Heat shock (37°C) was induced and fluorescence was measured with the Fluoroscan Ascent fluorometer (Thermo Labsystems, Bonn, Germany) every 30 min. To detect SYTOX green fluorescence, excitation wavelength was set at 485 nm and emission was measured at 538 nm. Nematodes were scored as dead at the second timepoint of significantly raised fluorescence over background values as verified by touch provocation when standardizing the assay.

2.5 qPCR

Total RNA was extracted from 10 000 worms using Trizol. One-step real-time PCR reactions were performed in triplicate using 1 μL of RNA template, Brilliant II SYBR Green QRT-PCR Mastermix-Kit and appropriate primers in a CFXTM Real-Time PCR Detection System (BioRad, München, Germany). Cycling conditions were 1 × (15 min 50°C), 1 × (10 min 95°C), 40 × (30 s 95°C, 15 s 53°C, 30 s 60°C), 1 × (1 min 95°C), 1 × (30 s 53°C), and 1 × (1 min 95°C). Changes in the target gene expression were calculated according to Pfaffl [30] using equation $2^{-\Delta\Delta CT}$. For each sample, the fold change in the target gene was normalized to 18S rRNA and relative to the control expression. For determination of RNAi efficiency, unique primer pairs recognizing only cDNA derived from endogenous mRNA were designed to avoid cross-reaction with genomic DNA and bacterially generated dsRNA. Primers used were as follows: 18S rRNA: 5′-ATGGTTGCAAAGC TGAAACT-3′ (fw), 5′-TCCCGTGTTGAGTCAAATTA-3′ (rev); nhr-14: 5´-AATCGGAAATGAAGAACAGCCGTC-3´ (fw), 5´-ACCTTGTCAGCAAGTGGTACTTCAG-3´(rev); vit-2: 5′-AGATGCCTTCCTTGAACTCC-3′ (fw), 5′-GTTCTTTG AGACCATTCAGCGTC-3′ (rev); vit-3: 5`-TCTGCTTCCCAC ATTCCACAATC-3` (fw), 5`-TCTTGGCGTTCTTGGACCAC TC-3`(rev); vit-5: 5`-CAATACTCTGCTTCCCACATTCCAC-3`(fw), 5´-TCTTGGACCACTCGGCATCTTC-3`; vit-6: 5′-AC CCCATGCTACTCCGTTCTC-3′ (fw), 5′-CAACCTTGACGT TCTTCCACTCC-3′ (rev).

2.6 Western blot

For detection of VIT-6, a YP88 rat-antivit-6 polyclonal antibody (kindly provided by Prof. Thomas Blumenthal, University of Colorado) at a 1:5000 dilution was used and β-actin, as a loading control, was detected with a rabbit-antiactin polyclonal antibody at 1:500. As secondary antibodies goat antirat IgG polyclonal at 1:5000 and goat-antirabbit-IgG polyclonal at 1:500, respectively, were used as HRP conjugate. Groups of 10 000 nematodes were treated with lysis buffer (saccharose 0.8 M, EDTA 1 mMm, Tris-HCL 10 mM, PMSF 0.5 mM) and twice freeze-thawed at −80°C and room temperature, respectively. Lysates were separated on 8.5% SDS-PAGE gels, and proteins were transferred to a PVDF membrane for 1 h at 1.0 mA/cm^2 membrane area. The blots were preblocked in 15 mL of 1% milk in TBS/0.05% Tween 20, and probed with antibodies diluted in blocking solution for 1 h. Following 2 × 5 min washes with TBS-T and 2 × 5 min TBS, and incubation in ECL solution according to the manufacturer's instructions, the radiographic film was exposed to detect the immunoreactive bands. Band intensities were quantified by using ImageJ (NIH).

2.7 Calculations and statistics

Results are presented as the means ± SD. For statistical analysis of differences between two groups, a Student's *t*-test (GraphPad Prism 5.0) was used. For each variable, at least three independent experiments were carried out. The Mantel–Haenszel log-rank test was performed to compare survival rates. All data were evaluated at the significance level $\alpha = 0.05$. Kaplan–Meier survival plots are shown in lifespan experiments. Statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad, La Jolla, CA).

3 Results

3.1 17β-estradiol increases immunity against *P. luminescens* by increased VIT-expression

17β-estradiol at 10 μM caused an increased VIT-expression at the mRNA (Fig. 1A) and protein levels (Fig. 1B). Exposure of the nematodes to *P. luminescens* resulted in a significant reduction of lifespan at 37°C that was almost completely prevented by the addition of 17β-estradiol (Fig. 1C). The estrogen displayed no influence on lifespan under heat-stress in worms fed on *E. coli* (Fig. 1C) demonstrating the specific effects of 17β-estradiol on immunological stress response. Vit-6 RNAi reduced the lifespan in presence of *P. luminescens* and moreover prevented completely the life-extending effects of 17β-estradiol (Fig. 1C). That vit-6 RNAi was functional at the transcript level and protein level was verified by qPCR and Western blotting, respectively (Fig. 1D and E). Moreover, in accordance with a lack of redundancy of VITs, RNAi for vit-2, vit-3, and vit-5 all prevented 17β-estradiol to exert its lifespan increasing effects when measured at 37°C (Table 1). Also in a *vit-2* mutant strain, 17β-estradiol was not able to increase the survival when exposed to *P. luminescens* and heat (Table 1).

3.2 The nuclear hormone receptor NHR-14 does not mediate the life-extending effects of 17β-estradiol in *P. luminescens*-infected nematodes

NHR-14 has been postulated to mediate the effects of 17β-estradiol on VIT-expression. We therefore tested its necessity for lifespan rescue in *P. luminescens*-infected *C. elegans* by 17β-estradiol. In our experiments, RNAi for nhr-14 caused no significant decrease of vit-6 expression at the transcript level (Fig. 2A) as well as at the protein level (Fig. 2B). RNAi for nhr-14 was effective as indicated by relative mRNA amounts

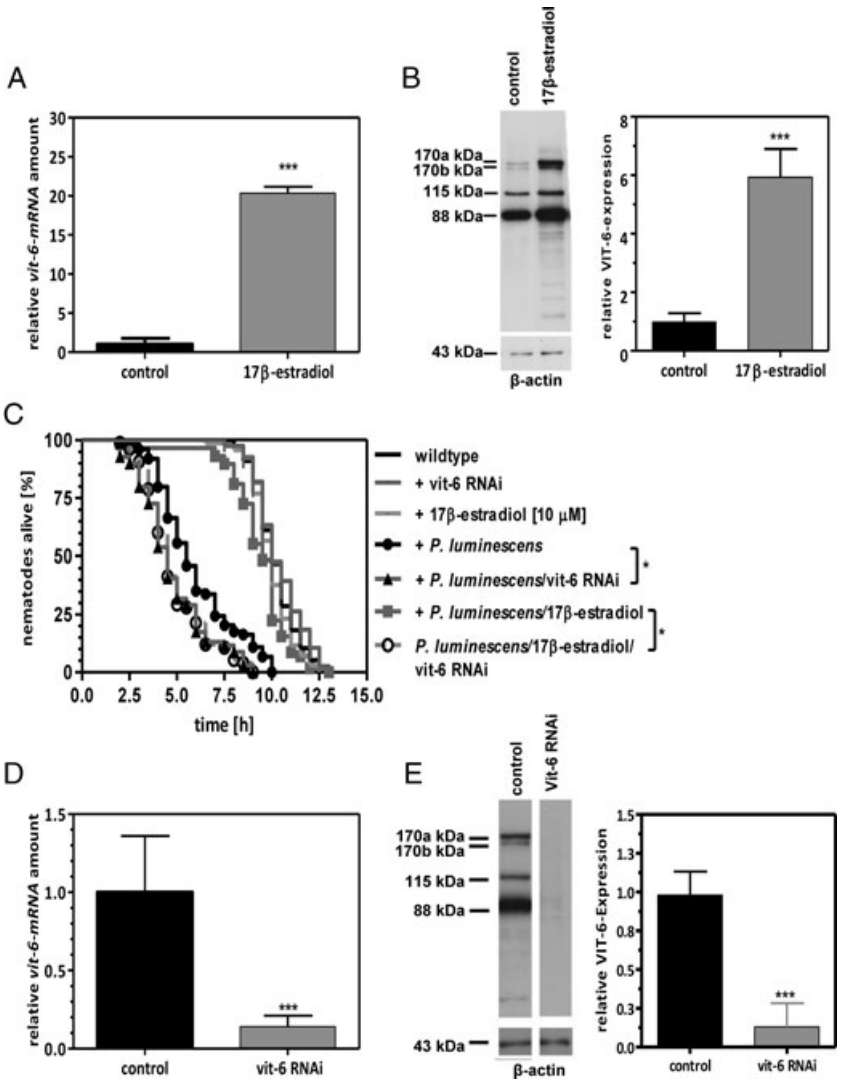

Figure 1. 17β-estradiol increases resistance versus *P. luminescens* through enhanced VIT-expression. (A) qPCR revealed a significantly increased vit-6 mRNA-expression due to exposure of *C. elegans* to 10 μM 17β-estradiol. (B) Western blotting results show increased VIT-expression also at the protein level. β-actin was detected as a loading control for the normalization of intensities of the bands between the different treatments. The vit-6 gene products are 88, 115, and 170 kDa. Quantification of three independent Western blots was carried out using Image J (NIH) software. (C) By incubating the *P. luminescens*-infected wild-type nematodes with 17β-estradiol a significant prolongation of lifespan could be observed. The life-extending effects of 17β-estradiol were no longer present in nematodes with knocked down vit-6. *Statistically significant, demonstrating that the two lifespan populations are different. That vit-6 knockdown is effective at the mRNA level and protein level is given in (D) and (E), respectively. *** $p < 0.001$ versus the control.

Table 1. RNAi of vits prevent the extension of lifespan caused by 17β-estradiol in *P. luminescens*-infected *C. elegans*

Strain	Exposure	PL	RNAi	ALS ± SD [h]	ALS [%]	*N*
Wild-type	–	–	Control	10.2 ± 1.1	100.0	77
N2	17β-estradiol	–	Control	10.5 ± 0.9	102.9	69
	-	+	Control	5.9 ± 2.0	57.8	74
	17β-estradiol	+	Control	8.1 ± 2.6	79.4	58
	-	+	Vit-2	4.8 ± 1.8[a)]	47.1	69
	17β-estradiol	+	Vit-2	4.9 ± 1.6[a)]	48.0	75
	-	+	Vit-3	5.1 ± 0.9[a)]	50.0	57
	17β-estradiol	+	Vit-3	5.3 ± 0.8[a)]	52.0	63
	-	+	Vit-5	4.3 ± 1.3[a)]	42.2	65
	17β-estradiol	+	Vit-5	4.4 ± 1.5[a)]	43.1	66
vit-2	-	–	–	8.9 ± 1.6	87.3	104
(RB2365)	17β-estradiol	–	–	9.3 ± 0.9	91.1	68
		+	–	4.0 ± 1.1[b)]	39.2	79
	17β-estradiol	+	–	3.9 ± 1.2[b)]	38.2	66

ALS ± SD [h] = average lifespan with standard derivation, ALS [%] = percentage change of average lifespan compared to the control, *N* = number of nematodes, ns = not statistically different to control, PL = *P. luminescens.*
a) Statistically significant versus nematodes in the presence of 17β-estradiol and infected with *P. luminescens*, *or*
b) statistically significant versus the vit-2 mutant in the absence of both, *P. luminescens* and 17β-estradiol.

of 0.25 ± 0.06 versus the control ($p = 0.0062$; data not shown). That VITs remained functionally unaffected by nhr-14 RNAi became evident in *P. luminescens*-exposed nematodes, which lived as long as in the absence of nhr-14 RNAi (Fig. 2C). More importantly, nhr-14 RNAi does not prevent the observed immune stimulation by 17β-estradiol, leading to a lifespan similar as observed in the uninfected control (Fig. 2C).

3.3 Genistein acts antiestrogenic in *C. elegans* and increases the sensitivity versus *P. luminescens*

Genistein at a concentration of 100 μM reduced the mRNA levels (Fig. 3A) and also protein levels (Fig. 3B) of VIT-6. Identical regulations of other vit-mRNAs by the phytoestrogen were verified by qPCR (Table 2). Whereas the diminished expression of VITs by genistein was not associated with effects

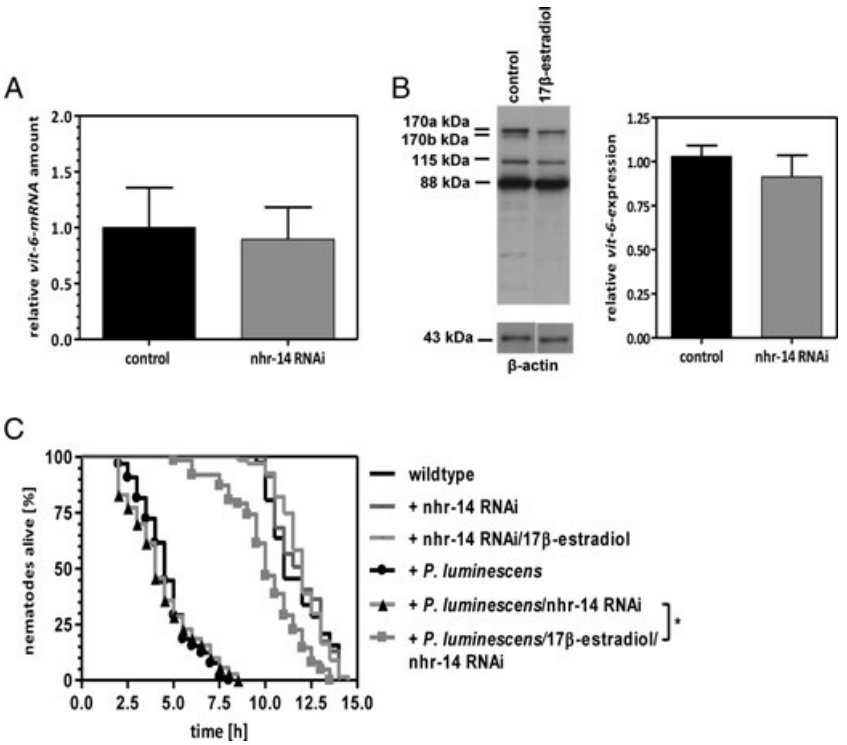

Figure 2. NHR-14 is not involved in the life-extending effects of 17β-estradiol in *P. luminescens*-infected *C. elegans*. (A) Transcript levels for vit-6 are reduced insignificantly as a consequence of nhr-14 RNAi in wild-type nematodes. (B) Western blot analysis revealed a reduction of VIT-6 by 9% at the protein level that did not reach statistical significance. (C) RNAi for nhr-14 displayed no effect on lifespan reduction caused by *P. luminescens* infection nor on lifespan extension due to application of 10 μM 17β-estradiol. *Statistically significant regarding their lifespans.

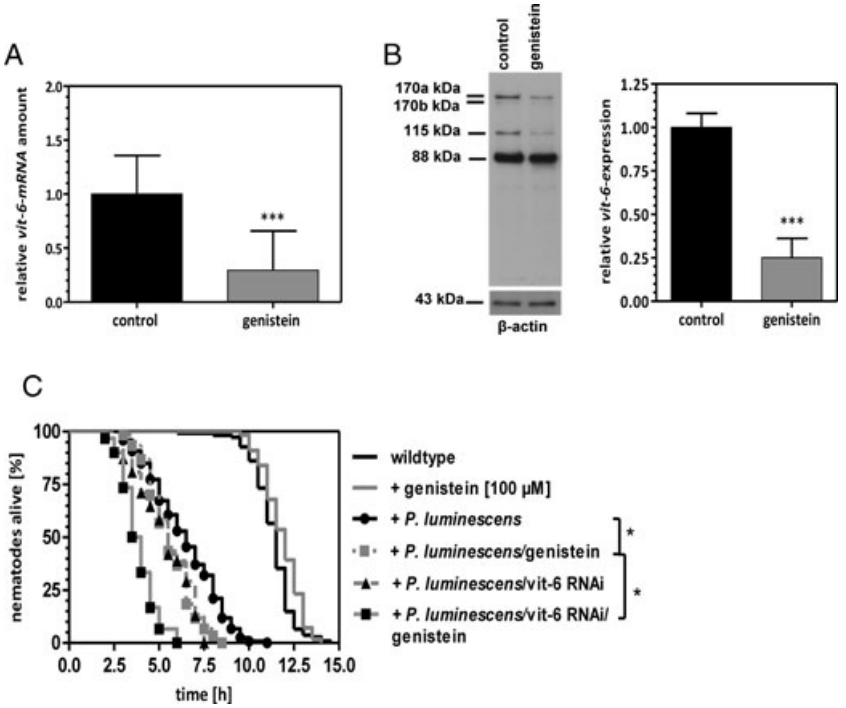

Figure 3. Genistein reduces VIT-6 expression and increases sensitivity versus *P. luminescens*. (A) qPCR analysis showed significantly reduced vit-6 mRNA levels as a consequence of incubation with 100 μM genistein. $^{***}p < 0.001$ versus the control. (B) Diminished VIT-6 expression due to genistein application was evident also at the protein level as assessed by Western blotting. Quantification of three independent Western blots was carried out using Image J (NIH) software. $^{***}p < 0.001$ versus the control. (C) In *P. luminescens*-infected wild-type nematodes, genistein significantly shortened the lifespan of the population to an identical extent as vit-6 RNAi did. Combination of both, genistein and vit-6 RNAi led to a further lifespan decrease. *Statistically different lifespan curves.

Table 2. Regulation of vit-mRNA levels by phytoestrogens genistein and daidzein in *C. elegans*

Exposure	mRNA quantified	$2^{\Delta\Delta CT} \pm$ SD	*N*
Control	Vit-2	1.0 ± 0.3	6
Control	Vit-3	1.0 ± 0.2	5
Control	Vit-5	1.0 ± 0.3	4
Genistein [100 μM]	Vit-2	0.3 ± 0.5*	7
Genistein [100 μM]	Vit-3	0.3 ± 0.2**	5
Genistein [100 μM]	Vit-5	0.4 ± 0.3**	4
Daidzein [100 μM]	Vit-2	4.0 ± 0.5***	5
Daidzein [100 μM]	Vit-3	3.6 ± 0.4***	4
Daidzein [100 μM]	Vit-5	5.1 ± 0.5***	5

N = number of experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the corresponding control.

on lifespan in wild-type nematodes fed on *E. coli*, genistein incubation resulted in a shortened lifespan in the presence of *P. luminescens* (Fig. 3C). The lifespan reduction was identical to that induced by vit-6 RNAi (Fig. 3C) under *P. luminescens* exposure and was reflected by the effects of both treatments on VIT-6 expression (Figs. 1D and E and 3A and B). Genistein application in vit-6 RNAi-treated worms caused an additionally increased sensitivity versus *P. luminescens* (Fig. 3C). The result may be explained at its best by a complete blockade under these conditions, which was verified at the mRNA level (0.05 ± 0.02, $p < 0.001$), whereas detection of VIT-6 at the protein level was no longer possible, as already observed under vit-6 RNAi alone (Fig. 1E).

3.4 Daidzein is protective against *P. luminescens* by increasing VIT-expression

In contrast to genistein, the isoflavone daidzein at a concentration of 100 μM increased VIT-expression significantly at the transcript (Fig. 4A) and protein levels (Fig. 4B). Similar expression stimulation by daidzein was shown for other vits at the mRNA level (Table 2). The estrogenic effects of daidzein, moreover, extended the lifespan at 37°C of *P. luminescens*-treated worms significantly, which was completely blunted by RNAi for vit-6 (Fig. 4C).

4 Discussion

VITs, invertebrate egg yolk proteins with a high grade of homology to mammalian ApoB-100, have emerged in the past as potential determinants of longevity in humans and in nematodes [31]. Since immunity is of central importance for an organism to survive in a harsh environment [12, 32], it appeared plausible to conclude that a link exists between VITs and longevity that is provided by potential effects on the immune status. Although *C. elegans* does not possess an adaptive immune system, the nematodes are able to cope with pathogen attack via their innate immune system [33]. Genetic experiments in *C. elegans* have led to the identification of numerous genes and signaling pathways that can modulate organismal lifespan and immune system function [34, 35]. Importantly, many of these signaling pathways exhibit conserved functions in multiple species, including mammals [36].

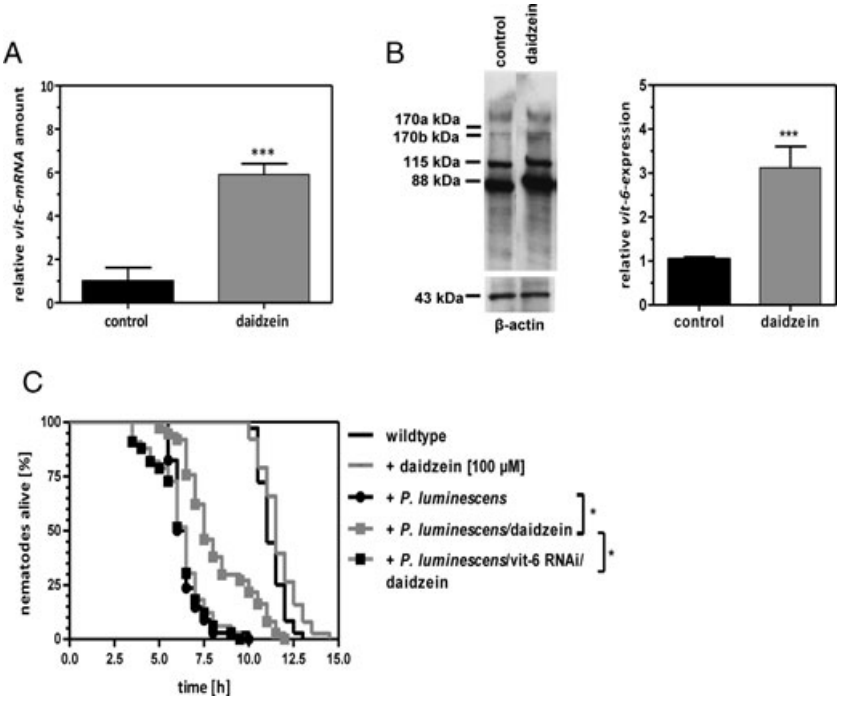

Figure 4. Daidzein acts as an estrogen in *C. elegans*. (A) qPCR analysis indicated a significantly increased vit-6 mRNA expression due to incubation of nematodes with 100 μM daidzein. RNA was isolated, reversely transcribed, and cDNA was analyzed by one-step qPCR using selective primers for vit-6 and 18S, respectively. $^{***}p < 0.001$ versus the control. (B) Western blotting experiments show that VIT-6 protein is increased in *C. elegans* by treatment with daidzein. $^{***}p < 0.001$ versus the control. (C) Lifespan analysis shows a large increase of resistance versus *P. luminescens* by daidzein. The daidzein-induced lifespan extension was completely prevented by vit-6 RNAi. *Statistically a significant difference between the two lifespan populations is indicated.

VITs encompass in *C. elegans* a family of six genes including the pseudogene *vit-1* [21]. In the present study, we show that *knockdown* of vit-6 increases the sensitivity of *C. elegans* versus a reduction of lifespan at 37°C induced by the nematode pathogenic bacterium *P. luminescens*. Identical results were obtained when vit-2, vit-3, or vit-5 was *knocked down*. According to high sequence homologies of VIT family members, especially between vit-2, vit-3, and vit-5, it must be considered that the *knockdown* of single *vit*-genes affects homologous mRNAs as well. Moreover, the functioning of VITs as heteromers [37] could explain the observed results since the cleavage products of VIT-6 to yp115 and yp88 both form complexes with yp170A, which corresponds to the polypeptide chains of VIT-3 to VIT-5 [38]. However, a lack of redundancy of different VITs cannot be excluded and provides the easiest explanation for the observed need of all the functional VITs for appropriate immunity.

Although the precise functional roles of VITs are described rather incompletely they are well known to possess estrogen-responsive elements (ERE) in their promoter gene regions [39]. The highly conserved ERE sequence shares great homology with human sequences and is present in vertebrates and invertebrates [40]. Accordingly, VITs can be properly expressed in the presence of estrogens, as was shown in *C. elegans* for instance [41].

Suggesting that VITs are necessary for an optimal immune status in *C. elegans*, it was the aim of the present study to see whether their induction by 17β-estradiol affects pathogen resistance versus *P. luminescens*. Based on the studies of Custodia et al. [41], we used 10 μM 17β-estradiol which resulted in our experiments in a 20-fold increased VIT-6 expression at the mRNA level and a six-fold increase at the protein level. That other VITs are affected in a similar manner becomes evident by the increased intensity of the bands of 170A and 170B in the Western blot experiments whose detection is due to cross-reactivity of the VIT-6 antibody with yp170A, i.e. VIT-3 to VIT-5, and yp170B, i.e. VIT-2 [35]. The increased expression of VITs provided almost complete protection from *P. luminescens*-induced lifespan reduction under heat-stress. This large prolongation of lifespan must be solely attributed to stimulation of the immunity by 17β-estradiol since the estrogen did not affect lifespan of wild-type nematodes at 37°C in the absence of *P. luminescens* at all. Moreover, the stimulated immunity seems to be mediated solely by enhanced expression of VITs as *knockdown* for vit-6 causes the effects of 17β-estradiol to completely disappear.

Although two classical receptors, ERα and ERβ, have been described to mediate the effects of 17β-estradiol, their activation or inactivation by so-called selective estrogen receptor modulators (SERMs), and their cooperation in different tissues have not been fully understood [42]. Regarding the expression of VITs in many species, ERα reveals to be the isoform necessary for adequate VIT-expression [43, 44]. Information about ERs in *C. elegans* is sparse and so far only NHR-14 has been suggested to function as an estrogenic hormone receptor [17]. The overall role of NHR-14 for an estrogenic response, however, must be questioned based on initial findings that VIT-expression in a nhr-14 mutant was only reduced by 20–30% at the mRNA level [17]. Moreover, using RNAi to *knockdown* nhr-14 in the present study caused no significant decrease of VIT-6 expression at the mRNA and protein levels. In accordance with the suggested

needlessness of NHR-14 for the effects of 17β-estradiol, the estrogen prolonged the thermotolerance in nematodes infected with *P. luminescens* independent on the presence of NHR-14.

Besides endogenous estrogens, those contained in food plants are of major interest as SERMs [44]. Probably the two most popular of those phytoestrogens are the soy isoflavones genistein and daidzein [45]. The effects of isoflavones on the transcriptional ER-activity in vivo must be considered as very complex. Genistein, e.g. dependent on tissue or ER-subtype and endogenous estrogen levels is able to act as estrogen agonist as well as estrogen antagonist [46]. In *C. elegans*, genistein displayed clearly antiestrogenic effects as was demonstrated by a diminished VIT-expression at the protein and also the transcript level. In accordance with the found functionality of VITs in immune response, genistein reduced the lifespan of infected nematodes at 37°C. Both VIT-expression and lifespan in the presence of *P. luminescens* were reduced by genistein and vit-6 RNAi to identical extents, whereas simultaneous application of the phytostrogen and vit-6 RNAi resulted in additive lifespan reduction under heat-stress. These results clearly indicate that finally the levels of VITs determine the extent of lifespan reduction or prolongation in the presence of the pathogen. In contrast to genistein, daidzein, differing from genistein only in the lack of a single hydroxyl group at position C5 in ring A of the phenylbenzopyrone structure, displayed significant estrogenic effects by causing increased VIT-expression at the transcript and protein level in association with increased resistance versus *P. luminescens*. Although various studies had shown different binding affinities of genistein and daidzein to the ERs, their effects were usually in the same directions [47, 48], suggesting that NHR responsible for estrogenic effects in *C. elegans* must be different, at least to some extent, from mammalian ERs.

In conclusion, our studies provide evidence for a significant role of an estrogenic response mediating bacterial immunity in the nematode *C. elegans*. The enhanced immunity as caused by 17β-estradiol was completely mediated by the induction of *vit*-genes. Moreover, the phytoestrogens daidzein and genistein were shown to act estrogenic and antiestrogenic, respectively, and by affecting VIT-expression in an opposite direction, genistein diminishes resistance versus *P. luminescens* whereas daidzein increases it.

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