1 Title: Disentangling the aging network of a termite queen

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13

14 Abstract

15 **Background:** Most insects are relatively short-lived, with a maximum lifespan of a few weeks, like
16 the aging model organism, the fruit-fly *Drosophila melanogaster*. By contrast, the queens of social
17 insects (termites, ants, some bees and wasps) can live for more than a decade. This makes social
18 insects promising new models in aging research providing insights into how a long reproductive life
19 can be achieved. Yet, aging studies on social insect reproductives are hampered by a lack of
20 quantitative data on age-dependent survival and time series analyses that cover the whole lifespan
21 of such long-lived individuals. We studied aging in queens of the drywood termite *Cryptotermes*
22 *secundus* by determining survival probabilities over more than 15 years and performed
23 transcriptome analyses for queens of known age that covered their whole lifespan.

24 **Results:** The maximum lifespan of *C. secundus* queens was 13 years with a median maximum
 25 longevity of 11.0 years. Time course and co-expression network analyses of gene expression
 26 patterns over time indicated a non-gradual aging pattern. It was characterized by networks of genes
 27 that became differentially expressed only late in life, namely after an age of 10 years, which
 28 associates well with the median maximum lifespan for queens. These old-age gene networks reflect
 29 processes of physiological upheaval. We detected strong signs of stress, decline, defence and repair
 30 at the transcriptional level of epigenetic control as well as at the post-transcriptional level with
 31 changes in transposable element activity and the proteostasis network. The latter depicts an
 32 upregulation of protein degradation, together with protein synthesis and protein folding, processes
 33 which are often down-regulated in old animals. The simultaneous upregulation of protein synthesis
 34 and autophagy is indicative for a stress-response mediated by the transcription factor *cnc*, a
 35 homolog of human *nrf* genes.

36 **Conclusion:** Our results show non-linear senescence with a rather sudden physiological upheaval at
 37 old-age. Most importantly, they point to a re-wiring in the proteostatis network as central for
 38 explaining the long life of social insect queens.

39

40 **Keywords**

41 RNASeq, transcriptomes, ageing, social insects, weighted gene co-expression networks, WGCNA,
 42 time series, termite, lifespan, senescence

43 Background

44 Almost all animals age, but at different pace [1]. The fruit fly *Drosophila melanogaster* lives only
 45 for around seven weeks [2], while the clam Ocean Quahog, *Arctica islandica*, can have a lifespan of
 46 more than 400 years [3]. Generally, organisms with large differences in rates of aging are found
 47 between widely divergent species [1,4], which makes controlled comparisons of the underlying
 48 aging mechanisms difficult.

49 Classical model organisms typically have a short lifespan and can be characterized by r-life history
 50 strategies ('live fast, have many offspring and die young') as exactly these traits make them good
 51 model organisms. Social insects such as termites, ants, or the honeybee, offer promising new
 52 insights into aging research because individuals with the same genetic background can differ by
 53 orders of magnitudes in lifespan. Within a social insect colony, which is generally a large family,
 54 the reproducing queen (and in termites, also the king) can reach lifespans of more than 20 years,
 55 while non-reproducing workers have a lifespan of a few months only [5-8]. However, quantitative
 56 demographic data covering the whole lifespan of queens are inherently rare and many reports on
 57 queen-longevity are more anecdotal. Thus, it is largely unknown for long-lived queens whether they
 58 age gradually or whether aging is a more sudden event.

59 During recent years, several pioneering studies, especially on the honeybee, revealed exciting new
 60 insights into the mechanisms of how queens can live so long. In the honeybee, juvenile hormone
 61 (JH) seems to have lost its direct gonadotropic function in adults so that queens have a high
 62 expression of the yolk precursor gene vitellogenin (*Vg*) without requiring high JH titres (*e.g.*,
 63 [9,10]). This result has led to the hypothesis that uncoupling JH and *Vg* expression might account
 64 for the long life of honeybee queens [9] as well as social insect queens more generally [11] because
 65 the life-time shortening consequences of high JH titers are absent. However, this re-wiring along the
 66 JH-*Vg* axis is not universal for all social Hymenoptera, since the queens of many other ant and bee

67 species require JH for vitellogenesis (*e.g.* [12] and references therein). For termites, fewer studies
68 exist, but JH is required for vitellogenesis [13,14]. Hence, other mechanisms must exist to explain
69 the long life of termite queens. Studies of the subterranean termite *Reticulitermes speratus*
70 implicated the involvement of a *breast cancer type 1 susceptibility (BRCA1)* homolog [15], which is
71 involved in DNA repair [16], and better protection against oxidative stress by superoxide
72 dismutases and catalases [17,18]. The latter have also been discussed for other social Hymenoptera,
73 including ants and the honeybee. Yet overall evidence of the role of oxidative stress is less clear
74 (reviewed in [19-21]). Furthermore, regulation of the activity of transposable elements (TEs) [22],
75 and changes in the insulin/insulin-like growth factor1 signalling (IIS) and target of rapamycin
76 (TOR) pathways [23] have been linked with caste-specific aging differences in termites. Both, the
77 TOR and IIS pathway, have been associated with longevity in model organisms from *D.*
78 *melanogaster* to mice and humans [24-26]. They are the most intensively studied aging related
79 pathways and they have also been associated with caste differences in social Hymenoptera (*e.g.*,
80 [27-31]). Yet, all studies on social insects suffer from a lack of time-series data to investigate
81 molecular changes across the lifespan of long-lived queens. Like the demographic life history data,
82 such data are inherently difficult to obtain due to the long lifespan of queens. However, they are
83 necessary (i) to understand the aging process, (ii) to work out potential changes compared to
84 solitary insects, and (iii) to identify the relevant age-classes for detailed studies. The latter is a
85 completely overlooked issue but highly relevant. If, for example, aging is a non-linear process,
86 differences across studies might just be consequences of none-comparable age-classes between
87 studies.

88 We studied aging in termite queens of known age across their entire lifespan to measure at the
89 ultimate, eco-evolutionary level age-dependent survival and at the proximate, mechanistic level
90 age-specific changes in gene expression. For the latter, we generated head and thorax

91 transcriptomes of queens of different age (for an outline of the workflow see Additional File 1,
92 Figure S1). We used field collected, newly established colonies of the wood-dwelling termite
93 *Cryptotermes secundus* (Hill, 1925) (Blattodea, Isoptera, Kalotermitidae) that were kept under
94 identical conditions in the laboratory for a time span of 15 years. Keeping them under constant and
95 optimal conditions allowed us to study intrinsic aging, disentangled from causes of extrinsic
96 mortality such as predation, food shortage, or disease. As typical for wood-dwelling termite species,
97 *C. secundus* colonies are founded in a piece of wood, which serves as food and shelter and which
98 workers never leave to forage outside. Such species have a low social complexity with small
99 colonies and totipotent workers that develop into sexuals.

100

101 **Results**

102 **Survival analysis**

103 Overall, we had surviving founding queens from an age of two years up to a maximum of 13 years.
104 The potentially 14- and 15-year old queens all had died as had most queens with a potential age of $\geq$
105 12 years. Out of eight queens in this ‘old-age’ class, only a single queen (13 years) had survived
106 (Fig. 1). The median longevity of the queens in the laboratory after successful colony establishment
107 was estimated with Kaplan Meier survival analysis to be 12.0 years (SE: ± 0.54) (mean longevity:
108 11.1 years, SE: ± 0.66) (Fig. 1).

109

110 **Identifying transcripts that change their expression with age: age-related DETs**

111 To study gene expression changes over the lifetime of queens we generated transcriptomes of head
112 and thorax from twelve queens with different chronological age since onset of reproduction, from
113 two until 13 years, covering the complete lifespan of *C. secundus* queens: 2, 3, 4, 5, 6, 7, 8, 9, 10
114 (two samples), 11, and 13 years (Additional File 2, Table S1).

115 A total of 169 transcripts were significantly differentially expressed (DETs) over time as revealed
 116 by Iso-MaSigPro time series analysis (Additional File 2, Table S2). According to their expression
 117 pattern, DETs were grouped into six Iso-MaSigPro clusters (hereafter, 'cluster') (Fig. 2). Cluster 1
 118 represented 44 DETs, which were slightly expressed in young queens followed by a decline at
 119 middle ages and a strong increase when queens became older. The 32 DETs of cluster 2
 120 characterized young queens with a declining expression with age. Clusters 3 and 5 comprised 31
 121 and 37 DETs, respectively, that were highly expressed in middle aged queens, while cluster 4 and
 122 cluster 6 (15 and 10 DETs) characterized old queens with no expression in young ones. Thus, in the
 123 following we referred to the DETs as young (cluster two), middle-aged (clusters three and five) and
 124 old DETs (clusters one, four and six). Details for all clusters are provided in Additional File 2,
 125 Table S2.

126

127 **Identifying modules of co-expressed transcripts**

128 To identify modules of co-expressed transcripts, we performed a weighted gene co-expression
 129 network analysis (WGCNA). It revealed a total of 254 modules of co-expressed transcripts. Based
 130 on eigengene values, 13 modules correlated significantly positively with age and 13 negatively (see
 131 Additional File 1, Figures S2 and S3; Additional File 3 (WGCNA module-age association, shown
 132 are eigengene values for all modules).

133

134 **Identifying transcript co-expression modules with age-related DETs**

135 Within the age-correlated WGCNA modules, we identified age-related DETs. The negatively age-
 136 correlated module 'seashell4' had the highest number of young DETs (10 DETs). No gene ontology
 137 (GO) term was enriched for this module. The highest number of old DETs were found in the
 138 positively age-correlated modules 'cyan' (89 DETs) and 'tan' (79 DETs) (Additional File 2, Table

139 S3 and S4). Only broad categories were enriched in the ‘cyan’ module (*e.g.* RNA metabolic process
140 and gene expression) and the ‘tan’ module was enriched for ribosomal and tRNA related functions
141 (Additional File 1, Figure S4).

142

143 **Extracting age-related subnetworks based on age-related DETs**

144 To generate subnetworks related to the age-related DETs, we located them in the WGCNA co-
145 expression network. These DETs and their one- and two-step neighbors (*i.e.*, the ‘second level
146 neighborhood’) were then extracted from the co-expression network, which resulted in 50
147 subnetworks of different sizes (for more details see Methods) (Additional File 1, Figure S5). Note,
148 DETs might be located at the boundaries of multiple WGCNA modules which means the
149 subnetworks obtained consist of fragments of multiple WGCNA modules. The resulting
150 subnetworks either contained young and middle-aged DETs or old DETs, with a single exception
151 where a middle-aged DET was in the periphery of the largest subnetwork containing old DETs. The
152 largest subnetwork containing either young and middle-aged DETs (hereafter, young subnetwork)
153 or old DETs (plus a single middle-age DET; hereafter old subnetwork) were further analyzed.

154

155 *Young subnetwork*

156 The largest young subnetwork comprised 164 transcripts (out of these 24 Iso-MaSigPro DETs) of
157 which only 12 (7%) were one-to-one orthologs to *D. melanogaster* genes (Additional File 2, Table
158 S3). The GO enrichment analysis of the young subnetwork showed multiple Biological Process
159 (BP) terms related to RNA catabolism, but these GO terms were not significant after correcting for
160 multiple testing (Additional File 2, Figure S6).

161

162 *TE activity and genome instability.* 53 DETs (32%) of the young subnetwork were related to TEs
 163 (Fig. 3 and Additional File 2, Table S3), comprising TEs and genes from TE defense pathways.
 164 This included one homolog of the gene *argonaute 2 (ago2)* (two transcripts), an essential gene of
 165 the endo-siRNA pathway which silences TEs [32], and *arsenite 2 (ars-2)* which is required for
 166 siRNA and miRNA-mediated TE silencing [33]. Additionally, we found two genes connected to
 167 DNA damage response and genome instability: *kin17* and *PIF1-like* gene.

168

169 *Other signatures.* From well-known aging pathways, we identified (i) *inositol polyphosphate*
 170 *phosphatase 2 (mipp2)* and (ii) *adenylyl cyclase 76E (ac76E)*. The former is part of the TOR
 171 pathway and has been associated with longevity [34] and the latter is activated by the transcription
 172 factor ‘Forkhead box O’ (*foxo*). Additionally, we found several fecundity related DETs. They
 173 included two transcripts of the gene *hu li tai shao (hts)* (one a DET of IsoMaSigPro cluster two) and
 174 one homolog of the gene *bällchen (ball)* (two transcripts) (one a DET of cluster five, Fig. 3)

175

176 *Old subnetwork*

177 The largest ‘old subnetwork’ comprised 1,098 transcripts (out of these 42 Iso-MaSigPro DETs).
 178 521 transcripts (47%) were identified as one-to-one orthologs of *D. melanogaster* genes (Additional
 179 File 2, Table S4). Iso-MaSigPro DETs in the old subnetwork belonged mainly to Iso-MaSigPro
 180 clusters 1 and 4. The second level neighborhoods of these DETs were connected in the network and
 181 a GO enrichment analysis revealed multiple GO terms associated with protein related functions,
 182 including translation, protein folding, unfolded protein binding, proteolysis involved in cellular
 183 protein catabolic process, protein targeting to ER, ribosome, and proteasome complex (Additional
 184 File 1, Figures S7 and S8). 198 transcripts of the old subnetwork (18%) were involved in protein
 185 translation, protein folding, and protein catabolism and proteolysis (Figs. 4 and 5 [35,36] and

186 Additional File 2, Table S4). Additionally, 61 transcripts (~6%) were related to TEs (Additional
187 File 2, Table S4).

188

189 *Epigenetic modifications, transcriptional regulation, and TE activity.* Many genes of the old
190 subnetwork are involved in de/acetylation and methylation of DNA, which are important epigenetic
191 modifications that regulate gene expression and genome stability [37-39] (Additional File 2, Table
192 S4).

193 Most strikingly, two crucial histone acetylation modifying complexes, the Tip60 acetyltransferase
194 complex and the male specific lethal (msl) complex were represented in the old subnetwork. The
195 former included the genes *dom*, *ing3*, *mrg15*, *pont* and *rept*, and the latter *msl-1*, *msl-3* and *mof*.

196 Genes involved in deacetylation of DNA were, for instance, *sirtuin 1 (sirt1)*, *histone deacetylase 3*
197 (*HDAC3*), and *histone deacetylase 6 (HDAC6)*. Genes linked to epigenetic histone methylation

198 included, for instance, *ash-1* and *lid*. Another well represented group of genes connected to

199 expression regulation in the old subnetwork were spliceosome components and splicing factors.

200 Additionally, we found in the old subnetwork important transcripts related to TE silencing: *dicer-2*,

201 *Hsc70-4*, *Hsc70-3*, *Hsp83*, *trsn*, *armi*, *Rm62*, *Gas2*, *Tudor-SN*, and *Hel25E*. Details are given in

202 Additional File 2, Table S4.

203

204 *Proteostasis and oxidative stress.* Related to proteostasis we detected a strong signal for protein

205 synthesis and degradation. Regarding protein synthesis, the old subnetwork comprised many

206 transcripts coding for initiation, elongation and termination factors, as well as many ribosomal

207 proteins and aminoacyl-tRNA synthetases (Fig. 4; Additional File 2, Table S4). Regarding protein

208 degradation, almost all subunits of the ubiquitin proteasome system (UPS) were present (Fig. 5) as

209 well as autophagy genes, heat shock proteins, and the transcription factor *xbp1*. *Xbp1* is involved in

210 the ‘unfolded protein response’ (UPR) and in the ER-associated protein degradation (ERAD)
 211 pathway [40,41].
 212 Additionally, *BRCA1* was also present in the old subnetwork. This gene is involved in oxidative
 213 stress response, and in the transcriptional activation of proteasomal genes by stabilizing the
 214 transcription factor *cnc/nrf-2* (*cap-n-collar/nuclear factor erythroid 2–related factor 2*) [42]. Other
 215 genes in the old subnetwork involved in oxidative stress response and transcriptionally activated by
 216 *nrf-2* were thioredoxin and S-glutathione transferase.

217

218 *Other signatures.* Additionally, the old subnetwork was characterized by a signature of ecdysone
 219 biosynthesis with *ecdysone receptor (EcR)*, *ecdysone-induced protein 75B (Eip75B)*, *phantom* and
 220 *disembodied*. The presence of *Phosphatidylinositol 3 kinase 68D (Pi3K92E)* links to the IIS
 221 pathway.

222

223 **Locating age-related co-expression modules in the age-related subnetworks**

224 Finally, we inspected those WGCNA modules with a large fraction of transcripts in the young and
 225 old subnetworks as well as those modules that were significantly associated with age.
 226 In the young subnetwork, WGCNA modules with a large fraction of transcripts were ‘saddlebrown’
 227 and ‘skyblue4’ which both did not significantly correlate with age. Significantly age-correlated co-
 228 expression modules were firebrick2, indianred1 and seashell4 (Additional File 1, Figure S2). No
 229 GO terms were significantly enriched for any of these modules.

230

231 In the old subnetwork, the modules with a large fraction of transcripts were ‘green’ and
 232 ‘paleturquoise’ which again did not significantly correlate with age. The old subnetwork contained
 233 transcripts of 13 significantly age-correlated co-expression modules (Additional File 1, Figure S3).

234 The GO enrichment analysis of these modules revealed several terms involved in protein related
235 functions, including ribosome biogenesis, rRNA processing, protein folding, translation, unfolded
236 protein binding, protein catabolic process, protein transport, tRNA aminoacylation for protein
237 translation, and proteasome core complex (Additional File 1, Figure S4, S9, S10 and Additional
238 File 4, Table S5).

239

240 **Discussion**

241 Our study revealed a median maximum reproductive longevity of *C. secundus* queens of 12 years
242 with a maximum lifespan of 13 years when excluding all causes of extrinsic mortality in the
243 laboratory. The curve suggests a rather sudden decline in live expectancy after an age of around 11
244 years; out of eight queens with a potential age ≥ 12 years, all had died, except one 13-year old
245 queen (Fig. 1). The survival curve indicates a type I survivorship, after queens have successfully
246 founded a colony and without extrinsic mortality.

247

248 Our transcriptome study identified six clusters of transcripts that were significantly differentially
249 expressed with age (DETs) (Fig. 2): one cluster for young queens (cluster 2), two for medium aged
250 queens (cluster 3 and 5) and three for old queens (cluster 1, 4, and 6). This implies that three
251 ‘molecular’ life stages can be distinguished in *C. secundus* queens with the third corresponding to
252 old, aged queens that will probably die soon as no queen reached a lifespan beyond 13 years. The
253 co-expression network analysis, which extracted subnetworks based on age-related DETs, resulted
254 in two main subnetworks, a young and an old subnetwork. This implies that there are two *age-*
255 *related* ‘molecular’ life stages, as DETs/genes of young and medium ages belonged to the same
256 young subnetwork.

257

258 **Young subnetwork**

259 The young subnetwork contained DETs characteristic for young and medium ages. This shows the
 260 similarity in expression of these two age stages. Not unexpectedly, the young subnetwork indicates
 261 an upregulation of transcripts linked with fecundity (*e.g.*, *hts*, *ball*) and of the TOR pathway
 262 (*mipp2*) which has been associated with longevity [34]. The upregulation of *Ac76E* may imply that
 263 the IIS pathway is down because this gene is activated by the transcription factor foxo which is
 264 inhibited by an upregulated IIS pathway. However, other evidence suggests that, like in other social
 265 insects (*e.g.* [12] and references therein), queens are characterized by an upregulated IIS pathway
 266 [23]. Additionally, we detected several upregulated TEs-related transcripts associated with signs of
 267 an upregulation of the endo-siRNA pathway (*e.g.* *ago2*, *ars*) which is a transcriptional and post-
 268 transcriptional TE-defence mechanism of the soma [32,33,43] (Kim, Lee and Carthew, 2007; Sabin
 269 *et al.*, 2009; Piatek and Werner, 2014).

270

271 **Old subnetwork**

272 The old subnetwork contained many more transcripts (1,098 vs. 164 in the young subnetwork). Our
 273 results imply a physiological stage of upheaval shortly before queens die. There were strong signs
 274 of decline and repair at the upstream transcriptional level of epigenetic control as well as at the
 275 posttranscriptional level of TE-activity and the proteostasis network.

276

277 *Epigenetic modifications*

278 An upregulation of genes modifying histone marks implied considerable epigenetic changes that
 279 lead to altered gene expression as is typical for aging organisms:

280 First, our results indicate dynamic changes of ‘active’ histone marks of euchromatin because many
 281 genes related to H3K4 and H3K36 de/methylation and H4K16 de/acetylation were present in the

old subnetwork (Additional File 2, Table S4). For instance, the Tip60- as well as the msl-complex were well represented. Both complexes are involved in acetylation of histones, including H4K16, which, for instance, is indicative for replicative aging in yeast [44]. The upregulation of *Sirt1* may function as an antagonist as it deacetylates H4K16. The same applies for the deacetylases HDAC6 and HDAC3, which can also deacetylate histones [39].

Second, there is also evidence for changes of repressive histone mark of heterochromatin (*e.g.* linked to H3K9 and H3K27 acetylation) (Additional File 2, Table S4). For instance, the old age transcript ALP1 is an antagonist of HP1, the latter is involved in the maintenance of heterochromatin. HP1 generally decreases with age, and its overexpression can lead to an increased lifespan in *D. melanogaster* [45,46].

292

293 *TE activity*

In line with a deregulation of repressive histone mark of heterochromatin, several TE-related transcripts were also connected with the old subnetwork (Additional File 2, Table S4). TEs often are accumulated in heterochromatin which silences their activity [47]. Yet, dysregulation of heterochromatin can allow them to become active and this has been associated with aging [48,49].

Also the upregulation of several genes from two TE-defense pathways - the endo-siRNA pathway (*e.g. dicer2, Hsc-70-4, trsn*) as well as the of piRNA pathways (*e.g., piRNA biosynthesis. armi, gasz, Hel25E, Rm62*; ping-pong cycle: *Tudor-SN, qin*) - support the notion of active TEs. Both pathways silence TEs posttranscriptionally [50-52]. TE activity and especially the piRNA pathway has also been associated with aging and the longevity of termite reproductives in another termite species [22].

304

305 *Loss of proteostasis*

Our results revealed a very strong proteostasis signal indicative for an upheaval in protein synthesis, protein folding and protein degradation in old queens. Many genes from the proteostasis network were detected in the old subnetwork (Additional File 2, Table S4). They indicate an upregulation of protein degradation, together with protein synthesis and protein folding (Figs. 4 and 5). This is unusual because old organisms are typically characterized by a downregulation of all these processes (Fig. 6).

Protein synthesis. Many transcripts coding for ribosomal proteins and aminoacyl-tRNA synthetases were found in the old subnetwork, indicative of upregulated protein synthesis (Additional File 2, Table S4). This is further supported by a strong signal of an active TORC1 system which promotes protein synthesis (Fig. 7 [53-55]). Thus, for instance, many downstream eukaryotic initiation factors (eIF4A, eIF4B, eIF4E, eIF4G) and eukaryotic elongation factor (eEF2) transcripts were found in the old subnetwork (Fig. 4). They activate, ribosome biogenesis, translational elongation, and cap-dependent translocation (Fig. 7).

Protein degradation. Normally, an active TORC1 system is associated with a downregulation of protein degradation as it inhibits proteolytic systems [54-56] and autophagy (*e.g.*, upregulated TORC1 inhibits ATG1, which is necessary for autophagy activation; Fig. 7). Surprisingly, however, we found strong evidence of upregulated protein degradation in the old subnetwork. Several transcripts linked to autophagy, almost all subunits of the UPS, the UPR-, and the ERAD pathway as well as heat shock proteins characterized the old subnetwork (Figs. 5 and 6; Additional File 2, Table S4).

Linking protein synthesis and degradation. The simultaneous upregulation of protein synthesis and autophagy might be explained by a stress response. In *D. melanogaster* as well as in humans, under stress conditions upregulated TORC1 enhances an oxidative stress response, controlled by the transcription factor *cnc*, a homolog of human *nrf* genes (Fig. 7). Ubiquitinated proteins and damaged mitochondria activate *cnc/nrf-2* via p62, supported by upregulated TORC1, which then activates oxidative stress response genes [57,58] (Ichimura *et al.*, 2013; Aramburu *et al.*, 2014). Additionally, *cnc* is known to activate chaperones (protein folding) and the proteasome [59], and this has been associated with lifespan expansion in *D. melanogaster* and *Caenorhabditis elegans* [60,61].

Support for the conclusion of a stress-associated, active *cnc* transcription factor in old queens comes from several transcripts in the old subnetwork (Fig. 7): (i) BRCA1, which indirectly activates *cnc* by inhibiting *cnc* inhibitor *Keap1*, and (ii) the *Tip 60* complex as well as genes that are transcriptionally activated by *cnc*, such as *thioredoxin*, *S-Glutathione transferases* and *UPS* genes (Fig. 7).

Hence, our results imply that old *C. secundus* queens are in a stage of stress. They have mounted stress response systems mediated by *cnc*, including protein degradation and protein folding. However, it is unusual that old queens can do this. In *D. melanogaster*, only young individuals can mount this stress response [59] (Tsakiri *et al.*, 2013). The constant activation of the proteasome in these very old queens may lead to their death (note, the studied queens had reached their maximum lifespan, we never had older queens) as the constant activation of the proteasome in transgenic flies was detrimental for survival [59].

Comparison with other social insects

There has been a debate about the role of oxidative stress to explain the long lifespan of social Hymenoptera queens; yet evidence is inconclusive (*e.g.* reviewed by [19-21]). For instance, markers of oxidative stress in the brain of honey bee workers do not increase with age, although they live shorter than queens [62]. In the ant *Lasius niger*, both workers and males show higher activity of the antioxidant superoxide dismutase than queens, but both live shorter than queens [63]. These studies have shown that higher expression of oxidative stress response genes do not necessarily correlate with longer lifespans. For the termite *Reticulitermes speratus*, it has been suggested that queens are better protected against oxidative stress as qRT-PCRs studies showed a higher expression of the antioxidants catalases and peroxiredoxins in queens compared to workers [17], while kings were characterized by a high expression of *BRCA1* (in the fat body) compared to workers [15]. Unfortunately, the age of the studied reproductives is not known. It would be of interest to study expression of these genes with age, as this would contribute to a better understanding of the aging process but such studies are rare.

Conclusion

Our results imply that *C. secundus* queens do not age gradually, rather at old age there is a physiological stage of upheaval, characterized by signs of stress (activity of TEs, active *crc*) and defense (piRNA pathways) / repair (protein degradation and synthesis) before the animals die. This apparently sudden decline is in line with the few life history records of social insect queens that exist. They also found no signs of gradual senescence but an abrupt death (*e.g.*, the ant *Cardiocondyla obscurior* [64]; see also Fig. 1). This stresses the importance of using queens of known age for aging studies as processes revealed from middle-aged versus old queens probably

376 differ considerably. Our study is the first addressing the aging process for a social insect by
377 studying the complete lifespan of queens.

378 **Methods**

379 Figure S1 (Additional File 1) provides a schematized workflow of the analyses described in the
380 following sections.

381

382 **Collection and colony maintenance**

383 From 2002 until 2016, *C. secundus* colonies were collected from mangroves near Palmerston -
384 Channel Island (12°30' S, 131°00' E; Northern Territory, Australia) when they were less than one
385 year old [65]. Colonies of an age of less than one year can be identified by the size and slightly
386 lighter sclerotization of the founding reproductives (primary reproductives), the presence of less
387 than 20 workers and short tunnel systems of a few centimeters. All collected colonies were
388 transferred to *Pinus radiata* wood blocks and transported to the laboratory in Germany, where they
389 were kept under standardized conditions in a climate room with a temperature of 28 °C, 70%
390 humidity and a 12h day/night rhythm (Additional File 2, Table S1). Under these conditions,
391 colonies develop like in the field (see [66]). Up until 2019 wood blocks were split and the colonies
392 were extracted to determine survival of reproductives.

393

394 **Survival analysis**

395 The survival of primary queens (and kings) was determined by their presence /absence. Founding
396 (primary) queens can be identified by their dark brown color, compound eyes and wing abscission
397 scars. If the primary reproductives had died and the colony was still alive, they had been replaced
398 by neotenic replacement reproductives which lack these traits. The median longevity of queens was
399 determined using Kaplan Meier survival analysis in SPSS 23 [67]. Only colonies that survived the
400 transfer from the field to the laboratory in Germany and the re-establishment in the new wood block
401 were used for this analysis. This resulted in a sample size of 41 colonies. Overall, we had surviving

primary queens from an age of one year up to a maximum of 13 years. The three potentially 14- and 15-year old queens were all dead.

Transcriptome study

RNA extraction and sequencing

RNA was extracted from twelve queens with different chronological age since onset of reproduction from two years until 13 years: 2, 3, 4, 5, 6, 7, 8, 9, 10 (two samples), 11, and 13 years. In colonies older than 13 years, the original queen was always replaced by a neotenic replacement queen.

An in-house protocol was followed for RNA extraction (see [23]). Individuals were placed on ice and the gut was removed and discarded. The head together with the thorax were used for RNA extraction. Samples were transferred into peqGOLD Tri Fast™ (PEQLAB) and homogenized in a Tissue Lyser II (QIAGEN). Chloroform was used for protein precipitation. From the aqueous phase, RNA was precipitated using Ambion isopropyl alcohol and then washed with 75% ethanol. Obtained pellets were solved in nuclease-free water. DNA was subsequently digested using the DNase I Amplification Grade kit (Sigma Aldrich, Cat. No. AMPD1). We performed an RNA Integrity Number Analysis (RIN Analysis) measuring the RNA concentration with the Agilent RNA 6000 Nano Kit using an Agilent 2100 Bioanalyzer (Agilent Technologies) for quality control. Samples with total RNA were sent on dry ice to Beijing Genomics Institute (BGI) Tech Solutions (HONGKONG) Co. and then to the BGI-Shenzhen (PR China) for sequencing. The preparation of the cDNA libraries was performed by BGI according to their internal and proprietary standard operating procedure. The cDNA libraries were paired-end sequenced (not-strand specific) on Illumina HiSeq 2500 and 4000 platforms (100 base pairs read length and about 4 Giga bases per sample). Index sequences from the machine reads were demultiplexed and a quality -check and

426 filtering of raw reads was done using the package soapuke (-n 0.05 -l 20 -q 0.2 -p 1 -i -Q 2 -G --
427 seqType 1 and -A 0.5, <http://soap.genomics.org.cn/>).

428

429 *Processing of RNASeq raw reads*

430 FastQC (v0.11.4) [68] was used to evaluate the quality of the cleaned raw reads. To obtain a
431 transcript count table, the cleaned raw reads were pseudo-aligned with Kallisto (default settings,
432 v0.43.0) [69] (Bray *et al.*, 2016) against a *C. secundus* transcriptome obtained from a draft version
433 of the *C. secundus* genome (with estimated gene and transcript models, see [23,70]. The counts
434 estimated by Kallisto were normalized using DESeq2 (v1.18.1, count/size factor) [71] in R (v3.4.4)
435 [72].

436

437 *Time course analysis to identify age-associated differentially expressed transcripts (DETs)*

438 The normalized counts were used as input for the R package Iso-MaSigPro (v1.50.0) [73] to test for
439 differentially expressed transcripts (DETs) across time. Iso-MaSigPro is designed for the analysis of
440 multiple time course transcriptome data. It implements negative binomial generalized linear models
441 (GLMs) [73,74]. Significantly differentially expressed transcripts (FDR corrected p-value set to
442 0.05) were clustered using the clustering algorithm mclust in Iso-MaSigPro [73] resulting into six
443 Iso-MaSigPro clusters (Additional File 2, Table S2).

444

445 *Weighted Gene Co-expression Network Analysis (WGCNA) to identify networks of co-expressed* 446 *transcripts*

447 To obtain networks of co-expressed transcripts that were categorized as modules we performed a
448 Weighted Gene Co-expression Network Analysis (WGCNA). The counts obtained with Kallisto
449 (v0.43.0) [69] were transformed using variance stabilizing transformation (vst) as implemented in

450 DESeq2 (v1.18.1) [71]. The vst transformed counts were used to perform a co-expression network
 451 analysis with the R package WGCNA (v1.63) [75]. For more details on the methodology, see [75-
 452 77]. In short, (Additional File 1, Figure S1, workflow, right side), a similarity matrix was built by
 453 calculating Pearson correlations between the expression values of all pairs of transcripts. Using the
 454 similarity matrix, a signed weighted adjacency matrix was obtained as described by the formula:

$$a_{ij} = \left(\frac{1}{2} (1 + \text{cor}_{ij}) \right)^{\beta}$$

455 Where cor_{ij} is the Pearson correlation between the expression pattern of transcript ‘i’ and transcript
 456 ‘j’ (the similarity value). The value of β was chosen based on the soft-thresholding approach [75].
 457 With this value of β , we obtained a weighted network with an approximate scale-free topology
 458 ($\beta=14$, scale-free topology $R^2 = 0.84$). In a signed weighted adjacency matrix negative and small
 459 positive correlations get negligibly small adjacency values shifting the focus on strong positive
 460 correlations. Seeing the adjacency matrix as a network, the nodes correspond to the transcripts and
 461 the connections between nodes correspond to the adjacency values (transformed correlation
 462 coefficients). A topological overlap matrix (TOM), which in addition to the adjacency matrix
 463 considers topological similarity (shared neighbors reinforce the connection strength between two
 464 nodes), was constructed using the adjacency matrix [78]. To define transcript modules, a
 465 hierarchical clustering tree was constructed using the dissimilarity measure (1-TOM). Transcript
 466 modules were defined by cutting the branches of the tree using the Dynamic Hybrid Tree Cut
 467 algorithm [79] and the minimum module size was set to 30 transcripts. Transcript modules with
 468 similar expression profile were merged by hierarchical clustering of the eigengene correlation
 469 values. Briefly, a hierarchical clustering tree was created with the eigengene dissimilarity measure
 470 (1-correlation coefficient of eigengenes) and a tree height cut of 0.20 was used (corresponds to a
 471 eigengene $\text{cor} \geq 0.80$). Eigengenes were calculated with the function `moduleEigengenes` (default
 472 settings) [75]. A module eigengene corresponds to the first principal component of the module and

473 can be seen as a weighted average expression profile [75]. To find significantly associated modules
474 with age, correlations between age and eigengenes of the merged modules were calculated. Each
475 module was named after a color by WGCNA.

476 The adjacency matrix of the WGCNA was visualized using Cytoscape (v3.7.1) [80], only including
477 pairs of nodes with a $cor_{ij} \geq 0.90$. The color of each module corresponds with the respective module
478 name (*e.g.*, saddlebrown color for the saddlebrown module).

479

480 To identify co-expression modules containing age-related DETs, we looked for age-related DETs
481 from the Iso-MaSigPro analysis in the WGCNA modules. Those modules which were significantly
482 correlated with age and which contained the highest number of Iso-MaSigPro DETs were further
483 inspected.

484

485 **Identifying/Extracting age-related subnetworks based on age-related DETs**

486 To identify age-related subnetworks within the co-expression network, we combined the results of
487 the Iso-MaSigPro analysis with those from the WGCNA and extracted subnetworks that were based
488 on age-related Iso-MaSigPro DETs. Therefore, we extracted 1st and 2nd neighbors of DETs based on
489 the WGCNA co-expression network (*i.e.*, the visual representation of the adjacency matrix). To do
490 this, we used the ‘First neighbors’ function of Cytoscape. We selected an age-related DET from Iso-
491 MaSigPro as transcript of interest. By calling the function, the neighboring transcripts were
492 selected, which were then extracted to form a subnetwork. By calling the function twice, one
493 obtains the one- and two-step neighbors (called ‘second level neighborhood’) of the transcript of
494 interest. This was done for each DET identified in IsoMaSigPro.

495

496 The obtained subnetworks were clearly separated in those containing young Iso-MaSigPro DETs
 497 (young subnetworks) and those containing old Iso-MaSigPro DETs (old subnetworks). The largest
 498 subnetwork obtained for each group was used for further analysis paying attention to both,
 499 transcript identity as well as WGCNA module content. Thus, we looked, for instance, for WGCNA
 500 modules that had been identified to be age-related within the global WGCNA in these subnetworks.
 501 The AutoAnnotate Cytoscape plug-in (v1.3) [81] was used to annotate the subnetworks using the
 502 clustering algorithm ‘Markov Cluster’ (MCL) [82] to define and visualize sub-clusters, and the
 503 labeling algorithm ‘Adjacent Words’ to label the sub-clusters. The Cytoscape plug-in BiNGO
 504 (v3.0.3) [83] was used for GO enrichment analysis. The p-values of the GO enrichment analysis
 505 were adjusted for multiple testing using the FDR approach [84]. Subnetworks were graphically
 506 processed with Inkscape (v0.91, www.inkscape.org).

507

508 Transcript (functional) annotation

509 Nucleotide and protein sequences were obtained from the draft version of *C. secundus* genome
 510 [23,70]. For annotation, the translated transcripts were searched against the Pfam database (Pfam A,
 511 release 30) [85] with the software *hmmsearch* (option `--cut_ga`, HMMer v.3.1b2) [86] and against the
 512 InterPro database with the software InterProScan (v5.17-56.0) [87]. Additionally, we did a
 513 BLASTX search (NCBI BLAST suite v. 2.3.0) [88] with an e-value of $1e^{-05}$ as cut-off against the
 514 protein coding sequences of the termite *Zootermopsis nevadensis* (official gene set version 2.2)
 515 [89]. To further assist the annotation, we inferred a set of clusters of orthologous sequence groups
 516 (COGs) from the official gene sets at the amino acid level of *C. secundus* (draft version) and *D.*
 517 *melanogaster*, and a BLASTP search of *C. secundus* sequences against the protein coding
 518 sequences (longest isoforms only) of *D. melanogaster* with a threshold e-value of $1e^{-05}$.

519 To detect possible TEs, transcripts were searched against the Dfam database (v2.0) [90] using
 520 nhmmer [91]. A transcript was considered TE related if there was a hit against the Dfam database
 521 and the other annotation sources (Pfam, Interpro and BLAST) were not pointing in the direction of
 522 a known gene.

523

524 **Gene identification and construction of gene trees**

525 In addition to the functional annotation, we inferred phylogenetic trees for selected transcripts
 526 (Supplementary Archive 1, DRYAD, doi available upon acceptance of the manuscript). Following
 527 the procedure described in [23], we retrieved protein coding sequences of the respective cluster of
 528 orthologous sequence groups (COGs) from OrthoDB v.9.1 [92] for the following species: *D.*
 529 *melanogaster* (DMEL), *Apis mellifera* (AMEL), *Cardiocondyla obscurior* (COBS), *Polistes*
 530 *canadensis* (PCAN), *Tribolium castaneum* (TCAS), *Z. nevadensis* (ZNEV) and *Blattella germanica*
 531 (BGER). COGs were identified using text search by searching for the gene name or IDs of *D.*
 532 *melanogaster*. In case Selenocysteine (U) was included in sequences, “U” was replaced by “X” to
 533 avoid problems in downstream analyses since many programs cannot handle Selenocysteine
 534 properly. Protein sequences of COGs of above listed species were separately aligned with MAFFT
 535 (v.7.294b) applying the G-INS-i algorithm and otherwise default options [93]. For each multiple
 536 sequence alignment, a profile hidden Markov model (pHMM) was built with hmmbuild (HMMER
 537 v.3.1b2) [86]. Then the pHMM was used to search (hmmsearch) for corresponding protein coding
 538 sequences in *C. secundus* and *Macrotermes natalensis* to identify orthologous candidate sequences
 539 for each COG in both species. For gene (transcript) tree inference, we only kept sequences with a
 540 threshold e-value of $\leq 1e^{-40}$. In addition, we annotated all candidate sequences identified in *C.*
 541 *secundus* and *M. natalensis* against the Pfam database (Pfam A, release 30) using hmmscan
 542 (HMMER v.3.1b2).

To infer phylogenetic gene trees, we merged for each COG the COGs (amino-acid level) of the seven species listed above with the putatively orthologous amino-acid sequences of *C. secundus* and *M. natalensis*. We generated multiple sequence alignments for a total of 29 genes of interest applying MAFFT (G-INS-i, see above). Ambiguously aligned sequence sections were identified with Aliscore (v. 2 [94,95]; settings: -r: 10000000 and otherwise defaults) and removed with Alicut (v. 2.3, <https://www.zfmk.de/en/research/research-centres-and-groups/utilities>; masked alignments provided as Supplementary Archive S1 (deposited at DRYAD, doi available upon acceptance of the manuscript). Phylogenetic trees were inferred with IQ-TREE (1.7-beta12 [96] for each gene. The best model was selected with the implemented ModelFinder [97] from all available nuclear models implemented in IQ-TREE plus the two protein mixture models LG4M and LG4X [98] based on the Bayesian Information Criterion (BIC). We applied default settings for rates and the number of rate categories. Statistical support was inferred from 2,000 non-parametric bootstrap replicates. Unrooted trees with the bootstrap support mapped were visualized with Seaview (v4.5.4 [99]) and provided in Newick Format with Supplementary Archive S1 at DRYAD (doi available upon acceptance of the manuscript).

558

559 **Ethics approval and consent to participate**

560 Not applicable

561 **Consent for publication**

562 Not applicable

563 **Competing interests**

564 The authors declare that they have no competing interests.

565

566 **Availability of data and materials**

567 Raw sequence reads are deposited on NCBI (Bioproject XXX, BioSample Accessions see
568 Additional File 2, Table S1 – Bioproject numbers and BioSample Accessions will be provided
569 when obtained from NCBI). Additional supplementary data are deposited on the Dryad digital
570 repository DRYAD (DOI provided upon acceptance of the final manuscript). For Supplementary
571 Material please contact: judith.korb@biologie.uni-freiburg.de

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576

577 **Contributions**

578 JK designed the study, JK and MMK collected and identified the termite samples, MMK performed
579 all transcriptome analyses, KM helped with gene identification, with data processing and inferred
580 gene trees, JK did survival analysis and supervised the study, all authors wrote the paper.

581

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592

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862 **FIGURE LEGENDS**

863 **Figure 1. Survival plot of *C. secundus* queens.**

864 Shown is the age-dependent survival probability of queens. The median longevity of queens in the
865 laboratory after successful colony establishment was estimated with Kaplan Meier survival analysis
866 to be 12 years (mean longevity: 11.1 years). The maximum lifespan was 13 years. After an age of
867 around 11 years, life expectancy declines rapidly; out of eight queens with a potential age ≥ 12
868 years, all had died, except one 13-year old queen.

869

870 **Figure 2. Median expression profiles of DETs assigned to Iso-MaSigPro clusters.**

871 Iso-MaSigPro grouped the differentially expressed transcripts (DETs) into six clusters. DETs of
872 cluster 1, 4 and 6 were especially highly expressed in old queens, while those of cluster 3 and 5
873 characterized middle-aged queens and those of cluster 2 young queens. The expression values
874 correspond to normalized counts (see Methods). The youngest queen (age: 2 years) was taken as
875 time step zero and each of the subsequent older queens (based on chronological age) were
876 considered to be one time step older. One age class (time step 8; age: 10 years) consisted of two
877 samples.

878

879 **Figure 3. Young subnetwork highlighting Iso-MaSigPro DETs.**

880 Shown is WGCNA-based co-expression network of transcripts, which contains DETs characterising
881 young and middle-aged queens and their one- and two-step neighbors (*i.e.*, young subnetwork; for
882 more information, see text and Additional File 1, Figure S1 and S2). Highlighted are the Iso-
883 MaSigPro DETs of cluster 2, 3, and 5, characterizing young and middle-aged queens (see insert;
884 Fig. 2). Node colors correspond to the WGCNA modules. Transposable element (TE) related
885 transcripts are highlighted with a ‘?’. Transcripts with an asterisk indicate 1:1 orthologs (*C.*

886 *secundus* and *D. melanogaster*). Connection length and width do not have a meaning. Red circles
887 indicate transcripts discussed in the text.

888

889 **Figure 4. Genes related to protein synthesis that were found in the old subnetwork.**

890 Shown are genes that have been related to various processes of protein synthesis, from initiation,
891 and elongation to termination. For all genes listed, corresponding transcripts were present in the old
892 subnetwork of *C. secundus* queens. Figure modified after [35].

893

894 **Figure 5. Genes related to the proteasome complex that were found in the old subnetwork.**

895 Shown are genes that have been related to the proteasome complex. The textbox in red indicates
896 subunits, for which we found transcripts in the old subnetwork. Figure modified after [36].

897

898 **Figure 6. Transcription- and proteostasis-related expression pattern in old *Cryptotermes***

899 *secundus* queens.

900 Depicted is the expression patterns of genes related to transcription and proteostasis for old *C.*
901 *secundus* queens (red arrows) in contrast to that reported for other species (grey arrows). Old *C.*
902 *secundus* queens were characterized by a very strong proteostasis signal indicative of an
903 upregulation of protein degradation, together with protein synthesis and protein folding. This is
904 unusual because old organisms are typically characterized by a downregulation of these processes.
905 The simultaneous activation of protein synthesis and degradation in old *C. secundus* queens can be
906 explained by the activity of the transcription factor cnc/Nrf-2 (for more details, see text). The inner
907 cycle arrows depict the protein life cycle; dashed arrows indicate the special case when mistakes/
908 errors occur. After a protein is degraded, its components are recycled.

909

910 **Figure 7. Aging signal of *C. secundus* queens in relation to known aging pathways.**

911 Shown are simplified IIS (insulin/insulin-like growth factor signaling; blue), TOR (target of
 912 rapamycin; green), and ecdysone (brown) pathways and their interactions with an emphasis on **A.**
 913 ecdysone biosynthesis and **B.** protein synthesis and degradation. Red encircled genes were members
 914 of the old subnetwork, and thus highly expressed in old queens. Important genes that regulate
 915 protein synthesis and degradation are depicted in white. In short, the TOR pathway controls cell
 916 growth and metabolism in response to amino acid availability. It is generally composed of two main
 917 complexes: TORC1 and TORC2 [55]. Activation of TORC1 promotes mRNA translation, for
 918 instance, via S6K /eIF4B / eIF-4a and 4E-BP / eIF4E. Additionally, active TORC1 inhibits
 919 autophagy by targeting upstream components necessary for autophagy activation, like Atg1. TOR
 920 interacts with IIS, which also regulates multiple physiological functions, including aging.
 921 Generally, an active IIS pathway can activate the TORC1 complex via phosphorylation and
 922 inactivation of Tsc2 by AKT. AKT inhibits the transcription factor foxo via phosphorylation, which
 923 results in the inhibition of transcription of many downstream genes, e.g. involved in lifespan
 924 extension, stress response and autophagy. Stress induced *Cnc* can activate TORC1 in a positive
 925 feedback loop (big dashed arrow). It may be responsible for the simultaneous upregulation of
 926 protein synthesis and degradation. For more information, see text. Figures are adapted after [53-55].

927

928

929 **Additional Files**

930 **Additional File 1 (Additional_File_1.pdf): Supplementary Figures S1-S10.**

931 Figure S1: Schematic workflow. Details are explained in the Methods.

932 Figure S2: WGCNA modules of co-expressed transcripts that negatively correlate with age.

933 Modules marked with an asterisk were found in the young subnetwork. Modules are named after
934 colors by WGCNA.

935 Figure S3: WGCNA modules of co-expressed transcripts that positively correlate with age.

936 Modules marked with an asterisk were found in the old subnetwork. Modules are named after colors
937 by WGCNA.

938 Figure S4: GO enrichment for the WGCNA module ‘tan’ that positively correlated with age and
939 which had many old-age DETs. Details are shown for BP (Biological Process), which revealed an
940 enrichment of transcripts for ribosomal and tRNA related functions.

941 Figure S5: Young and old transcript subnetworks corresponding to the second level neighborhood
942 of Iso-MaSigPro DETs. Age-related DETs were located in the WGCNA co-expression network and
943 these DETs and their one- and two-step neighbors (*i.e.*, the ‘second level neighborhood’) were then
944 extracted from the co-expression network to provide the shown networks.

945 Figure S6: BiNGO GO enrichment (Biological Process) for the young subnetwork. No terms were
946 significantly enriched after correcting for multiple testing (FDR).

947 Figure S7: BiNGO GO enrichment (Biological Process) for the old subnetwork. Colored nodes are
948 GO terms that were significantly enriched after correcting for multiple testing (FDR).

949 Figure S8: BiNGO GO enrichment of (Molecular Function; Cellular Component) for the old
950 subnetwork. Colored nodes are GO terms that were significantly enriched after correcting for
951 multiple testing (FDR).

952 Figure S9: BiNGO GO enrichment (Biological Process; Molecular Function; Cellular Component)
 953 for the ‘green’ WGCNA module, which is part of the old subnetwork. Colored nodes are GO terms
 954 that were significantly enriched after correcting for multiple testing (FDR).

955 Figure S10: BiNGO GO enrichment (Biological Process, Molecular Function; Cellular Component)
 956 for the ‘paleturquoise’ WGCNA module, which is part of the old subnetwork. Nodes in color are
 957 GO terms significantly enriched after correcting for multiple testing (FDR).

958

959 **Additional File 2 (Additional_File_2.xlsx): Supplementary Tables S1-S4.**

960 Table S1: Sample information of samples included in this study.

961 Table S2: Differentially expressed transcripts of the Iso-MaSigPro analysis, separately for cluster 1-
 962 6.

963 Table S3. Transcripts in the young subnetwork (SNW).

964 Transcripts in the young subnetwork (SNW) classified into major categories; TE-related transcripts
 965 of the young subnetwork (SNW).

966 Table S4. Transcripts in the old subnetwork (SNW).

967 Transcripts in the old subnetwork (SNW) classified into major categories; TE-related transcripts of
 968 the old subnetwork (SNW).

969

970 **Additional File 3 (Additional_File_3.pdf)**

971 WGCNA module-age associations. Listed are the eigengene values for each module.

972

973 **Additional File 4 (Additional_File_4.xlsx): Supplementary Table S5.**

974 Go terms for enriched differentially expressed transcripts (DETs) included in BiNGO module
 975 green; BiNGO module paleturquoise; BiNGO module tan; BiNGO module Thistle2; BiNGO

976 module snow; BiNGO module cyan; BiNGO module deeppink1; BiNGO module navajowhite;
 977 BiNGO module blue2_NS; BiNGO module cornflowerblue_NS; BiNGO module pink3_NS;
 978 BiNGO module steelblue4_NS.

979

980 **Supplementary Archive 1**

981 The Supplementary Archive includes i) masked multiple sequence alignments (MSAs) of 29
 982 selected genes (subdirectory “MSAs” used for ML gene tree inference in FASTA format) in IQ-
 983 TREE and ii) inferred ML gene trees (subdirectory “ML_gene_trees” in NEWICK format) for each
 984 of the 29 genes including non-parametric statistical bootstrap support. These gene trees additionally
 985 helped to ensure a proper assignment of transcripts to repective genes.

Cluster 1 **Cluster 2** **Cluster 3** **Cluster 4** **Cluster 5** **Cluster 6**

Initiation

Ribosome binds mRNA
at start codon

eIF3h eIF3f1 eIF2A eIF4A eIF3c eIF4B eIF3l eIF1 eIF5B eIF2gamma eIF2beta eIF3k eIF3d1 eIF3i eIF2Bepsilon eIF2alpha	eIF3j eIF6 eIF4G1
---	-------------------------

Elongation

Polypeptide chain elongates by
successively adding amino acids

eEF1gamma
eEF1beta
eEF1beta
eEF1delta

Termination

When a stop codon is
encountered, polypeptide is
released and ribosome
dissociates

eRF3

Ribosomal proteins

RpL28	RpL24-like	RpLP1	RpS13
RpL18A	RpS3A	RpS25	RpS15
RpS26	RpL4	RpS18	RpL6 (3)
RpL40	RpL5	RpL22	RpS17
RpLP0-like	RpL23A	RpS9	
RpL23	RpLP0	RpL27A	
RpL27	RpS15Aa	RpL10	
RpL34a	RpL18	RpL36	
RpL36A	RpL19	RpL26	
RpS30	RpL8	RpS23	

Aminoacyl-tRNA Synthetases

GluProRS	ThrRS
CysRS	LysRS
IleRS	HisRS
AsnRS	GlyRS
GlnRS	ValRS
alpha-PheRS	beta-PheRS
	AsnRS

Subunits in the "old" subnetwork

Rpn3, 7, 8, 9, 11, 12

Rpn2, 10(2)

Rpt1, 2, 3, 4, 5

3, 4, 5, 7

1-7

A

B