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**Aging Effects of *Caenorhabditis elegans* Ryanodine Receptor Variants
Corresponding to Human Myopathic Mutations**

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17 **Running title**

18 *C. elegans* RyR Variants and Aging

19

20 **Key words**

21 *Caenorhabditis elegans*; aging; ryanodine receptor; malignant hyperthermia;
22 muscle.

23

24

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30

31 **Abstract**

32 Delaying the decline in skeletal muscle function will be critical to better maintenance of an
33 active life-style in old age. The skeletal muscle ryanodine receptor, the major intracellular
34 membrane channel through which calcium ions pass to elicit muscle contraction, is central to
35 calcium ion balance, and is hypothesized to be a significant factor for age-related decline in
36 muscle function. The nematode *Caenorhabditis elegans* is a key model system for the study of
37 human aging and strains with modified *C. elegans* ryanodine receptors corresponding to human
38 myopathic variants linked with malignant hyperthermia and related conditions were generated.
39 The altered response of these strains to pharmacological agents reflected results of human
40 diagnostic tests for individuals with these pathogenic variants. Involvement of nerve cells in the
41 *C. elegans* responses may relate to rare medical symptoms concerning the central nervous system
42 that have been associated with ryanodine receptor variants. These single amino acid
43 modifications in *C. elegans* also conferred a reduction in lifespan and an accelerated decline in
44 muscle integrity with age, supporting the significance of ryanodine receptor function for human
45 aging.

46

47 **Article Summary**

48 Mutations in the human ryanodine receptor gene *RYR1* lead to muscle disorders such as
49 malignant hyperthermia. Equivalent changes in the corresponding *Caenorhabditis elegans* gene
50 led to alterations in movement after challenge with the pharmacological agents caffeine and
51 halothane, reflecting observations seen for humans. The single amino acid changes generated by

52 these mutations also led to reduced lifespan and accelerated muscle aging, supporting the
53 hypothesis that failure to maintain calcium ion balance in muscle cells appropriately contributes
54 to reduced mobility in human old age. The change in response to caffeine also depended upon a
55 neural component.

56

57 **Introduction**

58 To improve the health of the world's aging population we need a better understanding of the
59 aging processes, and age-related decline of skeletal muscle function is of key importance.
60 Defective excitation-contraction coupling (Payne and Delbono 2004) and reduced capacity of
61 Ca^{2+} homeostasis (Weisleder et al. 2006; Zhao et al. 2008) have been suggested to contribute to
62 the human muscle contractile dysfunction that occurs with age. The ryanodine receptor isoform 1
63 (RyR1) is the channel through which Ca^{2+} is released from the skeletal muscle sarcoplasmic
64 reticulum to elicit contraction. In the mouse there is an age-related increase in the ryanodine
65 receptor 'leakiness' (Anderson et al. 2011) and age-related decrease in both the number of
66 RyR1s and their degree of coupling to regulatory proteins (Ryan et al. 2000). Single-point
67 variants in human *RYR1* have been associated with the impairment of calcium handling in
68 malignant hyperthermia (MH) (Robinson et al. 2006; Bouchama and Knochel 2002; McCarthy et
69 al. 2000; Robinson et al. 2002; Tong et al. 1997; Jungbluth et al. 2009; Loseth et al. 2013; Nishio
70 et al. 2009). The clinical incidence of MH is age-dependent and there is evidence of premature
71 aging in MH mouse models (Boncompagni et al. 2009; Boncompagni et al. 2006). During an
72 MH episode the sensitised RyR1 is activated by inhalational anesthetics and remains open

73 without neural stimulation resulting in sustained muscle contraction across the body (Larach et
74 al. 1994), with death in the absence of a prompt and aggressive treatment regimen. The primary
75 method of diagnosing susceptibility to this condition is through an *in vitro* contracture test
76 (IVCT) (Ording et al. 1997) which measures the response of patients' muscle biopsies to the
77 inhalation anesthetic halothane and to caffeine.

78 Mammalian RyR1 is a very large tetrameric membrane protein (>5000 amino acid residues per
79 monomer) (Robinson et al. 2006) making it difficult to study. Similarly, the human *RYR1* gene,
80 with its many introns across such a large coding region, is awkward to manipulate. The
81 nematode *Caenorhabditis elegans*, however, has a compact genome and the *RYR1* orthologue,
82 *unc-68*, is only 30 kb (WormBase). Nevertheless, UNC-68 has approximately 40% amino acid
83 identity with the human RyR1, distributed along the entire length of the proteins, suggesting that
84 the mammalian and nematode ryanodine receptor operate and are controlled in similar fashion
85 (Sakube et al. 1997). The short life span and many other attributes of this species for
86 experimental study make *C. elegans* the ideal subject for investigating the contribution of human
87 RyR1 variants to aging.

88 **Materials and Methods**

89 **Recombineering**

90 Amino acid sequence alignment identified residues of RyR1 variants in human genetic
91 conditions but conserved in *C. elegans* UNC-68 (Table 1). Modification of the target gene, *unc-*
92 *68*, was achieved by a two-step counter-selection recombineering technique (Feng et al. 2012). A
93 PCR amplified variant-specific counter selection cassette was inserted into the target fosmid
94 (WRM069cA02) by bacterial transformation using positive selection for the cassette. The

95 cassette was then replaced, with incorporation of the desired point mutation in a second bacterial
96 transformation with a second PCR product bearing the desired sequence alteration, using
97 negative selection against the cassette. A dicistronic cassette was used containing the positive
98 marker *tetA(C)* conferring tetracycline resistance (Tc^R), and the negative marker *rpsL*⁺
99 conferring streptomycin sensitivity (Str^S) in the *rpsL*⁻ (thus Str^R) EL350 host, a recombineering
100 competent *Escherichia coli* strain (Feng et al. 2012). Confirmation of insertion and replacement
101 of the cassette was carried out using colony PCR. The final recombineered fosmid for each
102 variant was sequenced across the manipulated region and subjected to *EcoRI* restriction enzyme
103 digestion to confirm that the correct variant had been introduced into the fosmid, with at least no
104 substantial rearrangements

105 **Strains**

106 Manipulated and wild type *unc-68* fosmids were introduced into *unc-68(e540)* worms by
107 microinjection (Mello et al. 1991). *Unc-68(e540)* carries a point mutation towards the centre of
108 the gene and behaves genetically as a null (Maryon 1996). Those worms bearing the fosmid in an
109 extrachromosomal array encoding a functional *unc-68* display a wild type phenotype of
110 movement through which they could be selected and transgenic strains established. One fosmid
111 was also co-injected in a mixture with pRF4, a plasmid bearing *rol-6(su1006)*, which causes an
112 obvious dominant roller phenotype (Mello et al. 1991).

113 GFP-myosin strains were developed by mating N2 males with *unc-68(e540)* hermaphrodites to
114 generate male progeny heterozygous for *unc-68*. These males were mated with RW1596 (stEx30
115 (*myo-3::gfp, rol-6(su1006)*)). The resulting hermaphrodite progeny were then allowed to self-
116 fertilize to generate uncoordinated rollers, homozygous for *unc-68(e540)* and bearing the
117 extrachromosomal array containing *myo-3::gfp, rol-6(su1006)*. These worms were subjected to

118 UV mutagenesis and screened for uncoordinated rollers with progeny that are all rollers due to
119 the extrachromosomal array having been stably integrated into a chromosome (Mariol et al.
120 2013). Hermaphrodites from this new strain were mated with males from each of the *unc-68*
121 fosmid transgenic strains screening for worms with well-coordinated roller movement due to the
122 *unc-68* bearing extrachromosomal array and the chromosomally integrated *myo-3::gfp*, *rol-*
123 *6(su1006)* transgenes. Self-fertilization and selection for well-coordinated rollers yielded *myo-*
124 *3::gfp*, *rol-6(su1006)* homozygous strains bearing the *unc-68* transgenes.

125 **Strain maintenance**

126 All *C. elegans* strains were routinely maintained in culture at 20 °C on 50 mm plates of
127 Nematode Growth Medium (NGM) (51 mM NaCl, 1.7 % agar, 0.25 % Bacto-peptone in 1 L
128 H₂O, autoclaved, before addition of 1 ml 1 M CaCl₂, 1 ml 1 M MgSO₄, 25 ml 1 M KPO₄ pH 6.0,
129 1 ml cholesterol 5 mg/ml in ethanol) (Stiernagle 2006). NGM plates were seeded with *E. coli*
130 strain OP50. Transgenic strains were maintained by serial transfer of transgenic worms selected
131 for their altered phenotype.

132 **Age Synchronising**

133 Eggs were prepared by bleaching to synchronise worm populations for assay. Mixed stage
134 population of nematodes were washed from the NGM plates in 500 µl M9 buffer (20 mM
135 KH₂PO₄, 42 mM Na₂HPO₄, 86 mM NaCl, 1 mM MgSO₄) (Stiernagle 2006). 150 µl of
136 Sainsbury's thin bleach and 100 µl 4 M NaOH were added and the solution left at room
137 temperature for 5 minutes. After microcentrifugation at 13500 rpm for 30 seconds the
138 supernatant was removed and the pellet re-suspended in 1 ml fresh M9 buffer. Centrifugation
139 was repeated and the pellet re-suspended in approximately 50 µl of residual supernatant for
140 transfer to a freshly seeded NGM plate. This protocol kills all post-embryonic stages leaving the

141 eggs, which subsequently hatch across the 14 hours of embryogenesis, and then develop together
142 into adults, effectively ensuring that the worms assayed will be of approximately the same age in
143 days.

144 **Phenotyping assays**

145 The transgenic strains were assayed to determine their sensitivity to caffeine and halothane.
146 Individual adult worms 4 days after synchronisation, were selected from NGM plates using a
147 sterile worm pick and placed in 1 ml of 0, 1, 5, 10, 20, 40 or 80 mM caffeine dissolved in S-
148 medium (1 litre S Basal (5.85 g NaCl, 1 g K₂HPO₄, 6 g KH₂PO₄, 1 ml cholesterol (5 mg/ml in
149 ethanol), H₂O to 1 L and autoclaved), 10 ml 1 M potassium citrate pH 6, 10 ml trace metals
150 solution (1 L stock: 1.86 g Na₂ EDTA, 0.69 g FeSO₄ •7 H₂O, 0.2 g MnCl₂•4 H₂O, 0.29 g ZnSO₄
151 •7 H₂O, 0.025 g CuSO₄ •5 H₂O, H₂O to 1 litre, autoclaved and stored in the dark), 3 ml 1 M
152 CaCl₂, 3 ml 1 M MgSO₄) (Stiernagle 2006). After 5 minutes the effect of the chemical was
153 quantified by assessing the number of body bends in 30 seconds. Halothane assays were carried
154 out in a similar manner but using 1 ml of 0, 0.5, 1, 1.5, 2 and 2.5 mM halothane solution
155 (prepared from a 25 mM stock in DMSO and mixed into S-medium) and assaying after 1 minute
156 exposure. Fifty worms were assayed for each strain at each concentration for each reagent.

157 **RNAi assays**

158 Synchronized L1s were transferred to new NGM plates, including 1 mM IPTG and 50 µg/ml
159 Ampicillin, and seeded with *E. coli* (HT115) producing dsRNA for *cbd-1*, *osm-3* or *che-3*. *Cbd-1*
160 RNAi was used in the longevity assays to exclude progeny from the assay (Johnston et al. 2010).
161 *Cbd-1* is only required for eggshell production and *cbd-1* RNAi knockdown appears to have no
162 effect on longevity of hatched animals. Lifespan assays were initiated at the young adult stage

163 and populations were scored every day. Animals that were lost from the plates or died from
164 vulval extrusion were excluded from the analysis. *Osm-3* and *che-3* RNAi treated animals were
165 used in caffeine assays as described above, with controls treated identically except using HT115
166 containing L4440 RNAi plasmid lacking an insert so that no dsRNA was present.

167 **Muscle aging assays**

168 The transgenic strains expressing the *myo-3::gfp* were assayed on days 0, 2, 4, 6, 8, 10, 12 and
169 14 of adulthood. Day 0 was considered to be 3 days post-hatching. Only live worms were
170 selected for analysis. 20 worms were assayed for each strain at each time point. The extent of
171 muscle aging was quantified by direct observation using an aging scale of 1-5 (Figure 4). A score
172 of 1 indicates perfectly ordered myofilament structure, through to a score of 5 indicating total
173 disorder, with half scores for worms that lay between the defining states. Visualisation and image
174 capture was carried out using a Leica DMR fluorescence microscope and Improvion Openlab
175 software. In preparation for microscopy animals were immobilised using 5 mM levamisole and
176 placed in individual wells of an 8 well microscope slide. Each individual was scored for extent of
177 muscle aging at the head, vulva and tail regions of the body at 20x magnification. These scores
178 were combined to provide a whole-body score.

179 **Statistical Analysis**

180 Results of phenotyping assays were analysed to establish any potential differences in the rate of
181 body bends when the worms are subjected to halothane and caffeine. Each strain containing an
182 altered fosmid was compared to the strain containing the unaltered fosmid at each discrete
183 concentration of the reagent in question. A linear model was established describing body bends
184 being dependent upon presence of the variant and statistical significance was measured by
185 carrying out one-way analysis of variance on the linear model. Body bends data on RNAi treated

186 worms were also compared by one-way analysis of variance at each discrete concentration to
187 determine whether there was a significant difference in movement from a mock RNAi treatment
188 with the L4440 empty plasmid RNAi control.

189 Categorical whole-body muscle score data were analysed using ordered logistic regression with p
190 values calculated by comparing the T-statistic to the standard normal distribution. This enabled
191 examination of any differences between the strains with modified *unc-68* and the strains rescued
192 with the wild-type *unc-68*, evaluation of the effect of increasing age of the worm in days, and
193 interactions between these two variables. Differences between the scores for the regions of the
194 worm down the anterior-posterior axis were similarly carried out. All statistical analyses were
195 completed using RStudio version 3.0.2.

196 Comparison of life span data was subjected to survival analysis with curve comparison using the
197 log-rank test to determine significant differences between variant strains and the UL4140 wild
198 type control as well as testing for any difference between the UL4140 wild type control and N2.

199 **Reagent and Data Availability**

200 Strains and recombineered fosmids described are available upon request. File S1 contains the
201 raw data for the experiments presented.

202 **Results and Discussion**

203 **A single fosmid DNA clone contains the whole of *unc-68***

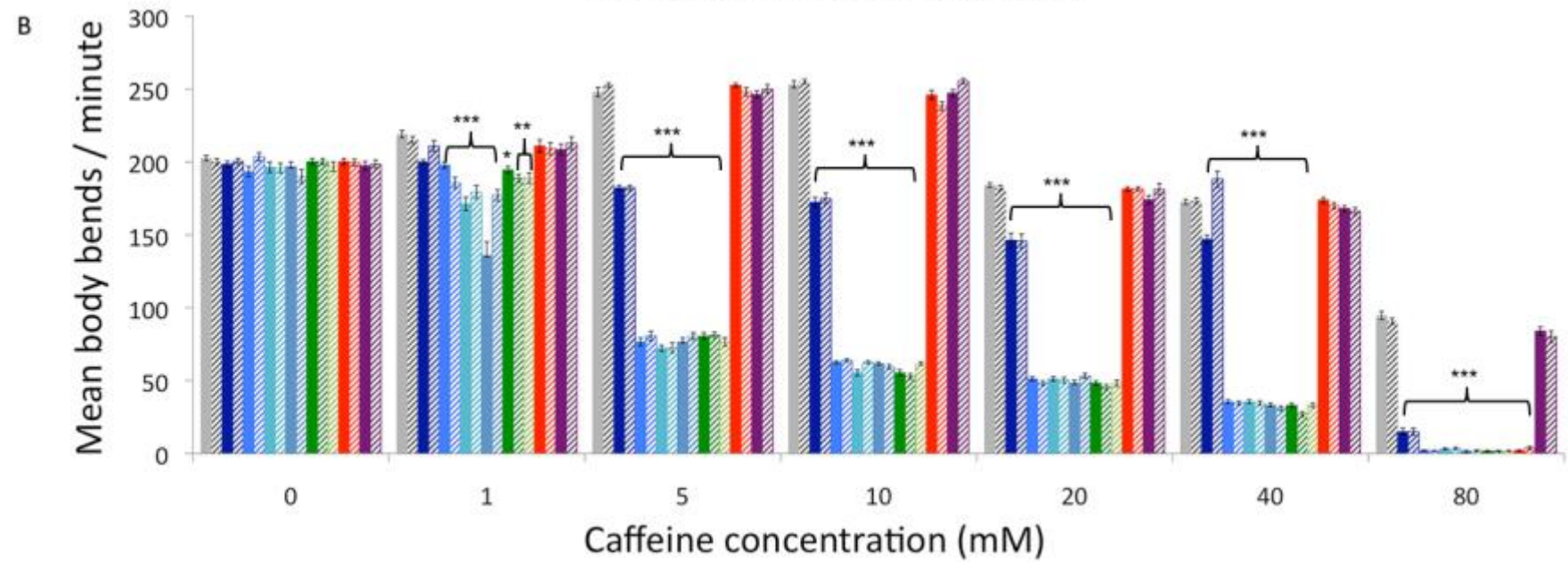
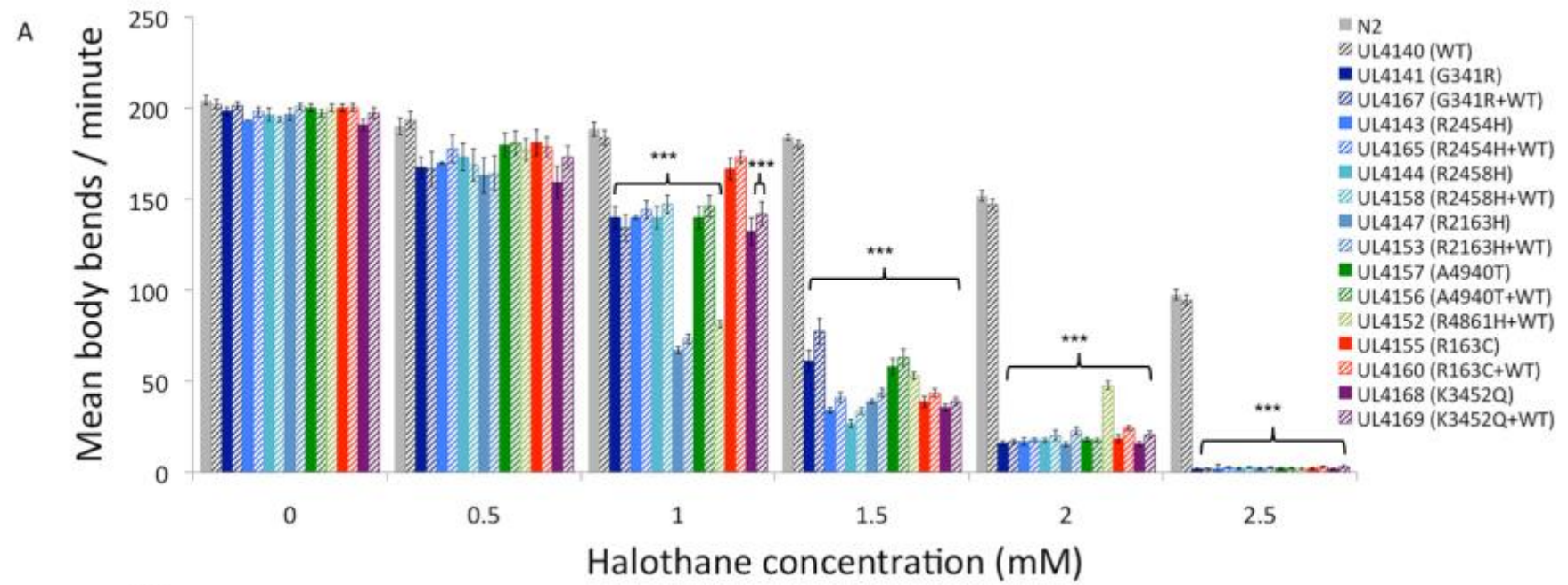
204 UNC-68, and release of Ca²⁺ from the sarcoplasmic reticulum, is needed for wild type
205 locomotion. *C. elegans* locomotion is achieved through the coordinated contraction and
206 relaxation of opposing dorsal and ventral muscle cells resulting in a sinusoidal wave passing

207 along the body propelling the worm forwards or backwards (Nicholas 1984). While mouse
208 mutants that lack RyR1 are not viable, *unc-68* null mutants survive (Sakube et al. 1997). The
209 relatively small size of the nematode's muscle cells means influx of calcium through the cell
210 membrane alone is sufficient for muscle contraction. However, muscle contractions of the *unc-*
211 *68* mutant are not as strong or rapid as in the wild type and consequently the overall appearance
212 of locomotion is affected (Sakube et al. 1997). Microinjection transformation of *unc-68* mutant
213 hermaphrodites with genomic DNA fosmid clone WRM069cA02 (WormBase) yielded progeny
214 with apparently wild type locomotion suggesting WRM069cA02 contains the entire *unc-68* gene
215 and all that is required for its expression. Each rescued line moved with around 200 body bends
216 per minute in liquid medium, considerably faster than the 60 mean body bends per minute of the
217 *unc-68* null mutant. Transmission of this rescued phenotype to subsequent generations
218 established that this fosmid, in an extrachromosomal transgenic array, provides appropriate
219 levels of expression of the ryanodine receptor and yielded the reference strain, UL4140, used in
220 subsequent comparisons, below, examining ryanodine receptor variants.

221

222 Not only did the wild type *unc-68* transgene in UL4140 fully rescue for locomotion but the strain
223 responded to increasing concentrations of halothane and caffeine, the drugs used in the MH
224 IVCT, in the same manner as the standard wild type strain, N2 (Figure 1). *C. elegans* responds to
225 inhalation anesthetics such as halothane in a similar manner to humans; initial excitation leads on
226 to lack of co-ordination, before complete cessation of movement, first with and then without
227 response to mechanical stimulation, with longer term exposure being fatal (Morgan and Carscobi
228 1985). Halothane inhibited UL4140 and N2 locomotion progressively and to the same degree
229 with increasing concentrations of the anesthetic, across concentrations tested (0.5 to 2.5 mM)

230 (Figure 1A). Previous work showed *unc-68* mutants to have altered responses to caffeine
231 (Adachi and Kagawa 2003), yet locomotion of both UL4140 and N2 individuals was stimulated
232 and then inhibited to the same degree by increasing concentrations of caffeine (from 1 to 80 mM)
233 (Figure 1B).



235

236 **Figure 1. Comparison of the rate of locomotion of *unc-68* variant strains and wild type**
237 ***C. elegans* in increasing concentrations of halothane or caffeine.** Mean body bends per
238 minute for 50 individuals in the presence of various concentrations of halothane (A) or caffeine
239 (B) are presented for each strain. *C. elegans* strains corresponding to Malignant Hyperthermia
240 (MH) associated variants are in shades of blue, to Central Core Disease (CCD) associated
241 variants are in shades of green, to the Exertional Heat Illness (EHI) associated variant is in red
242 and to the Late Onset Axial Myopathy (LOAM) associated variant is in purple. In the key, strain
243 names are provided, with the nature of the RyR1 variant and whether the wild type fosmid was
244 also present (WT) indicated in brackets. Transgenic strains generated using only a variant fosmid
245 are represented by solid bars, while strains generated using a variant fosmid and the wild type
246 fosmid are represented by striped bars, in adjacent corresponding pairs. The solid grey bars are
247 for the wild type N2 while the dashed grey bars are for the control transgenic strain, UL4140,
248 generated with just the wild type *unc-68*. Error bars are standard error of the mean. Significant
249 differences between variant strains and UL4140 are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
250 (ANOVA).

251

252 **UNC-68 variants equivalent to RyR1 myopathic variants retained ryanodine receptor**
253 **function**

254 Eight human *RYR1* variants were selected for study in *C. elegans* (Table 1). Selection of variants
255 took into account that some *RYR1* variants are implicated in other myopathic conditions beyond
256 MH; central core disease (CCD) (Robinson et al. 2002), exertional heat illness (EHI) (Davis et

257 al. 2002; Nishio et al. 2009) and late-onset axial myopathy (LOAM) (Jungbluth et al. 2009;
258 Loseth et al. 2013). CCD is a congenital myopathy that presents with progressive proximal
259 muscle weakness; type 1 skeletal muscle fibers exhibit cores with unstructured myofibrils
260 lacking mitochondria (Zhou et al. 2007). In EHI, individuals suffer potentially lethal
261 hyperthermic responses to exercise. Patients with LOAM exhibit lumbar hyperlordosis, scapular
262 winging and camptocornia, due to skeletal muscle defects, with onset between the ages of 30 and
263 70 years (Jungbluth et al. 2009; Loseth et al. 2013). Strikingly, the amino acid residues variant in
264 the different conditions are not segregated to distinct domains of the ryanodine receptor and are
265 distributed throughout the protein (Robinson et al. 2006).

266 The RyR1 variants selected for study were: G341R, R2163H R2454H and R2458H for MH
267 (Robinson et al. 2006); R4861H implicated in CCD only (Monnier et al. 2001); A4940T
268 implicated in CCD and MH (Kraeva et al. 2013); R163C implicated in EHI, CCD and MH
269 (Estève et al. 2010); and K3452Q implicated in LOAM (Jungbluth et al. 2009; Loseth et al.
270 2013). Single base pair changes were generated by recombineering of the *unc-68* fosmid
271 WRM069cA02 such that the change in the encoded UNC-68 would be precisely equivalent to
272 the single amino acid changes in these RyR1 variants.

273 Table 1. Amino acid alignment in region of all eight variants studied and the corresponding transgenic *C. elegans* strains generated.

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HUMAN CONDITION	RYR1 VARIANT	PROTEIN	ALIGNMENT	TRANSGENIC STRAIN WITH VARIANT FOSMID ONLY	TRANSGENIC STRAIN ALSO INCLUDING WILD TYPE FOSMID	
Malignant hyperthermia	p.G341R c.1021G>A	RyR1	KRDVEGMPPEIKYGESLFCFVQHVASGLW	UL4141 (UL4193)	UL4167 (UL4200)	
		UNC-68	EKEEEGGMGNATIRYGETNAFIQHVKTQLW			
	p.R2163H c.6488G>A	RyR1	DTMSLLECLGQIRSLIVQMGPQEEENLMI	UL4147 (UL4194)	UL4153 (UL4198)	
		UNC-68	DVTDFLVYLIQIRELLTVQFEHTEEAAILK			
	p.R2454H c.7361G>A	RyR1	CAPEMHLIQAGKGEALRIRAILRSLVPLE	UL4143 (UL4195)	UL4165 (UL4206)	
		UNC-68	CAPDPMAIQAGKGDLSLRARAILRSLISLD			
	p.R2458H c.7373G>A	RyR1	IQAGKGEALRIRAILRSLVPLEDLVGIIS	UL4144 (UL4201)	UL4158 (UL4197)	
		UNC-68	IQAGKGDLSLRARAILRSLISLDDLGQILA			
	Central Core Disease	p.R4861H 14582G>A	RyR1	VVYLYTVVAFNFFRKFY-NKSEDEDEPD	-	UL4152 (UL4205)
			UNC-68	VVYLYTVIAFNFFRKFYVQEGEGEEDPD		
		p.A4940T c.14820G>A	RyR1	FFFFVIVILLAI IQGLIIDAFGELRDQQE	UL4157 (UL4203)	UL4156 (UL4202)
			UNC-68	FFFFVIIILLAIMQGLIIDAFGELRDQQE		
Exertional Heat Illness	p.R163C c.487C>T	RyR1	CWWTMHPASKQRSEGEKVRVGDDIILVSV	UL4155 (UL4191)	UL4160 (UL4192)	
		UNC-68	CWWTIHPASKQRSEGEKVRVGDDVILVSV			
Late-onset axial myopathy	p.K3452Q c.10354A>C	RyR1	IFIYWSKSHNFKREEQN FVVQNEINNMSF	UL4168 (UL4196)	UL4169 (UL4199)	
		UNC-68	IFRIWSQSQHFKREELNYVAQFEEDAAAT			

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292 Human variant residues and corresponding *C. elegans* residues are in red. Other amino acids identical in RyR1 and UNC-68 are in blue. Strains
 293 in brackets also have the chromosomally-integrated *myo-3::gfp, rol-6(su1006)* transgenes. UL4140 is transgenic for the wild type *unc-68* fosmid
 294 and UL4190 is the corresponding strain with the chromosomally-integrated *myo-3::gfp, rol-6(su1006)* transgenes.

295

296 Multiple strains were generated by microinjection transformation of the *unc-68* mutant with each
297 modified fosmid. All the variants constructed, apart from that for R4861H, rescued the mutant
298 phenotype. Therefore, the identity of these particular amino acid residues, with the one
299 exception, is not critical for basic ryanodine receptor function, under normal conditions, despite
300 their evolutionary conservation from humans to nematodes. The progeny of *unc-68* null mutants
301 microinjected with the CCD associated R4861H variant all appeared to retain the *unc-68*
302 phenotype and transgenic progeny could not be distinguished from their non-transgenic siblings.
303 Co-injection of the fosmid for the R4861H variant, along with a distinct transformation marker,
304 yielded transgenic worms, recognizable from the roller phenotype, but still with a slow moving
305 *unc-68* mutant phenotype, suggesting that the modification equivalent to R4861H had indeed
306 inactivated, or at least severely compromised, UNC-68 function. The rolling phenotype means a
307 numeric comparison of body bends would not be meaningful. No human individuals
308 homozygous for R4861H are known and this amino acid substitution may inactivate the human
309 ryanodine receptor too.

310

311 **Increased halothane sensitivity was conferred by the UNC-68 variants**

312 All the rescued strains established with the modified *unc-68* fasmids exhibited an increased
313 sensitivity to halothane (Figure 1A, solid bars) revealing that these single amino acid changes did
314 modify the function of the ryanodine receptor and conferred an altered phenotype. An
315 approximately 10% decrease in the rate of body bends in liquid, in comparison to the control
316 strains with wild type *unc-68*, was apparent even at 0.5 mM halothane. At 1.5 mM halothane, a

317 concentration that has little effect on the wild type, the rate of movement in the variant strains
318 was reduced to 15% of that in the absence of halothane. The variant strains were completely
319 paralyzed by 2.5 mM halothane, while the wild type strains retained a reduced but still
320 substantial mobility. Previous extensive investigations identified genes such as *unc-79*, *unc-80*
321 and *gas-1* from mutations conferring halothane hypersensitivity in *C. elegans* (Kayser et al.
322 1999; Sedensky and Meneely 1987). Mutations in *unc-68* may not have been isolated in these
323 studies due to the need for specific point mutations that sensitize but do not eliminate ryanodine
324 receptor function.

325

326 **Increased caffeine sensitivity was conferred by some of the UNC-68 variants**

327 Young adults of the variant strains generated specific to MH or CCD also demonstrated a
328 modified response to caffeine across the range of concentrations assayed, while those for EHI
329 and LOAM did not (Figure 1B). The strains generated for the human *RYR1* variants G341R,
330 R2454H, R2458H, R2163H, A4940T and R4861H all failed to show the stimulation of
331 locomotion of the wild type as caffeine was increased from 1 to 10 mM. These same strains, with
332 the exception of that for variant G341R, actually displayed a substantial inhibition of locomotion
333 from 1 mM to 5 mM caffeine with a small progressive further inhibition from 10 to 40 mM, and
334 almost complete paralysis at 80 mM, a concentration at which the wild type still remains motile.
335 The G341R variant strain only shows a substantial inhibition of locomotion upon raising the
336 caffeine concentration to 80 mM, but this is still not quite to the same degree as the other variant
337 strains. The LOAM associated K3452Q variant strains showed a wild type response to all
338 concentrations of caffeine assayed, as did the EHI associated R163C variant strain with the

339 exception of almost total inhibition of locomotion specifically at the highest concentration of 80
340 mM.

341

342 **The modified *unc-68s* showed genetic dominance**

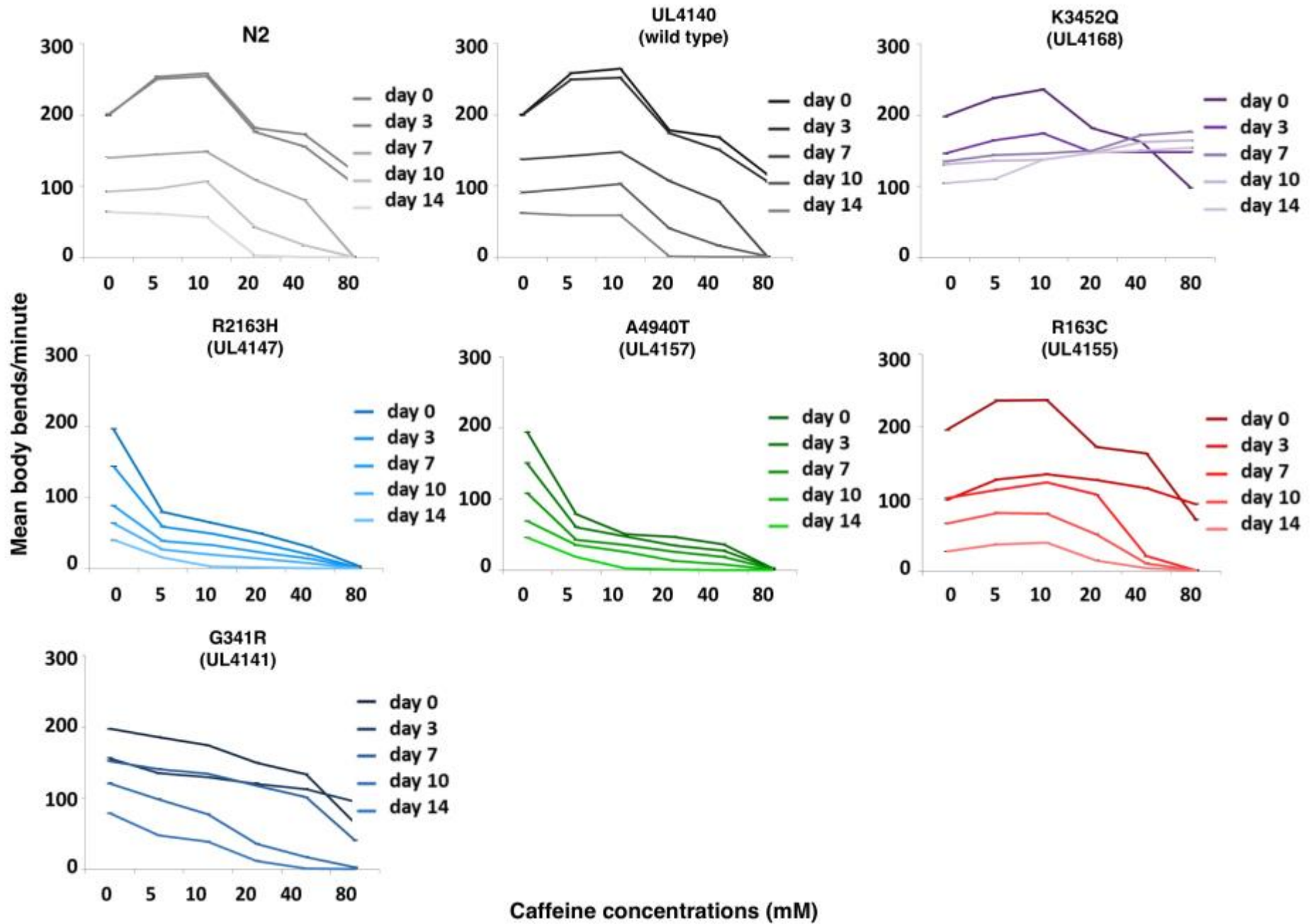
343 Genetic dominance, a striking property of many pathogenic human *RYR1* variants and all those
344 selected for study, was also demonstrated by the equivalent versions of *unc-68*. This was
345 revealed when transgenic strains of *C. elegans* were generated by microinjection transformation
346 using the modified *unc-68* fosmids but mixed with the unmodified fosmid, containing wild type
347 *unc-68* (Figure 1, striped bars). These strains all behaved in essentially the same manner as the
348 strains transformed with only the corresponding modified *unc-68* across the range of caffeine
349 and halothane concentrations assayed: i.e. variant UNC-68s that conferred a modified sensitivity
350 to caffeine or halothane did so even when wild type UNC-68 was also present. Remarkably,
351 when a wild type UNC-68 was also present, the *unc-68* variant for R4861H, the one variant
352 unable to rescue the *unc-68* mutant and apparently non-functional, nevertheless induced the same
353 modified response to caffeine and halothane (and the same aging effects – see below) as other
354 MH and CCD associated versions of UNC-68, like the corresponding human condition. This
355 could result from the non-functional variant being stably expressed and, in combination with the
356 wild type protein, generating a malfunctioning heteromeric ryanodine receptor.

357

358 **The modified *unc-68s* conferred age-related phenotypes**

359 Given the potential links between RyR1 variants and age related phenotypes in mammals, the
360 *unc-68* variant strains were examined for age-related phenotypes in *C. elegans*. First, a dramatic

361 age-related change in response to caffeine was found, for the LOAM (K3452Q) variant strain
362 (Figure 2). Young adults of the LOAM variant strain showed the same response to caffeine as
363 the wild type, with stimulation of locomotion at low concentrations and inhibition at higher
364 concentrations. All the other strains, i.e. those with other *unc-68* variants, showed simply a
365 progressive dampening of the rate of locomotion with age but the same general response profile,
366 with increasing concentrations of caffeine, characteristic of each strain (Figure 2). In contrast,
367 rather than an inhibition of locomotion, older LOAM variant strain adults showed a considerable
368 stimulation of locomotion, increasing with increasing caffeine concentrations, a response
369 attained progressively as the animals aged. It is tempting to link this striking effect in *C. elegans*
370 directly to the specific age-related symptoms characteristic of LOAM.



372 **Figure 2. Locomotion of *C. elegans* expressing the LOAM associated variant of *unc-68* is**
373 **specifically increasingly-stimulated by caffeine with age.** The locomotion of the strain for the
374 RyR1 variants G341R (UL4141), R2163H (UL4147), A4940T ((UL4157) R163C (UL4155) and
375 K3452Q (UL4168) are compared with the strain transgenic for only the wild type *unc-68*
376 (UL4140) and the standard wild type strain (N2). Mean body bends per minute in the presence of
377 increasing concentrations of caffeine are presented for 50 individuals at 0, 3, 7, 10, and 14 days
378 of adulthood. The colour coding from Figure 1 is retained with, broadly, blue for MH, green for
379 CCD, red for EHI and purple for LOAM. Error bars are standard error of the mean.

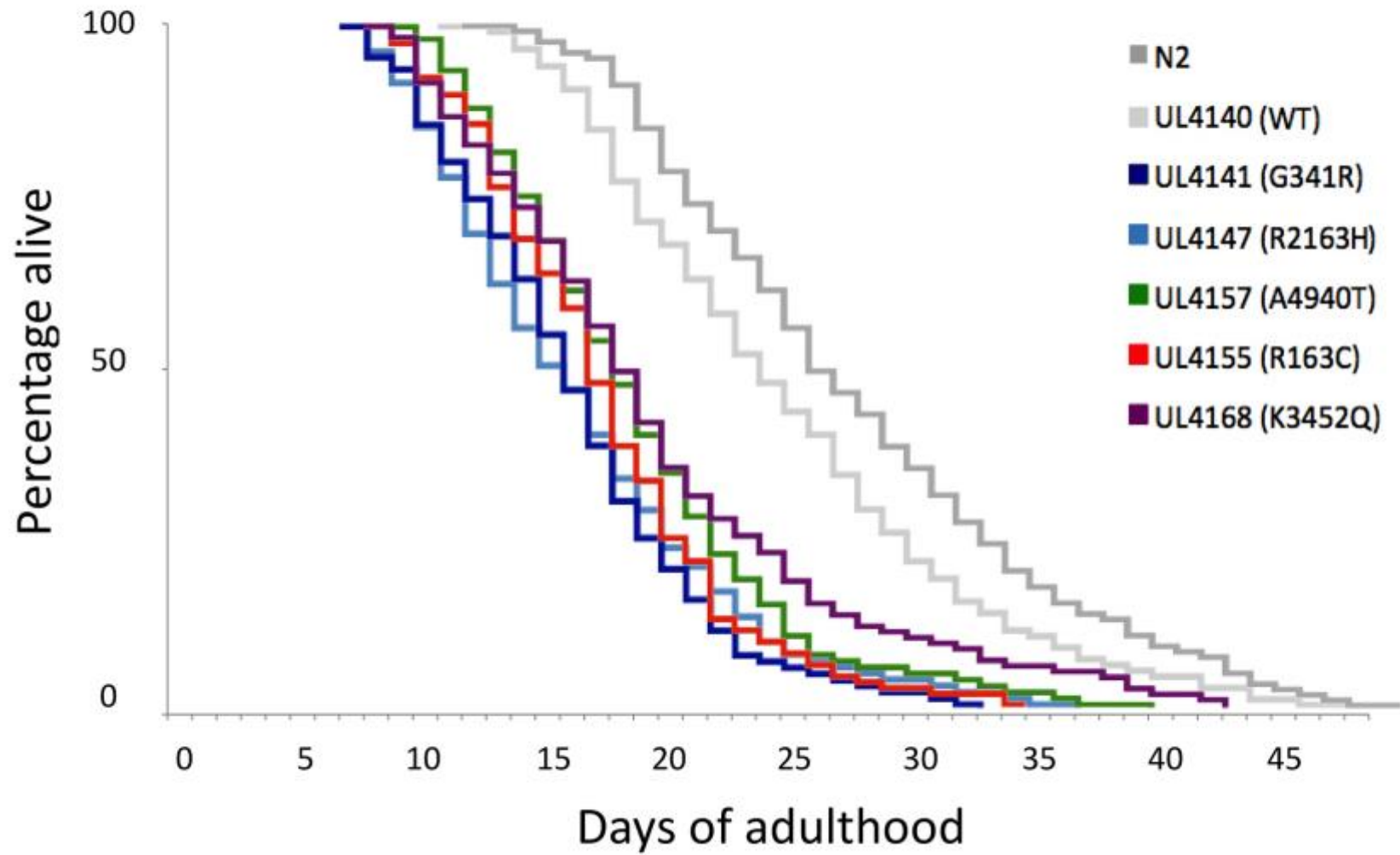
380

381 Second, the *unc-68* variants caused a decrease of median lifespan (Figure 3). All the *unc-68*
382 variant transformed strains which were assayed had median life-spans of 14 to 17 days into
383 adulthood, compared to the 22 or 24 days for the wild type control strains. A log-rank test on the
384 survival curves revealed that lifespans of strains transgenic for variant UNC-68s were
385 statistically shorter than the lifespan of UL4140, the control strain transgenic for the wild type
386 UNC-68 ($p < 0.0001$ in each case, File S1). Hence although the ryanodine receptor appeared to
387 remain functional, these single amino acid changes did shorten *C. elegans* lifespan.

388 It is also noted that the lifespan of UL4140 is significantly shorter than the lifespan of the non-
389 transgenic wild type control, N2, although the difference is not as marked ($p < 0.05$, File S1).

390 Presumably this small reduction in life span results from the difference in expression of *unc-68*
391 when present in multiple copies, as an extrachromosomal transgene, rather than in the
392 endogenous location, in single copy, within a chromosome.

393



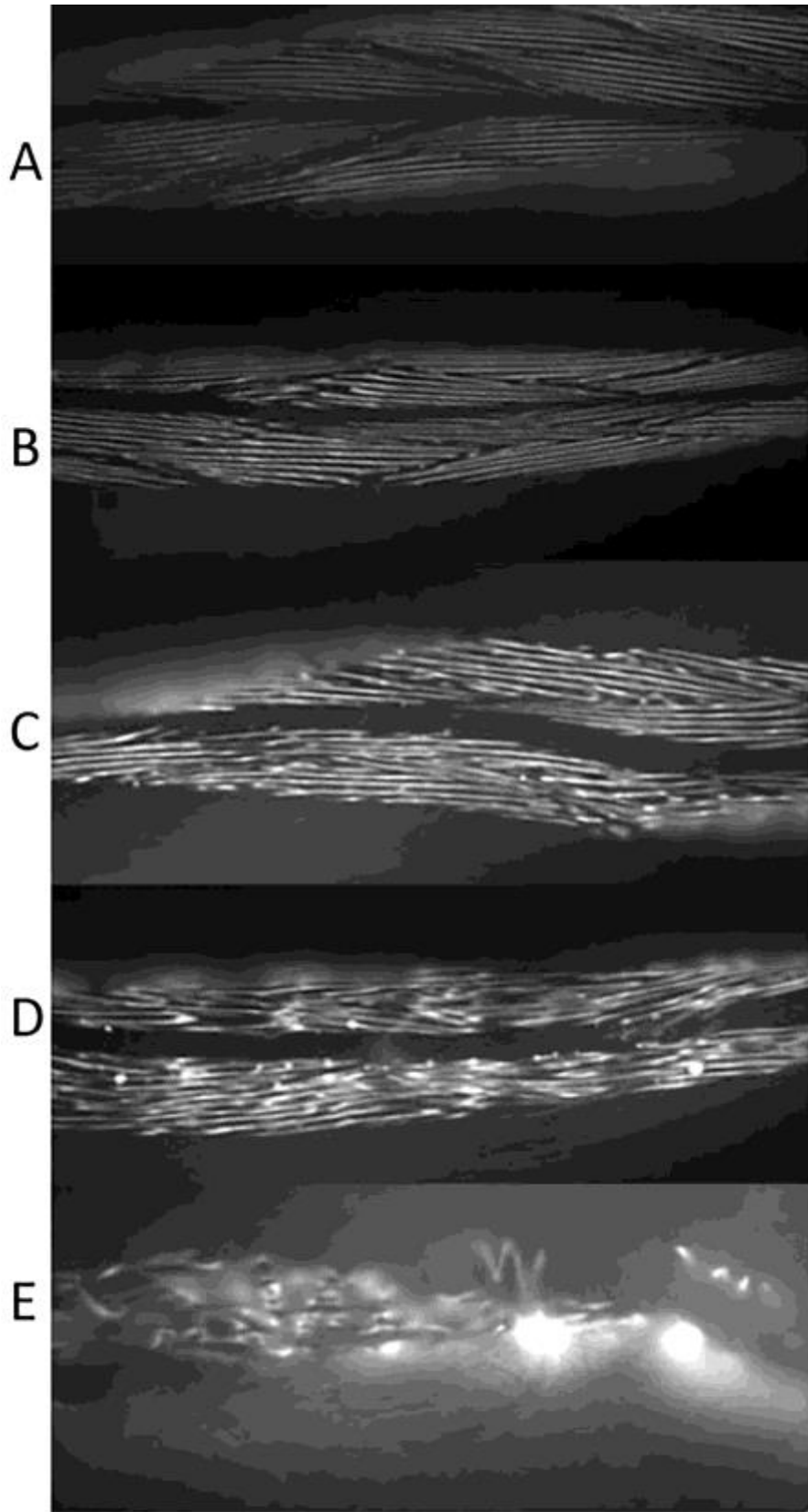
395 **Figure 3. Single amino acid changes in UNC-68 shorten *C. elegans* lifespan.** The percentage
396 of animals surviving on successive days of adulthood is presented for each strain: strains
397 transgenic for different *unc-68* variants; UL4140, transgenic for just the wild type *unc-68* (light
398 grey); and the non-transgenic wild type N2 (dark grey). In the key, strain names are provided,
399 with the nature of the RyR1 variant indicated in brackets. The colour coding from Figure 1 is
400 retained with, broadly, blue for MH, green for CCD, red for EHI and purple for LOAM.

401

402 Third, we found a specific aging effect of *unc-68* variants on *C. elegans* body-wall muscle. The
403 progressive disorganization of the sarcomeric structure with age can be followed in live
404 *C. elegans* by fluorescence microscopy using GFP tagged myosin (Herndon et al. 2002). An
405 extra-chromosomal *myo-3::gfp* transgene was chromosomally integrated, before introduction
406 into the strains bearing the various extra-chromosomal *unc-68* transgenes through mating. The
407 extent of muscle aging was quantified by comparison to a set of standard states on a scale of 1-5
408 (Figure 4), from 1 indicating fully-organized thick filament alignments through to 5 being totally
409 disorganized. As only live worms were scored and worms that die early are more likely to have
410 more disordered myofilaments, the degree of age-related structural decline will be underreported.
411 A whole-body score was derived from assessment of three regions along the anterior-posterior
412 axis in each individual. Differences in muscle aging rates between the strains with different *unc-*
413 *68* variants were small, but apparent upon statistical analysis by ordered logistic regression. No
414 significant difference in muscle aging was found between strain pairs, i.e. with versus without
415 wild type *unc-68* present, for specific *unc-68* variants. Therefore, these data were combined
416 when testing for differences in muscle aging that were specifically due to the change in UNC-68
417 sequence (Figure 5 and Table 2). Overall, the presence of an UNC-68 variant was found to

418 significantly affect the whole-body score ($p < 0.001$). No significant difference in whole-body
419 score on day 0 or day 2 of adulthood was found for any of the variant strains when compared to
420 the UL4140 based control, transformed with just wild-type *unc-68*. The whole-body score was
421 significantly increased compared to the control by day 4 for the R163C, A4940T and K3452Q
422 variant strains, by day 6 for R2454H and R2458H, by day 8 for G341R and by day 10 for
423 R2163H and R4861H, indicating that the single amino acid change in all *unc-68* variants
424 examined did induce faster muscle aging, although the age when this became statistically
425 significant varied.

426

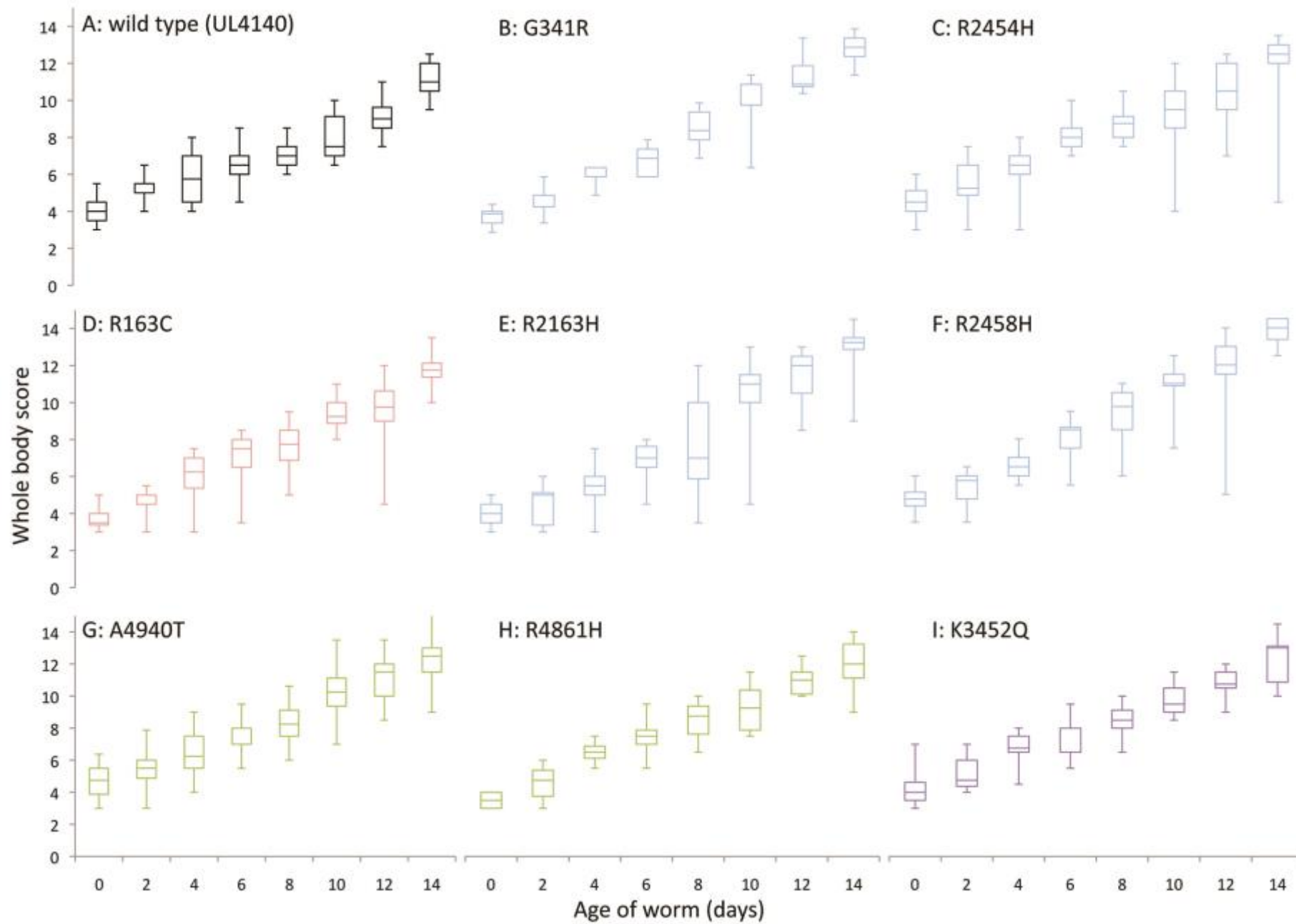


428 **Figure 4. Examples illustrating the 5 grades in the muscle disorganization scoring scale.**

429 The myosin::gfp fusion protein is localized to the thick filaments and so distribution of the
430 fluorescence reports on the regularity in the arrangement of the sarcomeres. Images captured by
431 fluorescence microscopy. A: Typical structure of a grade 1 muscle score; myosin filaments are
432 linear and well organised. B: Typical structure of a grade 2 muscle score; myosin filaments are
433 starting to show more bends but the pattern is still well organised. C: Typical structure of a grade
434 3 muscle score; myosin filaments are more fragmented and there are apparently overlapping
435 filaments. D: Typical structure of a grade 4 muscle score; myosin filaments are further
436 fragmented and the regularity of pattern is no longer clear. E: Typical example of grade 5 muscle
437 score; the pattern of myosin filaments is severely disorganized. Figure compiled by Matt Pipe.

438

439



441 **Figure 5. Comparison of increase in myofilament disorganization with age, for each *unc-68***
442 **variant.** The whole-body scores for extent of disorganization of the sarcomeric structure from 0
443 to 14 days of adulthood are presented for the strain transgenic for wild type *unc-68* (UL4140)
444 (A), and strains transgenic for *unc-68* with amino acid changes equivalent to RyR1 variants
445 associated with: MH in blue (G341R (B), R2454H (C), R2163H (E) and R2458H (F)); EHI in
446 red (R163C (D)); CCD in green (A4940T(G) and (R4861H (H)); and LOAM in purple (K3452Q
447 (I)). Each boxplot represents the median, inter-quartile range and minimum and maximum for
448 the whole-body scores.

449 Table 2. Comparison of strains transgenic for variant and wild type *unc-68*, in terms of the combined effects of variant and worm age
 450 on whole-body score of muscle organization.

		DAY of ADULTHOOD						
		2	4	6	8	10	12	14
V A R I A N T	G341R	-0.71 (0.48)	1.23 (0.22)	1.48 (0.14)	3.72 (0.0001)	5.33 (<0.0001)	4.83 (<0.0001)	4.53 (<0.0001)
	R2454H	-0.66 (0.51)	0.55 (0.58)	2.35 (0.018)	2.45 (0.014)	1.86 (0.063)	1.64 (0.0101)	2.07 (0.038)
	R2458H	-0.96 (0.34)	0.61 (0.54)	2.15 (0.018)	3.39 (<0.0001)	4.77 (<0.0001)	4.47 (<0.0001)	4.79 (<0.0001)
	R2163H	-1.65 (0.099)	-0.65 (0.515)	1.27 (0.204)	0.71 (0.478)	5.05 (<0.0001)	4.97 (<0.0001)	4.72 (<0.0001)
	R163C	-0.24 (0.81)	2.01 (0.04)	2.73 (0.006)	2.31 (0.02)	3.75 (0.0002)	2.75 (0.006)	2.75 (0.006)
	A4940T	-0.77 (0.85)	2.69 (0.007)	3.13 (0.002)	3.65 (<0.0001)	3.36 (<0.0001)	4.38 (<0.0001)	2.91 (0.0036)
	R4861H	0.19 (0.44)	0.27 (0.78)	0.67 (0.501)	1.19 (0.234)	2.61 (0.009)	2.71 (0.007)	1.38 (0.168)
	K3452Q	0.70 (0.48)	1.91 (0.055)	2.09 (0.036)	2.99 (0.003)	2.87 (0.004)	3.23 (0.001)	2.45 (0.01)

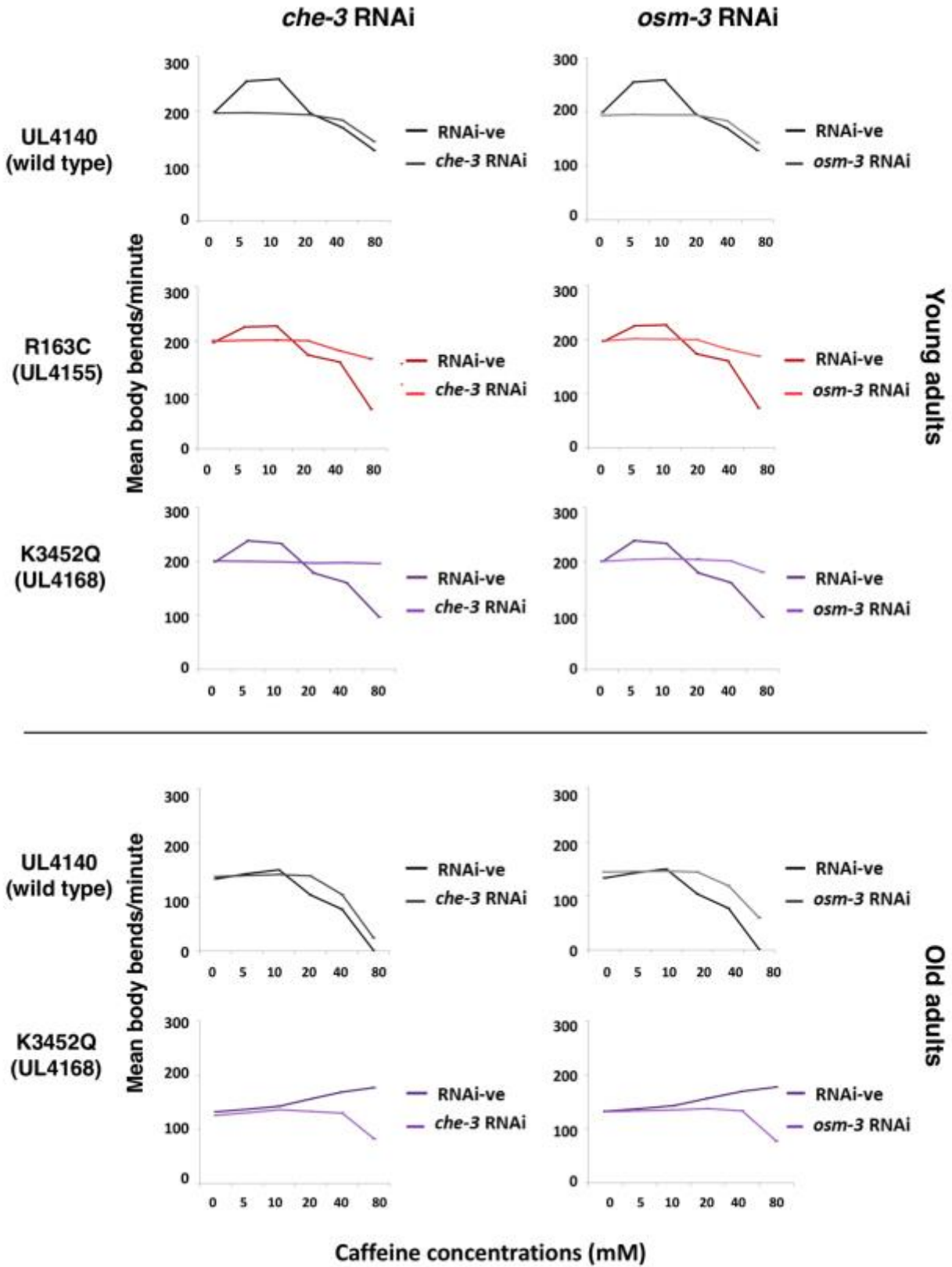
451 T-statistic is displayed correct to 2 decimal places with associated p-value in brackets. Day 2 represents 5 days after hatching.

452 **The modified responses to caffeine depended upon neural function**

453 While RyR1 is considered predominantly a skeletal muscle isoform and other isoforms are
454 expressed in other cell types, there is only a single ryanodine isoform in *C. elegans* UNC-68 and
455 this is likely to be a key intracellular Ca²⁺ channel in all cells, and critically in excitable cells.
456 Furthermore, caffeine resistant mutations have been localized to two *C. elegans* genes, *osm-3*
457 and *che-3*, specifically required for chemosensory nerve cell function (WormBase ; Hartman
458 1987). Therefore, the role of these genes in the differential response to caffeine attributed to the
459 *unc-68* variants was tested by RNAi knockdown (Figure 6 and Figure S1). In young adults of the
460 strain transgenic for only wild type *unc-68*, the stimulation of locomotion at low concentrations
461 of caffeine was completely lost, and the level of inhibition of locomotion at high concentrations
462 was reduced, upon *osm-3* and *che-3* RNAi knockdown. Exactly the same result was observed for
463 strains transgenic for the EHI associated variant R163C, for the LOAM associated variant
464 K3452Q, and for the standard wild type strain, N2. The inhibition of locomotion by caffeine,
465 across the entire range of concentrations assayed, in the strains transgenic for the MH associated
466 variant R2163H and the CCD associated variant A4940T was reduced by *osm-3* and *che-3*
467 knockdown, at each caffeine concentration. Furthermore, the dramatic progressive stimulation of
468 locomotion in response to increasing concentrations of caffeine in old adults with the LOAM
469 variant version of *unc-68* was also eliminated with knockdown of these genes. In summary, for
470 all strains examined, the locomotory response to each caffeine application was eliminated or
471 markedly reduced by *osm-3* and *che-3* RNAi. These results could indicate that the focus of
472 influence of the UNC-68 amino acid changes upon caffeine response is actually in these
473 chemosensory nerve cells. UNC-68 does have a function presynaptically at neuromuscular
474 junctions (Liu et al. 2005) and genetic analysis points to a presynaptic focus of anesthetic action

475 in *C. elegans* (Morgan et al. 2007). However, our results are also consistent with the primary site
476 of action of caffeine being in the chemosensory nerve cells with downstream consequences
477 dependent on UNC-68 in other cells, including or specifically muscle cells.

478



480 **Figure 6. The locomotory response to caffeine that is modified by amino acid changes in**
481 **UNC-68 is dependent upon the chemosensory neuron specific genes *che-3* and *osm-3*.** The
482 locomotion of strains transgenic for the wild type *unc-68* (UL4140), for the EHI associated
483 variant R163C (UL4155) and for the LOAM associated variant K3452Q (UL4168) was recorded
484 upon RNAi knockdown of *che-3* or *osm-3* or in precisely equivalent blank control RNAi
485 experiments. Mean body bends per minute in the presence of increasing concentrations of
486 caffeine are presented for 50 individuals at 0 and 7 days of adulthood, i.e. young and old adults
487 respectively. The colour coding from Figure 1 is retained with, broadly, red for EHI and purple
488 for LOAM. Error bars are standard error of the mean. Equivalent results for other strains are
489 presented in Figure S1.

490

491 That compromising chemosensory nerve cells eliminates the *unc-68* variant specific changes in
492 the response to caffeine may be pertinent to human biology. Central nervous system damage has
493 been reported occasionally after malignant hyperthermia episodes. The skeletal muscle-specific
494 effects of *RYR1* variants may be simply a reflection of the predominant tissue specific
495 distribution of this gene's expression. While *RYR2* is expressed predominantly in cardiac muscle
496 and *RYR3* is more broadly and weakly expressed, *RYR1* is also expressed in other tissues
497 including the central nervous system (De Crescenzo et al. 2012; Giannini et al. 1995). A clinical
498 report has linked *RYR1* variants to central nervous system damage in response to triggering
499 events (Forrest et al. 2015).

500

501 The response of *C. elegans* to caffeine and halothane, upon directed modification of *unc-68*, as
502 described here, emphasizes the conservation of functionality of the ryanodine receptor from
503 humans to nematodes. Sequence changes in UNC-68, equivalent to human disease causing
504 variants, conferred increased sensitivity of the whole organism to pharmacological agents of
505 direct relevance to the medical conditions. The variants even induced this increased sensitivity in
506 *C. elegans* in the presence of the wild type protein, mirroring the genetic dominance of the
507 human variant alleles. The distinction of increased caffeine sensitivity being seen for the *unc-68*
508 variants corresponding solely to MH or CCD, but not those implicated in EHI or LOAM, is
509 based on analysis of a limited number of variants. However, this finding may also be relevant to
510 muscle biopsies of EHI patients and some MH susceptible individuals responding to halothane
511 but not to caffeine in the diagnostic IVCT (Carpenter et al. 2009; Hopkins et al. 1991).

512

513 Processes determining animal lifespan appear remarkably conserved and studies on *C. elegans*
514 longevity have been instrumental in delivery of our current understanding on this subject
515 (Rodriguez et al. 2013). Here, single amino acid changes in the ryanodine receptor have been
516 shown to decrease lifespan and increase muscle aging, in *C. elegans*, adding further support to
517 the evidence suggesting equivalent effects in mammals. The progressive increase in sensitivity to
518 caffeine stimulation of locomotion with age in the strain with an *unc-68* variant equivalent to
519 LOAM may reflect changes that occur with age in humans and contribute to this condition.

520

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