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**Phenotypic covariance of longevity, immunity and stress
resistance in the *Caenorhabditis* nematodes**

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1 **Abstract**

2 **Background**

3 Ageing, immunity and stresstolerance are inherent characteristics of all
4 organisms. In animals, these traits are regulated, at least in part, by forkhead
5 transcription factors in response to upstream signals from the Insulin/Insulin-
6 like growth factor signalling (IIS) pathway. In the nematode *Caenorhabditis*
7 *elegans*, these phenotypes are molecularly linked such that activation of the
8 forkhead transcription factor DAF-16 both extends lifespan and
9 simultaneously increases immunity and stress resistance. It is known that
10 lifespan varies significantly among the *Caenorhabditis* species but, although
11 DAF-16 signalling is highly conserved, it is unclear whether this phenotypic
12 linkage occurs in other species. Here we investigate this phenotypic
13 covariance by comparing longevity, stress resistance and immunity in four
14 *Caenorhabditis* species.

15 **Methodology/Principal Findings**

16 We show using phenotypic analysis of DAF-16 influenced phenotypes that
17 among four closely related *Caenorhabditis* nematodes, the gonochoristic
18 species (*Caenorhabditis remanei* and *Caenorhabditis brenneri*) have diverged
19 significantly with a longer lifespan, improved stress resistance and higher
20 immunity than the hermaphroditic species (*C. elegans* and *Caenorhabditis*
21 *briggsae*). Interestingly, we also observe significant differences in expression
22 levels between the *daf-16* homologues in these species using Real-Time PCR,
23 which positively correlate with the observed phenotypes. Finally, we provide
24 additional evidence in support of a role for DAF-16 in regulating phenotypic
25 coupling by using a combination of wildtype isolates, constitutively active
26 *daf-16* mutants and bioinformatic analysis.

27 **Conclusions**

28 The gonochoristic species display a significantly longer lifespan ($p < 0.0001$)
29 and more robust immune and stress response ($p < 0.0001$, thermal stress;
30 $p < 0.01$, heavy metal stress; $p < 0.0001$, pathogenic stress) than the
31 hermaphroditic species. Our data suggests that divergence in DAF-16
32 mediated phenotypes may underlie many of the differences observed
33 between these four species of *Caenorhabditis* nematodes. These findings are
34 further supported by the correlative higher *daf-16* expression levels among the
35 gonochoristic species and significantly higher lifespan, immunity and stress
36 tolerance in the constitutively active *daf-16* hermaphroditic mutants.

37

38 **Introduction**

39

40 Longevity is a phenomenon shared by all living organisms but which varies
41 hugely across species and between different sexes of the same species. Several
42 evolutionary theories have been postulated to explain this phenomenon, but
43 the underlying biological regulators of longevity remained largely unknown
44 until pioneering genetic studies using the roundworm *Caenorhabditis elegans*
45 identified the first gene with a substantial role in determining lifespan [1,2,3].

46

47 Given that post-reproductive survival cannot evolve under direct selection,
48 diapause (the entry into a semi-dormant state with low metabolic turnover) is
49 generally perceived as being a by-product of a survival strategy triggered by
50 the organism to outlive harsh conditions so that, upon encountering a suitable
51 environment, reproduction can be resumed [4]. One such strategy employed
52 by the *Caenorhabditis* nematodes is to enter into a temporary, developmentally
53 arrested dauer stage. [5,6,7]. In *C. elegans*, this phenomenon is regulated by
54 the IIS (Insulin/Insulin-like growth factor (IGF) signalling) pathway, which

55 consists of a transmembrane protein DAF-2 [8], several intracellular kinases
56 and the DAF-16 transcription factor [9]. When inactivated, this pathway not
57 only extends lifespan but also regulates resistance to pathogens and abiotic
58 stresses [10,11,12]. Mutations in this pathway, such as inhibitory mutations in
59 *age-1* (a homologue of the mammalian phosphatidylinositol 3-OH kinase) or
60 *daf-2* (a homologue of the mammalian insulin receptor) result in the
61 relocation of the transcription factor DAF-16 into the nucleus where it
62 regulates a plethora of downstream genes [2,13,14,15,16].

63

64 The IIS pathway is highly conserved in organisms ranging from yeast to
65 humans and, in many cases, appears to retain its dual role as a major effector
66 of immunity and longevity [17,18,19,20]. Studies in *C. elegans* have explored
67 this coupling relationship between the *daf-16* determined phenotypes of
68 longevity, immunity and stress tolerance to a great extent, but little is known
69 about the corresponding phenotypes in other nematode species.

70

71 Here we provide experimental data to address this question by undertaking a
72 comprehensive analysis of immunity, stress response and longevity
73 phenotypes in several representative isolates of four nematode species within
74 the same genus. We demonstrate that, within this group of closely related
75 animals, there exists a high divergence with regards to traits such as lifespan
76 and stress tolerance and, intriguingly, in the expression of *daf-16*.
77 Furthermore, we investigated conservation in the DAF-16 downstream
78 regulon (target genes) by surveying the three available *Caenorhabditis*
79 genomes (*C. elegans*, *C. briggsae* and *C. remanei*) for genes containing the
80 known consensus sites for DAF-16. Based on orthologous sets of genes
81 containing the consensus sites, we asked whether certain biological processes
82 are more prevalent in one species than in others (divergent targets) and which
83 processes are shared between all three species. We also tested for adaptive

84 sequence evolution along the IIS pathway in these species. Finally, we use
85 classical genetics to constitutively activate the DAF-16 pathway in two
86 *Caenorhabditis* species in order to experimentally identify both conserved and
87 divergent downstream phenotypes.

88

89 **Results**

90 **Gonochoristic species are longer lived than the hermaphroditic species and** 91 **show higher levels of *daf-16* expression**

92 We and others have previously demonstrated that different *Caenorhabditis*
93 species exhibit significantly different lifespans [21,22]. Since different
94 laboratory isolates can exhibit variation in lifespan [23], we conducted parallel
95 longevity assays on our isolates of *C. elegans* N2, *C. briggsae* AF16, *C. remanei*
96 EM464 and *C. brenneri* CB5161. As previously reported [22], the two
97 gonochoristic species (*C. remanei* and *C. brenneri*) exhibit a significantly
98 ($p < 0.0001$; see Figure S1 for all p-values) longer lifespan than both
99 hermaphroditic species (*C. elegans* and *C. briggsae*) (Figure 1a). Additional
100 testing confirmed that this trend was highly conserved across multiple
101 wildtype isolates of each species (Figure 1b, Figure S2), as previously reported
102 [22]. The testing of several hermaphroditic wildtype isolates also ruled out the
103 possibility that these observations were due to the fixation of novel mutations
104 under the force of genetic drift in our laboratory *C. elegans* (N2) line.

105

106 Given the evolutionary conservation and critical role played by DAF-16 in
107 regulating lifespan in *C. elegans*, we quantified *daf-16* mRNA levels in both
108 mixed populations (nematodes at various stages of development) and staged
109 populations (L2-L3, L4 and adult stages) of all strains of the four
110 *Caenorhabditis* species. Whilst *C. briggsae* showed *daf-16* levels similar to those
111 in *C. elegans*, *daf-16* expression in the two gonochoristic species was between

112 seven (*C. brenneri*) and twelve (*C. remanei*) fold higher than *C. elegans* in the
113 mixed populations (Figure 2a). Higher *daf-16* expression levels among the
114 gonochoristic species was also observed throughout development in the
115 staged populations (Figure 2bi, 2bii & 2biii) with the difference being most
116 prominent in the L4 stage (Figure 2bii). Thus higher levels of *daf-16*
117 expression seem to positively correlate with longer lifespan.

118

119 We also tested for expression levels of *daf-16* in *C.elegans* males using two
120 independent reference genes (Figure S3) and found no significant
121 transcriptional difference in comparison to *C. elegans* hermaphrodites,
122 indicating that the absence or presence of males in a population has no effect
123 with regards to *daf-16* expression levels. We note that the presence of both
124 males and females within the gonochoristic population could mean that
125 enhanced *daf-16* expression may be restricted to one or other gender, but
126 given that both male and female animals in the gonochoristic species are
127 longer lived than either gender of *C. elegans* or *C. briggsae* [21], we regard it as
128 more likely that *daf-16* is highly expressed in both genders of the
129 gonochoristic species.

130

131 **Long-lived species are more resistant to abiotic stress than shorter-lived** 132 **species**

133 In *C. elegans*, DAF-16 activity substantially increases survival following
134 exposure to high temperature or heavy metals [10,24]. To test whether the
135 observed higher levels of *daf-16* in the gonochoristic species also correlate
136 with better survival to abiotic stress, we exposed multiple isolates of all four
137 species to prolonged high temperature of 37°C (Figure 3a) or toxic heavy
138 metals such as CuCl₂ (Figure 3b, Figure S4). In both cases, the gonochoristic
139 species showed significantly (Figure S1) higher survival than either

140 hermaphroditic species. The correlation of these phenotypes with *daf-16*
141 expression levels, together with prior knowledge from studies in *C. elegans*,
142 suggests that higher DAF-16 levels could potentially be driving both
143 increased lifespan and increased resistance to abiotic stress.

144

145 **Longer-lived species in general are more resistant to biotic stress factors**
146 **than the shorter-lived species**

147 Numerous human pathogens are now known to be lethal towards *C. elegans*
148 [25,26,27,28,29,30,31]. Since DAF-16 activity contributes towards stress
149 resistance during infection [32], we assessed whether the four *Caenorhabditis*
150 species varied in resistance to a range of pathogens. Interestingly, type
151 strains of the two long-lived, gonochoristic species (EM464 and CB5161)
152 showed significantly higher resistance to the Gram-negative bacterium
153 *Pseudomonas aeruginosa* (Figure 4a), the Gram-positive bacterium
154 *Staphylococcus aureus* (Figure 4b) and the fungus *Cryptococcus neoformans* [21]
155 than type strains of the two hermaphroditic species (AF16 and N2). However,
156 all four species showed similar sensitivity to the Gram-negative pathogen
157 *Salmonella typhimurium* (Figure 4c). To ensure that these differences were not
158 isolate dependent, we tested multiple additional isolates of each species for
159 resistance to *S. aureus*. In all cases, gonochoristic isolates exhibit substantially
160 higher resistance to killing by this pathogen (Figure S2 and S5), suggesting
161 that the higher DAF-16 levels in the two gonochoristic species may potentially
162 drive enhanced resistance to some, but not all, pathogens.

163

164 Since progeny production and the consequent risk of matricidal killing has
165 previously been shown to shorten *C. elegans* lifespan, particularly when
166 exposed to pathogens, [30,33,34] we considered the possibility that the
167 enhanced survival of gonochoristic species may result from the absence of

168 matricidal killing. To test this, we exposed feminised (and thus infertile when
169 singled) *C. elegans* animals (BA17, fem-1(hc17)) to the pathogenic bacteria *S.*
170 *aureus*. As previously reported, feminised *C. elegans* exhibited improved
171 survival under pathogenic conditions (Figure S6), but this increase is nowhere
172 as significant as the increase in lifespan seen in the higher *daf-16* producing
173 gonochoristic species on *S. aureus*. Thus the enhanced survival of
174 gonochoristic species is not attributable to the lack of progeny production.

175

176 **Manipulation of the DAF-16 pathway.**

177 *C. elegans* DAF-16 activity can be dramatically enhanced by loss-of-function
178 mutations in the upstream insulin-like growth factor receptor DAF-2 [35,36].
179 We investigated whether this phenomenon is conserved in *C. briggsae*, which,
180 like *C. elegans*, has low basal levels of DAF-16 (Figure 2a), by comparing *daf-2*
181 loss-of-function mutants in both species. As previously reported [7] we
182 observed that *C. briggsae* (*daf-2*) mutants, have increased longevity relative to
183 wildtype animals (Figure 5a). In addition, inactivation of *daf-2* in *C. briggsae*
184 enhances resistance to high temperature (Figure 5b) and heavy metal toxicity
185 (Figure 5c), as it does in *C. elegans* [10,37]. Interestingly, *C. briggsae* *daf-2*
186 mutants show enhanced resistance towards *S. aureus* ($p < 0.02$, Figure S7) and
187 *P. aeruginosa* ($p < 0.0001$, Figure S7), but the magnitude of the increase is
188 substantially smaller than that for *C. elegans* *daf-2* mutants (Figures 5d and 5e).
189 Finally, loss of *daf-2* did not enhance resistance to *S. typhimurium* in either *C.*
190 *elegans* or *C. briggsae* (Figure 5f). Regrettably, genetic mutants in *daf-2* or *daf-*
191 *16* are not available for either gonochoristic species, nor is RNA interference
192 efficient enough in these species to allow direct manipulation of the IIS
193 pathway in a similar manner. However, should such studies become feasible
194 in the future, then our data would predict that loss of *daf-2* would likely have

195 only a minimal effect on lifespan and stress resistance in the gonochoristic
196 species.

197

198 **Comparative analysis of the DAF-16 regulon in *C. elegans*, *C. briggsae* and**
199 ***C. remanei***

200 We considered the possibility that the DAF-16 pathway itself may have
201 become modified during the diversification of the Caenorhabditid nematodes.
202 However, calculation of Ka/Ks ratios for all of the components in the IIS
203 signalling pathway (*daf-2*, *age-1*, *pdk-1*, *akt-1*, and *daf-16*) between *C. elegans*, *C.*
204 *briggsae* and *C. remanei* showed no evidence for positive selection in any of the
205 genes (Figure S8).

206

207 Given that the IIS pathway itself does not appear to have been modified
208 during the evolution of these species, we next investigated whether the
209 downstream targets of DAF-16 differed between the three sequenced
210 nematode species (*C. elegans*, *C. briggsae* and *C. remanei*). We searched for the
211 presence of perfectly matched DAF-16 canonical consensus sites
212 (ttatttac/gtaaataa, ttgtttac/gtaaacaa) in the 3kb upstream of every predicted
213 gene in *C. elegans*, *C. briggsae* and *C. remanei*. In *C. elegans* our approach
214 yielded 6293 genes (31.2% of the genome) containing either one or both of the
215 known sites in their 3kb upstream region. In comparison, only 23.4% (5,150
216 genes) in *C. briggsae* and 26.7% (8,456 genes) in *C. remanei* contained at least
217 one of the consensus sites. We note that the short length and relative
218 variability of the DAF-16 consensus sequence means that this approach
219 inherently overestimates the number of DAF-16 binding sites in the genome.
220 However, given the absence of experimental techniques (such as chromatin
221 immunoprecipitation) in the non-*elegans* species, such a bioinformatic

222 approach is, at present, the only way of obtaining an approximate estimate of
223 genome-wide differences in the IIS pathway within this group of organisms.
224 Based on this analysis, the number of orthologous genes that contain perfect
225 matches to the DAF-16 consensus binding sites appears similar between *C.*
226 *elegans* and *C. briggsae* (1900 genes), *C. elegans* and *C. remanei* (2111 genes) and
227 *C. briggsae* and *C. remanei* (2165 genes). However, although *C. elegans*, *C.*
228 *briggsae* and *C. remanei* have 13,015 genes in common (64.4% of the *C. elegans*
229 genome) only 913 of these contain the DAF-16 binding elements in all three
230 species, a group that we define as the core DAF-16 regulon (Figure S9).

231

232 Based on these gene sets, we asked whether the core DAF-16 regulon and the
233 species-specific DAF-16 regulons differ in the type of genes they contain by
234 testing whether particular gene ontology (GO) terms (using GOTERM
235 BP_ALL and GOTERM BP_2) are overrepresented (Figure S10 and file S11).
236 The DAF-16 core regulon shows, amongst others, enrichment for genes that
237 are involved in lifespan regulation, immune response and responses to
238 chemical stimuli (including detoxification and stress response) (Figure S10).
239 Intriguingly, whilst both the *C. elegans*-specific and *C. remanei*-specific DAF-16
240 regulons also show overrepresentation of genes involved in immunity (11
241 genes in *C. elegans*, 13 genes in *C. remanei*) and stress responses (38 genes in *C.*
242 *elegans*, 14 genes in *C. remanei*) these groups are not overrepresented in the *C.*
243 *briggsae*-specific DAF-16 regulon (Figure S10).

244

245 In order to reduce the number of false positives in our *C. elegans* dataset we
246 compared it to a gene list containing all putative DAF-16 targets recently
247 identified in *C. elegans* via a range of other approaches by Oh [38], Murphy
248 [14], Halaschek-Wiener [39], Lee [16], McElwee [40] and Dong [41].
249 Altogether, 1746 genes were identified as putative DAF-16 targets in at least
250 one of these other datasets and 678 of these were also identified by our

251 approach, a group we refer to as the adjusted dataset. Of the 678 potential *C.*
252 *elegans* DAF-16 target genes, 283 overlap with the *C. brigssae* dataset and 274
253 genes were found in the *C. remanei* list. The adjusted DAF-16 core regulon
254 (genes found in all three species) contains 145 genes (Figure 6a).

255

256 Partitioning the adjusted DAF-16 core regulon using the GOTERM BP_ALL
257 and GOTERM BP_2 gene categories revealed significant enrichment for genes
258 involved in the regulation of lifespan, stress response, transport, localization
259 and metabolism (Figure 6b and Figure S12). As expected the outcomes of the
260 analyses of the unadjusted and the adjusted datasets differ slightly. However,
261 the overall pattern is the same between the two approaches for both the core
262 regulon as well as the species-specific regulon.

263

264 Finally, we compared the list of putative *C. elegans* DAF_16 targets identified
265 by Oh and colleagues via a direct, chromatin immunoprecipitation (ChIP)
266 approach [38] with those identified via microarray or bioinformatic
267 approaches in the other studies (Murphy [14], Halaschek-Wiener [39], Lee
268 [16], McElwee [40] and Dong [41]) or our own dataset. (Table 2).
269 Interestingly, there is very little overlap between DAF-16 targets identified by
270 ChIP and those inferred from microarray or bioinformatic analysis, with the
271 exception of 11 genes shared between Oh et al and McElwee et al and 30
272 genes shared between Oh et al and our dataset. Thus there is likely to be
273 considerable benefit in combining a range of experimental approaches in
274 order to narrow down the list of true DAF-16 target genes.

275

276 **Discussion**

277 It is now clear that the lifespan of an organism is determined by a
278 combination of environmental conditions, stochastic factors (such as lifestyle)

279 and genetic background. Numerous studies have demonstrated that the
280 evolutionarily conserved transcription factor DAF-16 is a critical gene
281 regulator that controls the transcription of hundreds of genes involved in
282 immunity, stress responses and longevity in *C. elegans* [42]. The homologues
283 of *daf-16* in other organisms have been shown to perform similar functions
284 [18] and yet species differ significantly in terms of lifespan and immunity,
285 raising the question of how such DAF-16 mediated phenotypes have changed
286 through evolutionary time.

287

288 Here we show covariance of three DAF-16 mediated phenotypes, longevity,
289 immunity and stress response, across the *Caenorhabditis* genus. Strikingly, the
290 two gonochoristic species (*C. remanei* and *C. brenneri*) show significantly
291 higher basal expression of DAF-16 than the shorter-lived hermaphroditic
292 species *C. elegans* and *C. briggsae*. Thus, enhanced expression and/or activation
293 of DAF-16 may be an important mechanism by which species regulate a
294 combination of phenotypes that enhance resistance to abiotic and biotic
295 stresses and hence favour a longer life. The fact that this pattern is seen in
296 multiple isolates of two gonochoristic species may reflect their need to search
297 for a partner to mate, a lifestyle that increases the chance of encountering
298 stressful conditions (eg. pathogens, high temperature) and is likely to favour
299 the evolution of a longer lifespan in order to increase mating opportunities.
300 In addition, since we know very little about the natural ecology of the
301 *Caenorhabditis* nematodes [43], it is possible that differences in the niches
302 inhabited by these species may impose extrinsic stresses that have led to the
303 evolution of improved stress tolerance via the over-expression of DAF-16.

304

305 It is interesting to note that susceptibility to several pathogens correlates with
306 other DAF-16 mediated effects, with the exception of the Gram-negative
307 bacterium *S. typhimurium*, which shows similar lethality in all four species

308 and the two *daf-2* mutants. Since *S. typhimurium* is one of the few human
309 pathogens thus far shown to establish a truly persistent infection in the worm
310 due to its resistance towards antimicrobial peptides [25,44], this finding may
311 indicate that DAF-16 plays little or no role in dealing with gut-colonising
312 pathogens.

313

314 The insulin-like signalling pathway contributes to both innate immune
315 responses and stress responses in *C. elegans*. Our data suggests that this may
316 also hold true in closely related nematode species. In line with this, we show
317 that the components of this pathway do not show evidence of adaptive
318 sequence evolution during the diversification of these species whereas the
319 complement of putative downstream targets controlled by DAF-16 appear to
320 vary between these species. All three sequenced species share a core DAF-16
321 regulon comprised of genes functioning in longevity, stress response and
322 other biological processes. However, whilst *C. elegans* and *C. remanei* contain
323 a similar set of target types in their species-specific DAF-16 regulons, the
324 species-specific DAF-16 regulon of *C. briggsae* lacks genes involved in
325 immunity and stress response. Interestingly, in line with this finding, we
326 observed that a *daf-2* mutant in *C. briggsae* is long-lived and resistant to abiotic
327 stress, but only moderately resistant to killing by a range of pathogens.

328

329 The majority of enriched genes identified by our approach are associated
330 with other biological processes such as metabolism, transport and other
331 functions, in line with previous studies that have identified downstream
332 targets of DAF-16 in *C. elegans* [14,38,39,40,45,46]. We note, however, that such
333 bioinformatic analyses are susceptible to false positive (due to the chance
334 occurrence of DAF-16 consensus sequences) and false negative (due to its
335 reliance on perfect-match sequence motifs) errors. Indeed, depending on the
336 approach used, others have estimated that up to 78% of *C. elegans* genes might

337 be potential DAF-16 downstream targets [42]. As such, we would emphasize
338 that our bioinformatic analysis is intended only as a guide for future
339 experimental analyses once tools become available.

340

341 In conclusion, we demonstrate covariance of DAF-16 mediated phenotypes in
342 the four most well-characterized species of the *Caenorhabditis* clade. We note
343 that our data are correlative but, as yet, cannot prove a causative influence of
344 *daf-16* expression level on these phenotypes in the gonochoristic species.
345 Currently, demonstrating a direct role for DAF-16 in phenotypic covariance in
346 the gonochoristic nematodes is not technically feasible. Very few genetic
347 mutants have been made in these species, RNA interference is of low
348 efficiency and no antibodies exist for chromatin immunoprecipitation
349 approaches. However, many groups are currently attempting to develop
350 such tools for these species and, as such, we hope that a full mechanistic
351 investigation of the IIS pathway in non-elegans species will be feasible within
352 the next few years.

353

354 **Materials and Methods**

355 **Bacterial strains and growth conditions**

356 *Escherichia coli* OP50 [47], *Salmonella typhimurium* SL1344 [48], *Pseudomonas*
357 *aeruginosa* PA01 and *Staphylococcus aureus* NCTC8532 were grown in nutrient
358 rich Luria-Bertani (LB) broth overnight with shaking at 37°. The bacterial
359 culture was then seeded onto standard Nematode Growth agar Medium
360 (NGM[47,49]) plates. These plates were incubated overnight at 37° (~16hrs)
361 followed by storage at 4°. Plates were always equilibrated to room
362 temperature before use.

363

364 **Worm Strains**

365 Worm strains N2, RC301, CB4856 (wildtype *Caenorhabditis elegans*), AF16,
366 ED3033, ED3034 (wildtype *Caenorhabditis briggsae*), EM464, JU1082, JU1084
367 (wildtype *Caenorhabditis remanei*), CB5161, LKC28, SB129 (wildtype
368 *Caenorhabditis brenneri*), CB1370 [*C. elegans daf-2(e1370)*], PS5531 [*C. briggsae*
369 *daf-2(sy5445)*] and BA17 [*C.elegans fem-1 (hc17)*] were grown on standard
370 NGM plates seeded with OP50 strain of *E. coli* bacteria as a food source
371 [47,49]. All strains except BA17 (which was grown at 25°C to induce
372 feminisation) were grown at 20°C. Fourth larval stage hermaphrodites from
373 the hermaphroditic species and females from the gonochoristic species were
374 used for all the phenotypic assays performed.

375

376 **Longevity/Pathogen Assays**

377 The hermaphroditic and gonochoristic strains of worms were bleached [49] to
378 produce age-synchronous L4 molt populations. Between 80 and 250 L4
379 worms from the hermaphroditic species, or females in the case of
380 gonochoristic species, were transferred onto NGM plates (~30 worms per
381 plate, yielding up to 10 replicates) seeded with OP50 for longevity assays and
382 SL1344, PA01 and NCTC8532 for pathogen assays as food source. Plates with
383 OP50 were incubated at 20°C with the rest being incubated at 25°C. Worms
384 grown at 25°C on OP50 have been shown to have a significantly longer
385 lifespan than those grown on pathogenic bacteria such as *S. typhimurium* [25]
386 which eliminates the negative effects of heat as an experimental determinant.
387 The worms on all these plates were scored for survival every 24hrs. Animals
388 were considered dead when they failed to respond to prodding by a platinum
389 wire. The worms were transferred onto new plates every one to two days
390 until they stopped egg laying, in order to prevent F1 progeny from interfering
391 with the experiment.

392

393 **Heat Shock Assay**

394 L4 worms from the hermaphroditic species, and females in the case of
395 gonochoristic species, were transferred onto NGM plates with OP50 that had
396 been prewarmed to 37°. These plates were then incubated at 37°C and the
397 worms were scored for survival at hourly intervals.

398

399 **Metallotolerance Assay**

400 Age synchronous L4 worms were transferred from NGM plates into 24-well
401 tissue culture plates containing copper chloride (7mM) dissolved in K
402 medium (53mM NaCl, 32mM KCl) [10,50]. The plate was incubated at 20°C
403 and the worms were scored for survival every hour.

404

405 **Statistical Analysis**

406 Survival curves were produced based on the Kaplan-Meier method using MS-
407 Excel and the significance was calculated using the non-parametric log-rank
408 method. Assays were then corrected for multiple testing using the Bonferroni
409 correction.

410

411

412

413 **Preparation of total nematode mRNA and Quantitative RT PCR**

414 For qRT-PCR, RNA was isolated from a mixed larval stage population of each
415 of the four species of worms. These worms were grown on NGM plates with
416 OP50 as food source at 20°. Animals were then washed off the plates using
417 M9 buffer, followed by repeated washes again with M9 buffer before being
418 homogenized using the Precellys 24 machine (Stretton Scientific). The RNA
419 was then isolated from these worm samples using the Qiagen RNeasy Mini
420 kit (cat. No. 74140) using the manufacturer's protocol. RNA was then reverse

421 transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen)
422 according to the manufacturer's instructions. Real-time quantitative RT-PCR
423 was performed (7300 Real Time PCR System; Applied Bio Systems) on this
424 cDNA using the SYBR Green PCR kit (Quantace) to determine the expression
425 levels of *daf-16* across the four species. Primers for this were designed
426 manually and tested for maximum efficiency with their respective cDNA
427 prior to qRT-PCR. Primers used include *daf-16* primers for *C. elegans*, *C.*
428 *briggsae*, *C. remanei* and *C. brenneri* (Table 1).

429

430 The RT PCR levels were normalized to the housekeeping gene,
431 Glyceraldehyde 3-Phosphate Dehydrogenase (*gpd-3*). The primers for this
432 gene were; Primer Fwd – TGAAGGGAATTCTCGCTTACACC and Primer
433 Rev – GAGTATCCGAACTCGTTATCGTAC. We confirmed that our results
434 were not due to variation in *gpd-3* by cross checking RT PCR levels against
435 another reference gene, 18sRNA, the primers for which were; Primer Fwd -
436 TTCTTCCATGTCCGGGATAG and Primer Rev -
437 CCCCACTTCTCGAATCAG. To assess the efficacy of the primers and the
438 sensitivity of the qPCR assay, 2-fold dilution series of the template DNA for
439 all the species tested were prepared and subjected to qPCR amplification. The
440 results obtained were extrapolated to produce standard curves by linear
441 regression analysis between threshold cycle (Ct) and sample dilution that
442 gave coefficients of determination (r^2) that exceeded 0.95 for all
443 template/primer combinations (Figure S13). Once amplification efficiencies of
444 the target and the reference were determined to be approximately equal, RT
445 PCRs were carried out for all the experimental conditions. These results were
446 analysed using the ΔC_T method with the *gpd-3* and 18S RNA levels as controls
447 for normalization and expressed as fold change compared to *C. elegans* [51].

448

449 **Bioinformatic analysis of DAF-16 downstream targets**

450 The complete genomes of *C. elegans* (20,189 genes), *C. briggsae* (21,976 genes)
451 and *C. remanei* (31,614 genes) were downloaded from Wormbase release WS
452 197 (www.wormbase.org). We surveyed a 3000bp upstream flanking region
453 of each gene (upstream of the lead ATG) for the presence of the two known
454 canonical DAF-16 binding sites (ttatttac/gtaaataa, ttgtttac/gtaaacaa; [52]). We
455 applied a perfect match approach using the dna-pattern tool implemented in
456 the freely available software package Regulatory Sequence Analysis Tools
457 (RSAT; <http://rsat.bigre.ulb.ac.be/rsat/>; [53]). Only genes with upstream
458 flanking region containing one or more perfect matches to the consensus sites
459 were included in further analyses. From this set of genes we then retrieved a
460 subset of genes for each species that are orthologous either between *C. elegans*
461 and *C. briggsae*, *C. elegans* and *C. remanei* or between all three species. Based on
462 these orthologous gene sets we defined the following classes: i) a species-
463 specific DAF-16-regulon, consisting of orthologs that contain the consensus
464 site in only one of the species, ii) the species-shared DAF-16 regulon,
465 consisting of orthologous genes that contain the consensus site in two of the
466 species and iii) the core-DAF-16 regulon, consisting of orthologs that contain
467 the consensus site in all three species. These gene subsets were subsequently
468 analysed in order to identify enriched functional gene groups. This analysis
469 was performed using the functional annotation tools available from the non-
470 commercial bioinformatic database DAVID (Database for Annotation,
471 Visualization and Integrated Discovery) [54,55].

472 Furthermore we compared the resulting *C. elegans* gene list to the available
473 datasets of Oh [38], Murphy [14], Halaschek-Wiener [39], Lee [16], McElwee
474 [40] and Dong [41]. These datasets were combined, duplicates removed and
475 subsequently run against the *C. elegans* gene list (containing all genes with a
476 perfect match to one of the binding motifs). The resulting gene list was then
477 compared to the lists obtained for *C. briggsae* and for *C. remanei*. The three

478 resulting lists were analysed using the functional annotation tools in DAVID.
479 Finally, we looked whether there was any overlap between the dataset of Oh
480 and the datasets of Murphy, McElwee, Lee, Halaschek-Wiener, Dong.

481

482 We determined the number of genes that were enriched within the functional
483 annotation category Gene Ontology GOTERM BP_ALL and especially
484 enriched in GOTERM BP_2. The results were obtained by using the
485 Functional Annotation Chart tool. The GOTERMS BP are available from the
486 DAVID database. The p-value obtained in this analysis is equivalent to the
487 EASE score, which uses a conservative adjustment of the Fisher's exact
488 probability, and was applied to identify significantly enriched gene
489 categories. DAVID provides several methods to correct for multiple testing
490 which include Bonferroni adjustment of the p-value, and the Benjamini-
491 Hochberg approach to control for family-wide false positive rate. The fold
492 enrichment value measures the magnitude of enrichment and is considered
493 significant if 1.5 or above [54] . For more statistical details and detailed
494 description of the annotation methods used in DAVID please refer to the cited
495 references above and references therein.

496 For all orthologs, the corresponding WormBase IDs of *C. elegans* genes were
497 used as input files. Orthologs between *C. remanei* and *C. briggsae* but not
498 occurring in *C. elegans* could not be addressed with this approach. All
499 orthologous genes with a duplicate output in one of the species were counted
500 only as one gene.

501

502 **Adaptive sequence evolution**

503 Adaptive sequence evolution along the IIS pathway was studied in *C. elegans*,
504 *C. briggsae* and *C. remanei*. Protein sequences and DNA sequences of the
505 coding regions ranging from *daf-2*, *age-1*, *pdk-1*, *akt-1*, and *daf-16* were obtained

506 from WormBase WS197 (www.wormbase.org). Protein sequences and DNA
507 coding regions were aligned using ClustalX2 [56]. For each gene of interest,
508 the presence of adaptive sequence evolution (ratio between synonymous [K_S]
509 and non-synonymous [K_A] substitutions) was calculated between a pair of
510 sequences (*C. elegans* and *C. briggsae*; *C. elegans* and *C. remanei*; *C. briggsae* and
511 *C. remanei*) using PAL2NAL [57]. PAL2NAL calculates K_S and K_A by the
512 codeml program in PAML. Briefly, pairwise protein alignments in CLUSTAL
513 format and the corresponding DNA sequence alignments in FASTA format
514 were used as input files. The following option settings were used. (i) Codon
515 table: "universal". (ii) Remove gaps and inframe stop codons: "Yes". (iii)
516 Calculate K_S and K_A: "Yes". (iv) Remove mismatches: "No".

517

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522

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692 **Figures Legends**

693 **Figure 1 – Lifespan analysis**

694 (a) Hermaphrodite (N2, AF16) or female (EM464, CB5161) animals at the
695 fourth larval stage (L4) were transferred onto plates pre-seeded with OP50
696 and monitored for survival over fifty days. Whilst hermaphrodite animals
697 show 100% lethality over this period, survival is significantly higher for both
698 gonochoristic species ($p < 0.0001$, Figure S1), with more than 50% of animals
699 surviving longer than twenty days. (b) This effect is conserved across multiple
700 wildtype isolates of each species.

701 **Figure 2 – Quantitation of *daf-16* gene expression**

702 (a) mRNA from a mixed population of hermaphroditic (N2, AF16) and
703 gonochoristic (EM464, CB5161) animals was extracted and *daf-16* gene
704 expression was quantified relative to the housekeeping gene *gpd-3*. Data
705 represent the mean of three experiments, error bars show standard deviation.
706 (b) *daf-16* gene expression for the same species but measured at various stages
707 of development that include L2-L3 stage (bi), L4 stage (bii) and the adult stage
708 (biii)

709 **Figure 3 – Survival analysis following exposure to abiotic stress (heat and 710 heavy metal)**

711 L4 Hermaphrodite (N2, AF16) or female (EM464, CB5161) animals were
712 monitored for survival (a) at 37°C or (b) during exposure to 7 mM copper
713 chloride. *C. elegans* and *C. briggsae* show significantly higher susceptibility to
714 both high temperature ($p < 0.0001$, Figure S1) and heavy metal toxicity ($p <$
715 0.01 , Figure S1).

716 **Figure 4 – Survival analysis following exposure to biotic stress (three**
717 **species of pathogenic bacteria)**

718 Survival of L4-stage hermaphrodites or female animals during exposure to
719 pathogenic bacteria. *C. remanei* and *C. brenneri* are significantly more resistant
720 to *Pseudomonas aeruginosa* (Figure 4a, $p < 0.0001$, Figure S1) and *Staphylococcus*
721 *aureus* (Figure 4b, $p < 0.0001$, Figure S1). However, all four species show
722 similar susceptibility to *Salmonella typhimurium* (SL1344) (Figure 4c, $p > 0.05$,
723 Figure S1).

724 **Figure 5 – The effect of *daf-2* mutations on lifespan and resistance to abiotic**
725 **and biotic stress.**

726 *daf-2* mutations in *C.elegans* (CB1370) and *C.briggsae* (PS5531) result in
727 enhanced lifespan (Figure 5a) and resistance to high temperature (37°C,
728 Figure 5b) or 7mM copper chloride (Figure 5c). Both mutants also show
729 significantly higher resistance to killing by *Staphylococcus aureus* (Figure 5d)
730 and *Pseudomonas aeruginosa* (Figure 5e), although the magnitude of the
731 resistance is significantly lower for *C. briggsae daf-2* than for the equivalent
732 mutation in *C. elegans*. In contrast, the *daf-2* mutation does not enhance the
733 resistance of either species to *Salmonella typhimurium* (Figure 5f).

734 **Figure 6 – The DAF-16 regulon based on an adjusted *C. elegans* dataset.**

735 (a) Venn diagram of putative DAF-16 target genes in the three species. 145
736 orthologous genes have an upstream DAF-16 binding site in all three species,
737 a group we define as the core DAF-16 regulon. (b) The genes within the core
738 DAF-16 regulon were tested for over-representation of particular annotation
739 categories within GOTERM BP_2 provided by the database DAVID. In
740 brackets are the number of genes in the core DAF-16 regulon which are
741 associated with a particular GO term within GOTERM BP_2.

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759 **Tables**

760 **Table 1 - List and sequence of primers used for studying daf-16 expression**
761 **levels using Real Time PCR**

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Gene	Species	Forward Primer 5'	Reverse Primer 3'
<i>daf-16</i>	<i>C. elegans</i>	GCGAATCGGTTCCAGCAATTCCAA	ATCCACGGACACTGTTCAACTCGT
<i>daf-16</i>	<i>C. briggsae</i>	AGAAGGCTACCACTAGAACCAACG	TCCATCCAGCGGAACTGTTCGAAT
<i>daf-16</i>	<i>C. remanei</i>	CGACGGCAATACTCATGTCAATGG	ACGGTTTGAAGTTGGTGCTTGGCA
<i>daf-16</i>	<i>C. brenneri</i>	CCTTAGTAGTGGCCTCAATGGTGT	CACAACCTATCACTTCACTCTCGC
<i>gpd-3</i>	All species	TGAAGGGAATTCTCGCTTACACC	GAGTATCCGAACTCGTTATCGTAC

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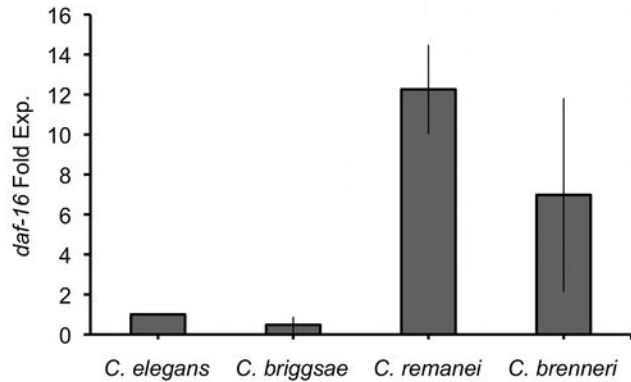
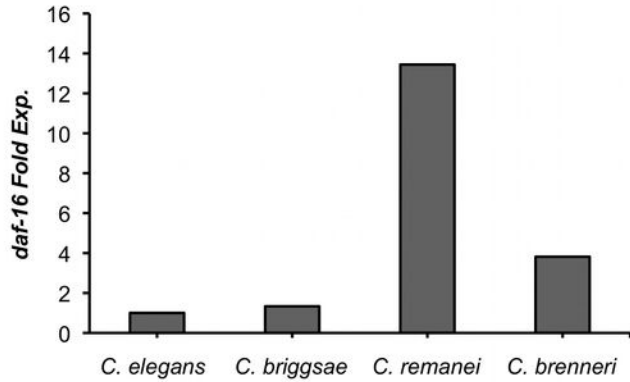
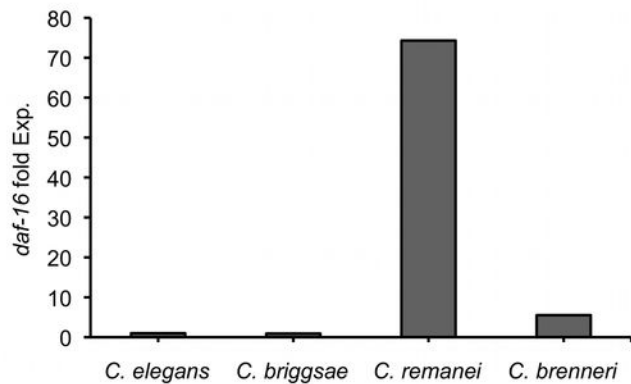
768

769 **Table 2 - Overlap of the number of potential DAF-16 targets.**

	Lee	Murphy	Wiener	McElwee	Dong	This study
Overlapping with Oh	1/81	2/473	1/317	11/953	1/93	30/6293

770

771 This table shows the overlap between the reference dataset of Oh and other
772 datasets obtained by Murphy [14], Halaschek-Wiener [39], Lee [16], McElwee
773 [40] and Dong [41] and the *C. elegans* gene list of this study. The first number
774 gives the number of genes that are shared between Oh and the dataset of
775 comparison. The second number stands for the total number of genes
776 identified as potential downstream targets of DAF-16 in the corresponding
777 study.

A**Bi****Bii****Biii**