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1 # A point-of–no-return leading to death during heat-shock in *C. elegans*

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12 # Abstract

13 Longevity is a complex trait determined by genes, the environment, and their interactions. There is
14 considerable insight into the genes associated with longevity and the interplay with environmental
15 conditions. Most genes involved in the stress response play a major role in determining longevity.
16 Yet, there is limited understanding of the mechanisms that determine how long stress can be tolerated
17 before death becomes inevitable. Here, we leveraged the detection of an irreversible switch to death
18 by studying global gene expression profiles in combination with survivorship following heat stress in
19 the nematode *C. elegans*. By analysing the transcriptional response in a high-resolution time series of
20 increasing stress exposures, we found a distinct shift in gene expression patterns between 3-4 hours
21 into the stress response, separating an initially highly dynamic phase from a later mostly stagnant
22 phase. Remarkably, this turning point in expression dynamics coincided with a phenotypic point of no
23 return, as shown by a strong decrease in movement, survival and, progeny count in the days after;
24 ultimately leading to death.

25

26 # Keywords

27 Transcriptome, Stress response, Time-series, Heat-shock, Recovery, *C. elegans*

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28 # Introduction

29 Longevity differs strongly within and between species. Over the past few decades detailed insight has
30 been obtained about the genetic and environmental factors that determine longevity. Many genes have
31 been identified that play a key role in lifespan determination, many of which are involved in
32 environmental stress response pathways. Environmental stress is a potentially harmful condition
33 beyond the optimum range of the organism, for example through shifts in temperature and exposure to
34 oxidants, or toxic compounds [1,2]. On a cellular level, these environmental stressors can interfere
35 with protein homeostasis, leading to an accumulation of misfolded proteins and protein aggregates
36 [3]. To avoid the detrimental effects of misfolded proteins and protein aggregates, multiple stress
37 response systems have evolved as a first line of defence to maintain proteostasis, of which the highly-
38 conserved heat-shock response (HSR) pathway is prominent [3,4]. The accumulation of misfolded
39 proteins is a hallmark of aging and age-related diseases such as Alzheimer's and Parkinson's disease
40 [5–7]. The connection between the processes involved in stress and aging is further substantiated by
41 the fact that several components of the stress response pathways were found to function as regulators
42 of lifespan [8,9]. For example, the evolutionary highly conserved transcription factor HSF-1 is a key
43 component in the initiation of the HSR, as well as a regulator of lifespan [10]. Therefore,
44 understanding how an organism perceives and handles stress is fundamental for understanding the
45 molecular mechanisms that underlie aging [6].

46 The nematode *Caenorhabditis elegans* is an established metazoan model for studying the
47 effect of - and response to - stress and aging *in vivo* [6,8–10]. One of the most widely studied stress
48 responses in *C. elegans* is acute heat stress, which can be easily applied by exposing the animal to
49 temperatures between 33-37ºC [10–12]. Often, the effects of the stress are quantified on a phenotypic
50 level by recording complex traits such as survival rate, mobility, and reproduction [12–14]. Generally,
51 the inflicted damage accumulates with increasing temperature and exposure time. For example, brood
52 size decreases with moderate increases in temperature beyond the optimum [14,15], whereas a strong
53 decrease in survival rates is only observed after prolonged exposures to heat stress [12,16,17]. It was
54 shown that *C. elegans* detects and responds to heat stress via transient receptor potential channels and

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55 a neuropeptide signaling pathway [18]. At the level of the transcriptome, a heat shock induces a
56 strong response. Genome wide gene expression analysis in *C. elegans* shows that a two hour exposure
57 to 35ºC affects genes associated with development, reproduction and metabolism [19]. Furthermore,
58 an exposure of 30 minutes to 33°C already induced a massive global gene expression shift highly
59 dependent on HSF-1, affecting genes associated with a wide range of functions such as cuticle
60 structure, development, stress response, and metabolism [20].

61 Yet, there is limited understanding of the mechanisms that determine how long stress can be
62 tolerated before death becomes inevitable. Given the range of phenotypic effects, it is to be expected
63 that the transcriptional response during heat stress is highly dynamic. For example, the initial
64 transcriptional response to heat shock probably does not resemble the transcriptome after a lethal
65 exposure to heat stress. To gain more insight into the underlying dynamics of the stress response, we
66 have generated a high-resolution time-series of transcriptomic and phenotypic data of *C. elegans*
67 exposed to heat stress conditions at 35ºC for 0-12h. Transcriptomic analysis revealed a global shift in
68 expression dynamics occurring between 3 and 4 hours into the heat exposure. The shift marks the end
69 of an initially highly dynamic transcriptional response to heat stress that plateaus at longer exposures.
70 On a phenotypic level, longer exposures (> 4h) were associated with low chances of recovery, thus
71 indicating that the critical shift observed in the global gene expression marks a point of no return
72 ultimately leading to death.

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73 # Results

74 ***Transcriptional variation during prolonged heat stress***

75 We first assessed the impact of heat stress durations on genome-wide expression levels. Wild type
76 Bristol N2 populations were exposed to heat stress conditions at 35°C for increased exposure
77 durations between 0.5-12 hours (**Figure 1A**). To find the main sources of variation during the
78 transcriptional response to heat-shock, we used principal component analysis (PCA). The first two
79 principal components (PCs) captured 77% (1st 57%, 2nd 20%) of the total variation (**Figure 1B**). The
80 first PC sorted the time points in chronological order, showing that variation in gene expression
81 between samples was largely due to the increasing length of heat exposure. Furthermore, the distance
82 along the 1st PC was larger for early time points in comparison to later exposure times, indicating that
83 a large part of the changes in gene expression occurred early in the stress response. Together, the 1st
84 and 2nd PCs indicated 3-4 hours of heat exposure as a turning point in transcriptional patterns during
85 the prolonged stress response.

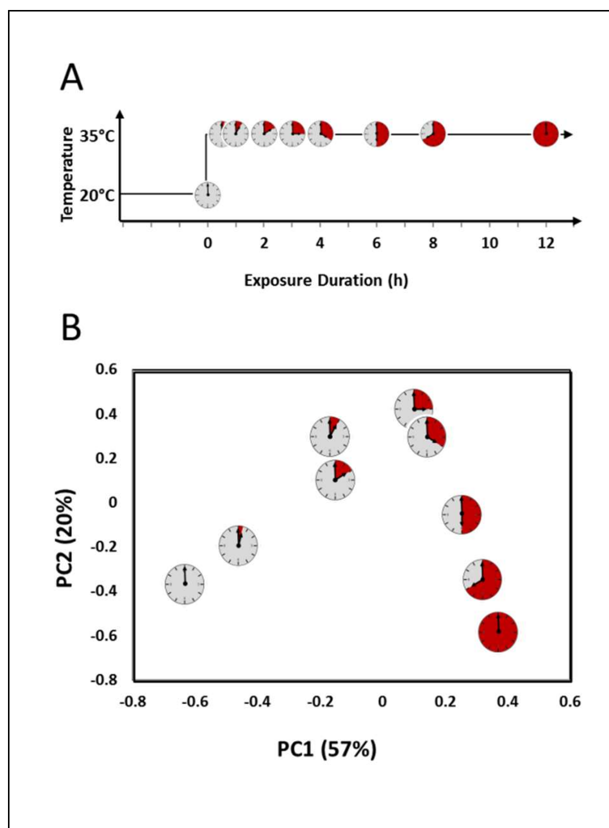

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Figure 1: Experimental set-up and principal component analysis. (A) mRNA sampling schedule. Bristol N2 populations were grown at 20°C for 46 hours before the start of the heat-shock at 35°C. Clock-symbols indicate the time of sampling for subsequent transcriptome analysis of the dynamic stress response. Each time point (0, 0.5, 1, 2, 3, 4, 6, 8, and 12 hours) was sampled 3-5 times. **(B)** Principal component analysis of gene expression data averaged per time point. The first two components retain 77% of the variation in the data set, and placed the exposure duration (as indicated by the clock symbol) in chronological order.

86

87 ***Early transcriptional activation of heat shock proteins***

88 Having identified a turning point in transcriptional patterns, we further investigated the temporal
89 dynamics of expression changes for a set of previously associated heat stress response genes. For *C.*
90 *elegans*, the Gene Ontology database listed 72 genes with a role in the 'response to heat stress'
91 (GO:0009408, WormBase version 257). Most of these genes show minor transcriptional changes in
92 the course of the 12-hour heat exposure **(Supplementary Figure S1).** This is not surprising, since
93 many components of the (heat) stress response are constituently expressed [21].

94 The fastest transcriptional response was found for five heat shock proteins, which are part of
95 the heat shock response pathway activated by HSF-1 upon stress exposure. Within 30 minutes of
96 stress exposure, *hsp-16.1*, *hsp-16.2*, *hsp-16.41*, *hsp-16.48*, and *hsp-70* showed a ~16-fold increase in
97 expression levels **(Supplementary Figure S1)**. The expression levels of these genes peak 4 hours into
98 the stress exposure, corresponding with the turning point identified in the PCA. Correlation analysis
99 was used to identify genes that have not previously been associated with the heat-stress response, but
100 show a similarly immediate and strong response to stress as the heat-shock proteins. Two correlated
101 genes were found that have not previously been listed in the GO-term 'response to heat stress':
102 F13E9.1 (ortholog of human NISCH) and F44E5.5 (member of the *hsp-70* family). Together, this
103 small set of seven genes presented the strongest, first and immediate reaction of the transcriptional
104 response to stress.

105

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106 ***Changes in gene expression reach a plateau***

107 We further investigated the temporal dynamics of global transcriptome changes. About ~6200 (~30%)
108 genes contributed significantly (q-value < 0.01) to the variation explained by the first two principal
109 components. This sub-set was used as input for k-means clustering to extract common patterns in
110 gene expression changes, identifying six distinct stress-response groups (**Figure 2A-B**). Cluster 1, 4,
111 and 6 (representing ~3790 (60%) of the genes) contained genes downregulated during exposure to
112 heat (**Figure 2A**), and cluster 2, 3, and 5 contained upregulated genes (~2450 (40%) of genes; **Figure**
113 **2B**). The largest changes were found in cluster 1 and 3 with an average 5-fold down- and 32-fold up-
114 regulation, respectively.

115 Within these clusters, the initial in- or decrease in transcript levels started rapidly, between
116 0.5-1 hour after initiation of the heat stress exposure, and reached a plateau before 3 hours into the
117 stress response. One exception is cluster 1, consisting of 420 genes that were down-regulated after 4
118 hours. Interestingly, this was the only pattern clearly distinguishing the later (>4h) time points. As
119 previously indicated by the PCA, the transcriptional patterns reveal a global change in expression
120 dynamics after approximately 3-4 hours into the stress response, starting with a highly dynamic
121 adaptive phase and ending with a plateau phase of minimal overall changes.

122 To explore the biological functions associated with the gene sets within the individual
123 response clusters, an GO-enrichment analysis was performed (**Figure 2C-D; Supplementary table**
124 **S1**). Overall, the down-regulated clusters were enriched with structural constituents of the cuticle,
125 particularly collagens (*col, dpy, rol, sqt*), as well as genes associated with transcription (*nhr*),
126 metabolic processes, and locomotion (**Figure 2C)**. In the upregulated clusters, genes involved in
127 nucleosome assembly (*his*) were found to be overrepresented, as well as those regulating embryo and
128 larval development (**Figure 2D)**. Cluster 3, the smallest group (54 genes), had an immediate and
129 strong reaction to the stress. This cluster could not be associated with an enrichment term. Half of the
130 genes within this cluster have not previously been classified with any GO term yet are very likely
131 involved in the response to heat stress.

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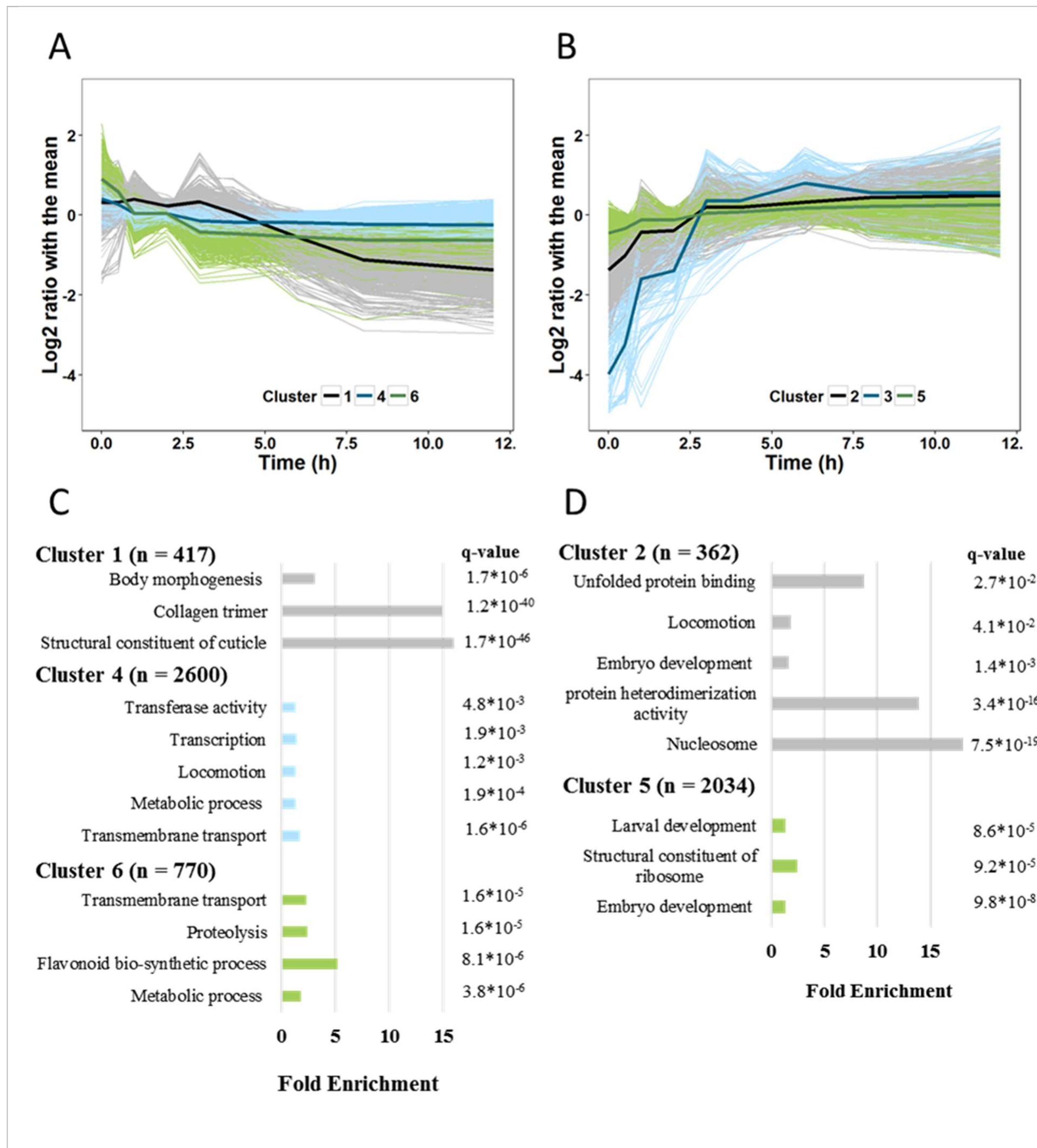

Figure 2: Temporal dynamics and functional enrichments of gene expression in response to continuous heat stress at 35°C. Genes with similar patterns in expression (log2 ratio with the mean) were grouped by k-means clustering. Dark coloured bold lines present the average expression of the individual clusters; lighter corresponding colours present the expression of individual genes. Enrichment analysis of gene clusters was performed with DAVID 6.8. **(A)** Cluster 1, 4, and 6 showed a downward trend in gene expression during heat stress. **(B)** Cluster 2, 3, and 5 were upregulated in response to heat stress. **(C)** Enrichment of downregulated gene clusters. **(D)** Enrichment of upregulated gene clusters 2 and 5. Analysis of cluster 3 did not result in a significant enrichment.

132

133 ***A point of no return leading to death***

134 Through transcriptome analysis, we identified a critical time point around 3-4 hours into the stress

135 response, separating an initially highly dynamic phase from a later mostly stagnant phase. Next, we

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136 tested how these observed transcriptional patterns correlate with the effects of increasing heat stress
137 durations on the phenotypic recovery of the animals. To measure the effects, we observed survival,
138 progeny count, and movement in populations that were allowed to recover at 20°C after different heat
139 stress durations (**Figure 3).** Since it has previously been shown that it can take three days after the
140 exposure to a transient lethal heat-shock to observe the fatal effects in the survival scores of *C.*
141 *elegans* [12], we recorded daily phenotypic observations over a four day recovery period following
142 the stress.

143 The heat exposure durations resulted in three phenotypically distinct groups. First, for
144 survival, the animals exposed to heat for up to two hours show high survival chances equal to the
145 control **(Figure 3A)**. An intermediary group was formed by animals exposed to heat for 3-4 hours
146 with about 80% surviving the first day, which steadily declined to ~60% survival by day 4. It is of
147 note that the exposure duration of this group coincides with the critical time point (3-4h) identified in
148 the transcriptomic data. In the third group, with heat-exposures over 6 hours, survival chances were
149 already drastically reduced after the first day (<20%).

150 Analogous to the 3 distinct survivorship groups found for short-, intermediate- and long-term
151 stress exposures, this division was also present in the fraction of nematodes regaining a healthy
152 movement during the recovery period, as well as regaining a normal number of progeny (**Figure 3B**
153 **and 3C**). The movement in populations exposed to a short heat stress (< 3 hours) did not differ from
154 that of control populations. While the heat stress initially causes slightly lower numbers of progeny,
155 the reproduction peeked 2-3 days after the heat stress together with the control population. For
156 intermediate exposure durations (3-4 hours), 60-70% of animals displayed a normal movement, yet
157 reproduction was further reduced and delayed in these populations. For longer heat exposures (>4
158 hours), the few surviving individuals commonly presented abnormal, slow, and sporadic movement.

159 Overall, the transcriptional patterns during heat stress changed dramatically around 3-4 hours
160 which coincided with a 'point of no return', as shown by the drastic decrease in movement, viable
161 offspring, and recovery chances in the days after.

162

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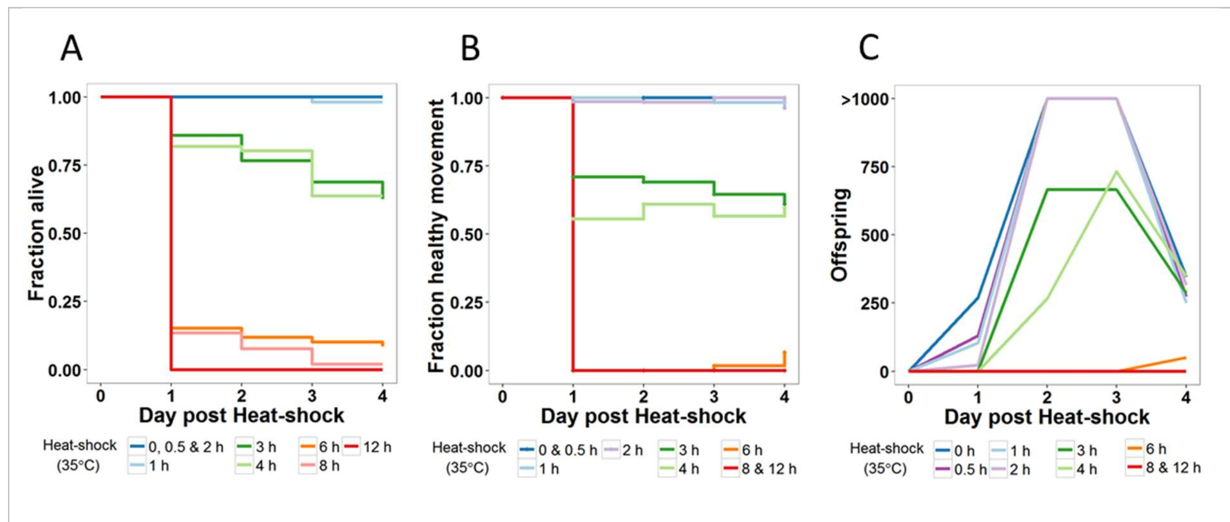

Figure 3: Effect of increasing heat-shock durations on selected phenotypes. After exposing N2 populations in the L4 stage to increasing heat-shock durations at 35°C, populations were maintained at 20°C. A total of ~65 individuals divided over 3 biological replicas per treatment group were observed. The following phenotypes were scored on the four consecutive days following the heat-shock: **(A)** fraction alive, **(B)** fraction of worms with a healthy movement phenotype (i.e. sinusoidal, constant and unprovoked movement), **(C)** the average number of viable offspring produced per population with a cut-off point set at 1000 offspring.

163

164

165 ***Heat stress disrupts major developmental processes***

166 To investigate the underlying causes of the 'point of no return', we first looked at how normal
167 developmental processes progress under heat stress conditions. A previous study by Snoek *et al.* has
168 dissected the temporal patterns of global transcript levels of *C. elegans* spanning the entire 4th larval
169 stage [22], which corresponds to the time frame used in this study. We analysed the heat stress
170 expression patterns in relation to developmental gene expression. First, we selected gene clusters
171 strongly upregulated during L4 development at 20°C (see Materials and Methods for details; **Figure**
172 **4A**). These genes showed little change in heat stress conditions at 35°C. Likewise, genes with a strong
173 transcriptional response to heat stress (cluster 3) displayed few expression differences during
174 development. Next, we selected gene clusters with a strong decrease in expression levels (**Figure 4B**).
175 While most of the transcriptional patterns differed between development and heat stress conditions,
176 about 20% of genes were present in both groups. An enrichment analysis of these genes found a
177 strong overrepresentation of genes associated with the cuticle structure and locomotion.

178 Together, these results showed that heat stress disrupted the major transcriptional changes
179 normally occurring during L4 development, indicative for the heat stress induced developmental

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180 delay. Furthermore, it shows that the animal almost fully shifts its transcriptional program to deal with
181 the acute heat stress conditions.

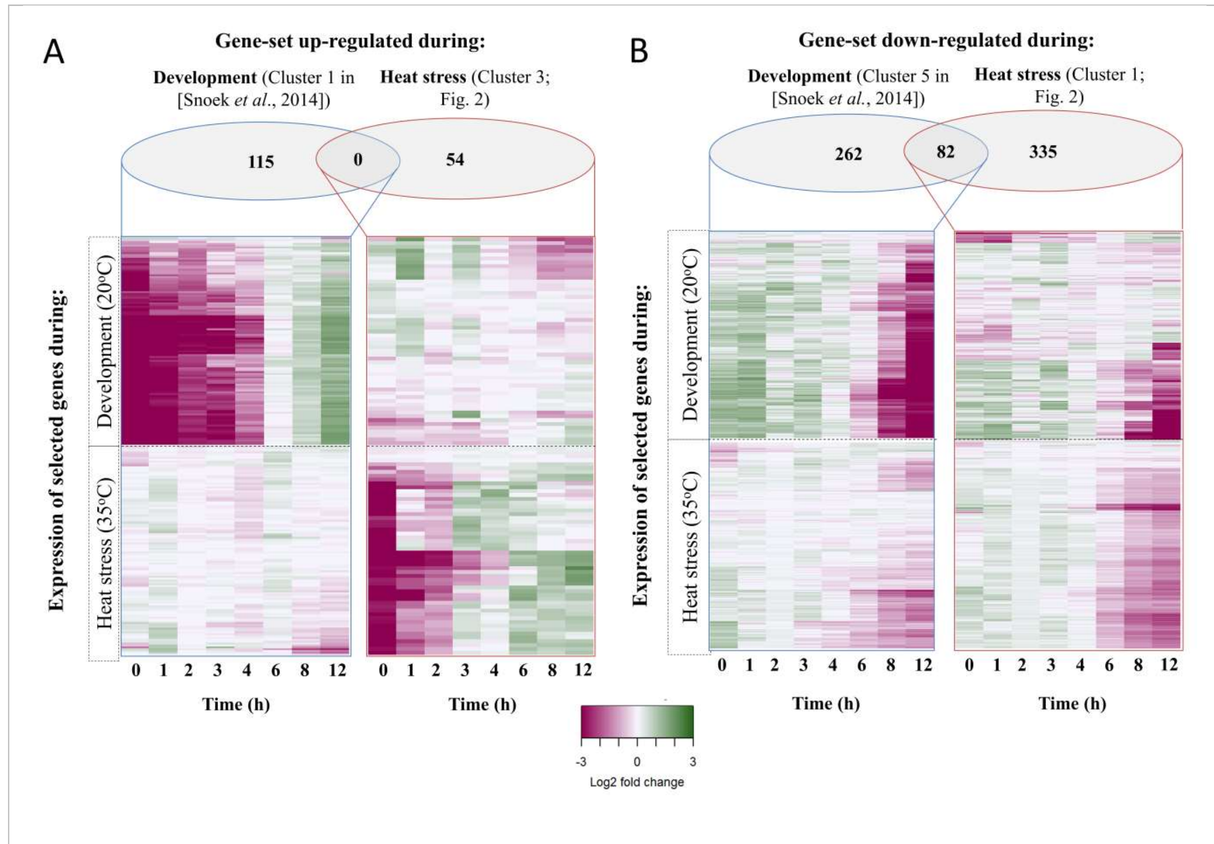

Figure 4: Comparison of expression dynamics during development (20ºC; upper panel) and heat stress (35ºC; lower panel) based on gene clusters with strong transcriptional patterns. The log2 transformed gene expression is indicated by the colour scale. Developmental gene expression data obtained from [22]. Time was measured beginning 46 hours post age-synchronization. The order of genes within each heat map was retrieved through hierarchical clustering, and is therefore not the same between the upper and lower panels. **(A)** Gene clusters with a strong up-regulation during development (left panel; cluster 1 in [22]) or during heat stress (right panel; cluster 3). **(B)** Gene clusters with a strong down-regulation during development (left panel; cluster 5 in [22]) or during heat stress (right panel; cluster 1). Venn diagrams presents the number of genes within each group.

182

183 ***Genes differently expressed after the point of no return***

184 To identify the genes involved in the sharp decrease in survival chances after the ‘point of no return’,
185 we compared gene expression levels of samples taken between 2-4 hours into the heat-shock with the
186 samples taken at the last three time points (6, 8 and 12 hours). We found 262 upregulated and 667
187 downregulated genes (q-value < 0.0001; **Supplementary table S1, Supplementary figure S2**).
188 Enrichment analysis revealed an overrepresentation of genes involved with cuticle development and
189 metabolic processes in the late heat stress down-regulated group. Genes involved in reproduction,
190 development, and locomotion were enriched in the late heat stress up-regulated group, possibly

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191 showing the continuation of the normal developmental processes after the initial slow down.
192 However, it should be noted that upregulation occurred with very low effect sizes, which could
193 indicate a hampering of the transcriptional processes after the point of no return.

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194 # Discussion

195 By analysing a series of stress exposure times in *C. elegans* we detected a shift in gene expression
196 patterns between 3-4 hours into the stress response, separating an initially highly dynamic phase from
197 a later mostly stagnant phase. Survival, progeny count, and movement revealed that exposure to a heat
198 stress lasting longer than 4 hours resulted in irreversible damage. Overall, the heat stress response
199 could be divided into three distinct phases: i) an early highly dynamic phase up to 2 hours of stress
200 exposure (including a very early upregulation of heat shock proteins), ii) an intermediate phase in
201 which the transcriptional response attenuates presenting a turning point in dynamics (3-4 hours), iii) a
202 late phase with gradual transcriptomic changes (6-12 hours). Phenotypically, each phase corresponds
203 with distinct trends in the ability to recover from the stress, presenting a 'point of no return' as seen
204 by a drastic decline in survival rates, the ability to recover normal movement, and to produce viable
205 offspring in the four days of recovery following the stress.

206 To our knowledge, this is the first study that links the dynamics of heat stress response at the
207 transcriptome level to the ability to recover. Gene expression regulation under stress conditions is
208 strictly controlled, its kinetics are rapid and very often it is reversible. This allows for extremely rapid
209 adaptation of cells and tissues in response to general stress, in particular heat stress, and for returning
210 to a baseline level [21]. We analysed the phenotypic recovery from these rapid adaptive changes
211 occurring during stress and found that already a relatively early response to heat stress abruptly
212 changes development.

213

214 *Early dynamic response to heat-stress disrupts development*

215 During the early phase of heat stress, the transcriptional response is highly dynamic. About 400 genes
216 (cluster 2 and 5) are highly upregulated. Comparison with transcriptional patterns normally occurring
217 during development has shown that this gene-set uniquely reacts in the response to stress.
218 Furthermore, genes highly active during development show low transcriptional changes during stress
219 conditions. These results indicate that the animal almost fully switches its transcriptional focus on
220 counteracting the adverse effects of heat stress. In this context, it is not surprising that heat shock

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221 proteins are the first set of genes to show a strong and rapid increase in transcript levels. Shortly after,
222 histones and genes associated with the nucleosome assembly are highly enriched in upregulated gene
223 clusters. Nucleosome remodelling has previously been shown to be an important part of the stress
224 response, *e.g.* by allowing access to transcription sites of stress responsive genes [21,23,24]. In *C.*
225 *elegans*, depletion of a nucleosome remodelling complex leads to a higher thermal sensitivity [25].
226 Packaging of DNA into nucleosomes could be an additional protective mechanism during the stress
227 response.

228 Phenotypic analysis of the recovery process indicates that the disruption of normal
229 transcriptional processes is fully reversible up to the 'point of no return'. After short exposures to
230 stress, the animals recovered a healthy movement phenotype and started reproducing, indicating that
231 the protective mechanisms put in place by the early transcriptional heat shock response are sufficient
232 in this time frame. However, the disruption of normal transcriptional development could be one of the
233 causes for the observed delay in reproduction. A study in which a two hour 35°C heat-shock was
234 compared to two hour recovery of that heat-shock showed that the transcriptional patterns in the
235 recovery population had still not returned to normal [19]. Also, a delay in reproduction has previously
236 been shown in heat-shocked pre-gravid adult *C. elegans* exposed to temperatures between 30-32°C
237 [15]. Arresting reproduction ensured limited damage to reproductive compartments during stress
238 conditions. In the early heat stress response, we found this delay on a transcriptional level as
239 development and reproduction related genes did not show their normal up regulation.

240

241 *Attenuation of dynamic response*

242 At medium-to-long exposures, the transcriptional stress response attenuates corresponding
243 phenotypes, *i.e.* a ~40% decrease in survival and an increased occurrence of animals with an abnormal
244 movement phenotype. The attenuation of the heat shock response has mostly been studied in several
245 cell lines [26,27]. An integral part is the activation and subsequent suppression of the HSF-1
246 transcription factor activity through a negative feedback loop, which is partially mediated by those
247 chaperones that are transcriptionally induced by HSF-1, such as HSP-70 [28]. The attenuation of the
248 heat shock response is believed to serve a protective function, as cell lines with defects in the process

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249 display lower growth rates and reduced fitness [21,27]. In *C. elegans,* it was shown that a gain-of-
250 function mutation in a negative regulator of the heat-shock response (HSB-1) results in severe effects
251 on survival after heat stress [26]. In our data set, transcripts of chaperones induced by HSF-1 increase
252 immensely within the first 30 minutes of the stress response (**Supplementary Figure S1**). The drastic
253 increase slows down until peak levels are reached at 4 hours into the stress response, followed by a
254 small decrease and complete attenuation. It is unclear if the observed global transcriptional slowing
255 down is due to an actively regulated process, such as the HSF-1 feed-back loop, or due to a passive
256 process, such as the accumulation of damage to key cellular processes. Another explanation might be
257 a developmental cue. During normal development without stress, the *C. elegans* transcriptome is
258 highly dynamic, marked by a pronounced shift at 50 hours, which overlaps in time with our point of
259 no return [22]. Passing this point of attenuation might result in the strong decrease in recovery
260 chances. Although the progression of survival rates in the four days following the heat exposure
261 implies that the heat shock does not kill nematodes immediately, the profound damage cannot be
262 repaired.

263 At long exposures, after passing the 'point of no return', recovery chances are drastically
264 reduced. While most transcript level have reached a plateau, a distinct exception is the pronounced
265 decrease in expression of a set of genes highly enriched with collagen related genes. Collagens are
266 key components of the nematode cuticle, which is critical for protection and locomotion [29]. During
267 development, the transcription of cuticle collagens is tightly regulated between any of the four molts
268 [29,30]. Comparison with normal development (**Supplementary figure S3**), shows a disruption of
269 these patterns and a general downregulation of all cuticle genes. More recently, gene expression
270 studies in *C. elegans* have shown that collagen genes are highly expressed in short heat stress
271 exposure and during oxidative stress [20,31]. The strongly reduced survival changes after longer
272 exposure might be caused be the later reduction of transcript levels of these cuticle genes in our
273 experiment.

274 Overall, our study links a strong shift in transcriptional dynamics upon exposure to heat stress
275 with an inability to recover from the stress response. The inability to recover was reflected in a

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276 decrease in worm activity, progeny count, and survival in the days after. Therefore, we think this
277 critical shift in the dynamics of gene expression marks a point of no return ultimately leading to death.
278

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279 # Materials and Methods

280 **Nematode Culturing and heat-shock treatment**

281 Hermaphrodites of the *Caenorhabditis elegans* strain Bristol N2 were used for all experiments and
282 kept under standard culturing conditions at 20ºC on Nematode Growth Medium (NGM) seeded with
283 *Escherichia coli* strain OP50 as food source [32]. For the experiments, starved populations were
284 placed onto fresh NGM dishes seeded with *E. coli* OP50 by transferring a piece of agar and
285 subsequently grown at 20ºC for 3-4 days until sufficient gravid adults had developed. Age-
286 synchronized populations were obtained by bleaching according to standard protocols using a
287 hypochlorite solution [32]. After bleaching, eggs were transferred to fresh 9 cm NGM dishes and
288 maintained at 20ºC.
289

290 **Heat-shock treatment**

291 The heat shock treatments were performed in an incubator set to 35ºC. N2 populations were exposed
292 to the heat stress treatment starting 46 hours after age-synchronization. Samples were taken at several
293 time points during the stress period: 0.5, 1, 2, 3, 4, 6, 8, or 12 hours. In total, 3-5 samples were
294 collected for each time point. As preparation for the transcriptome analysis, the populations were
295 washed off the plate with M9 buffer, collected in Eppendorf tubes and flash-frozen in liquid nitrogen
296 and stored at -80ºC until further use. For phenotypic observations, the N2 populations were
297 transferred back to pre-heat shock maintenance conditions at 20ºC.
298

299 **Phenotyping**

300 The selected traits (movement, survival, and progeny count) were observed using a stereomicroscope
301 at approximately 24, 48, 72, and 96 hours post heat-shock. To allow for accurate scoring of all
302 individual animals, the population size per dish was kept at a maximum of 25 animals at the start of
303 the experiment. In total, 3 dishes per heat-shock duration were scored, which amounts to a total of
304 approximately 60 animals per treatment. Animals were transferred to fresh NGM dishes every day
305 during the reproductive phase using a platinum wire. Bagging and suicidal animals were censored.

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306

307 ***Movement and Survival***

308 Movement was scored based on classification systems that have previously been described in
309 association with aging studies, where it acts as a measure of the biological age [33,34]. These systems
310 were combined and adapted to score the impact of the heat-shock. Healthy nematodes are actively
311 moving in a sinusoidal pattern (Hosono: type I; Herndon: Class A). As a result of the heat shock, a
312 proportion of the animals deviated from the healthy phenotype in varying degrees such as visibly
313 lower levels of activity, low responsiveness to touch with a platinum wire and/or an irregular shape of
314 movement (for example due to a partially paralysed tail). This is corresponding to Class B and C of
315 Herndon or Type II and III of Hosono). Worms were scored as dead, when no head movement was
316 observed after 3 touches with a platinum wire.

317

318 ***Progeny count***

319 It has previously been shown that *C. elegans* can lay non-viable eggs after heat shock [12]. For this
320 reason, the progeny count was measured, defined as the absolute number of living offspring per
321 population. We counted the progeny one day after transferring the adults of the experimental
322 populations to fresh dishes, at which time viable eggs have hatched. For populations with a high level
323 of reproduction, the total number of life offspring was estimated based on the count of a quarter of the
324 dish.

325

326 **<u>Transcriptome profile</u>**

327 ***RNA isolation***

328 RNA was isolated from the flash frozen samples using the Maxwell® 16 AS2000 instrument with a
329 Maxwell® 16 LEV simplyRNA Tissue Kit (both Promega Corporation, Madison, WI, USA). The
330 mRNA was isolated according to protocol with a modified lysis step. Here, 200 µl homogenization
331 buffer, 200 µl lysis buffer and 10 µl of a 20 mg/ml stock solution of proteinase K were added to each
332 sample. The samples were then incubated for 10 minutes at 65°C and 1000 rpm in a Thermomixer

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333 (Eppendorf, Hamburg, Germany). After cooling on ice for 1 minute, the samples were pipetted into
334 the cartridges resuming with the standard protocol.
335
336 ***Sample preparation and scanning***
337 For cDNA synthesis, labelling and the hybridization reaction, the 'Two-Color Microarray-Based Gene
338 Expression Analysis; Low Input Quick Amp Labeling' - protocol, version 6.0 from Agilent (Agilent
339 Technologies, Santa Clara, CA, USA) was followed. The *C. elegans* (V2) Gene Expression
340 Microarray 4X44K slides manufactured by Agilent were used. The microarrays were scanned with an
341 Agilent High Resolution C Scanner using the settings as recommended. Data was extracted with the
342 Agilent Feature Extraction Software (version 10.5) following the manufacturers' guidelines.
343
344 ***Data pre-processing***
345 Data was analysed using the 'R' statistical programming software (version 3.3.2 64-bit). For
346 normalization, the Limma package was used with the recommended settings for Agilent [35].
347 Normalization within and between arrays was done with the Loess and Quantile method, respectively
348 [36]. The obtained normalized intensities were log2 transformed and outliers were removed. Batch
349 effects within the data set were calculated with a linear model and removed as previously described
350 [22]. For further analysis, the expression values of biological replicas were averaged. To analyse
351 temporal expression dynamics independent from absolute expression values, the individual intensities
352 measured at each timepoint for each gene were rescaled to the average expression in time per gene.
353 The obtained values were log2 transformed and are further referred to as the log2 ratio with the mean.
354
355 ***Data analysis***
356 Principal component analysis (PCA) was performed on the log2 ratio with the mean to explore the
357 source of underlying variation in gene expression. PCA scores of the first and second component were
358 used to select genes with a significant contribution to the variation in expression dynamics. Selection
359 was based on a significance level $q < 0.01$ in a linear model relating expression values with PCA
360 scores for the separate components. q-values were determined by the Benjamini-Hochberg correction

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361 for multiple testing (FDR-adjusted p-value; R package: 'stats' version 3.1.3). To explore overall
362 trends in gene expression dynamics, gene clusters were extracted by k-means with 200 iterations on
363 10 different starting sets. Six clusters were sufficient to visualise distinct patterns in gene expression
364 changes.

365 Differently expressed genes between time-points were deducted by a linear model using the
366 log2 expression of individual samples (3-5 samples per heat-shock duration). In cases where multiple
367 time points were compared, they were grouped into one factor, e.g. Group 1 (2, 3, 4 hours) vs Group 2
368 (6, 8, 12 hours). A high significance level of $q < 0.0001$ was chosen.

369 To extract genes with similar expression patterns to heat-shock proteins, we used spearman
370 correlation analysis on the log2 ratio with the mean averaged for hsp-16.1, hsp-16.2, and hsp-16.41.
371 Genes were selected with a log2 change >2 within the first 30 minutes of heat exposure.

372

373 ***Enrichment analysis***

374 To explore the biological functions associated with selected gene sets, we used the functional
375 annotation tool provided by DAVID 6.8 [37,38]. For the enrichment analysis (functional annotation
376 chart), settings were limited to Gene Ontology terms (GOTERM_BP_DIRECT,
377 GOTERM_MF_DIRECT, GOTERM_CC_DIRECT).

378

379 ***Developmental Data***

380 List of genes within developmental cluster 1 and 5 (strongly up- and downregulated, respectively)
381 were obtained from Snoek et al. [22]. The normalized developmental expression data set was
382 retrieved from WormQTL [39,40]. From the developmental time series, a subset of samples were
383 selected that correspond to the heat shock time series (i.e.: of the developmental time series 46h, 47h,
384 48h, 49h, 50h, 52h, 54h, and 58h corresponding with the time points in the heat shock time series 0h,
385 1h, 2h, 3h, 4h, 6h, 8h, and 12h, respectively). Expression data of replicates was averaged. To compare
386 expression dynamics between the time series obtained during development and in heat stress
387 conditions, we selected the expression data of subsets of genes with strong expression patterns during
388 development (cluster 1 and 5, Snoek 2014) and heat stress (cluster 1 and 3, Fig. 2, **Supplementary**

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389 **table S1**). Heatmaps (R package: 'gplots' version 3.0.1) of the log2 ratio with the mean are used to
390 visualize the comparison of the expression dynamics during development and in heat stress conditions
391 for each subset of genes.

392

393 Data accessibility

394 The microarray datasets supporting this article have been deposited in the ArrayExpress database at
395 EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5753.

396 Competing interests

397 We have no competing interests.

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403

404 Authors' contributions

405 The study was conceived by JEK and LBS. The investigation was designed by KJ, MGS, JEK, and
406 LBS. Experiments were performed by KJ, MGS, RPJB, MR, and JAGR. The data was analysed by
407 KJ, MGS, and LBS. SA provided resources and support. Manuscript was discussed and written by KJ,
408 MGS, JEK, JG, and LBS.

409

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410 # Supplementary information

411 **Figure S1:** Temporal expression patterns of heat stress responsive genes during 12 hours of heat
412 stress (35ºC). Genes were selected based on the information provided by the Gene Ontology database
413 for the GO term 'response to heat stress' (GO:0009408, WormBase version 257). Expression levels of
414 individual genes are presented as the Log2 ratio with the mean.

415 **Figure S2:** Volcano plot showing the difference per gene in log2 transformed gene expression levels
416 between medium exposure durations (2, 3, and 4 hours) and long exposure durations (6, 8, and 12
417 hours). P-values were inferred from a linear model comparing the two groups, and corrected for
418 multiple testing by the Benjamini-Hochberg method. The red line indicates the selected significance
419 level resulting in the selection of 262 upregulated (positive effect size) and 667 downregulated
420 (negative effect size) genes.

421 **Figure S3:** Heatmap of temporal expression patterns of structural constituents of the cuticle during
422 development and under heat stress conditions.

423 **Table S1:** Gene lists used for GO enrichment analysis, and detailed output of the enrichment analysis
424 performed with the functional annotation tool provided by DAVID 6.8.

425

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