

1 **Comparative transcriptomics across nematode life cycles reveal gene**
2 **expression conservation and correlated evolution in adjacent**
3 **developmental stages**

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10 **Abstract**

11 Nematodes are highly abundant animals with diverse habitats and lifestyles. Some are
12 free-living while others parasitize animals or plants, and among the latter, infection abilities
13 change across developmental stages to infect hosts and complete life cycles. Although
14 parasitism has independently arisen multiple times over evolutionary history, common
15 pressures of parasitism—such as adapting to the host environment, evading and subverting
16 the host immune system, and changing environments across life cycles—have led phenotypes
17 and developmental stages among parasites to converge. To determine the relationship
18 between transcriptome evolution and morphological divergences among nematodes, we
19 compared 48 transcriptomes of different developmental stages across eight nematode species.
20 The transcriptomes were clustered broadly into embryo, larva, and adult stages, suggesting
21 that gene expression is conserved to some extent across the entire nematode life cycle. Such
22 patterns were partly accounted for by tissue-specific genes—such as those in oocytes and the
23 hypodermis—being expressed at different proportions. Although nematodes typically have 3-
24 5 larval stages, the transcriptomes for these stages were found to be highly correlated within
25 each species, suggesting high similarity among larval stages across species. For the
26 *Caenorhabditis elegans*-*C. briggsae* and *Strongyloides stercoralis*-*S. venezuelensis*
27 comparisons, we found that around 50% of genes were expressed at multiple stages, whereas
28 half of their orthologues were also expressed in multiple but different stages. Such frequent
29 changes in expression have resulted in concerted transcriptome evolution across adjacent

30 stages, thus generating species-specific transcriptomes over the course of nematode evolution.
31 Our study provides a first insight into the evolution of nematode transcriptomes beyond
32 embryonic development.

33

34 **Introduction**

35 Nematodes represent the largest animal phylum on earth and display a vast diversity,
36 with 25,000 species described and about 10,000,000 estimated (Poinar 2011). Their extensive
37 morphological diversity is a reflection of their trophic resources, lifestyles, reproductive
38 strategies, and living environments. Of the free-living nematodes, *Caenorhabditis elegans* is
39 the best-studied model organism in molecular and developmental biology. *Caenorhabditis*
40 *briggsae*—closely related to *C. elegans* and with an almost identical morphology (Grün et al.
41 2014)—is also widely used in comparative studies on nematode evolution and development.
42 Additionally, parasitism is ubiquitous in nematodes and has independently arisen at least 18
43 times during the group’s evolutionary trajectory (Blaxter et al. 1998; Blaxter and
44 Koutsovoulos 2015; Zarowiecki and Berriman 2015; Weinstein and Kuris 2016). Another
45 nematode genus of particular interests is *Strongyloides*, the species of which have a unique
46 life cycle in which they alternate between free-living and parasitic generations. Such
47 alterations make *Strongyloides* a unique and attractive model for studying the evolution of
48 parasitism. A previous study showed that parasitism-associated genes that are expanded and
49 specific to parasitic stages are clustered in specific chromosomal regions, suggesting that they
50 contribute to the regulatory mechanisms of parasite development (Hunt et al. 2016). Some of
51 these parasitism genes expanded across different clades of parasites, indicating convergent
52 evolution at the genomic level (Coghlan et al. 2019); however, the evolutionary relationships
53 among transcriptomes at different stages and parasitic or free-living nematodes remain to be
54 elucidated.

55 Evolutionary changes occur frequently in organisms through the co-opting of existing
56 traits for new purposes. Which co-opted features change with the emergence of species-
57 specific stages and how they do so at the genetic and regulatory levels are essential questions
58 in evolutionary developmental biology. One theory, the developmental constraint concept,
59 argues that these features limit phenotypic variability and the composition or dynamics of the
60 developmental system (Smith et al. 1985). In nematodes and arthropods, the morphologies

61 and transcriptomes were conserved during mid-embryogenesis between species within the
62 same phylum (Kalinka et al. 2010; Levin et al. 2012). These observations coalesced into the
63 hourglass model. Since evolution and development are two intertwined processes, constraints
64 and variations in a species' development may have significant impacts on that species'
65 evolutionary trajectory. A recent study suggested that some gene expressions changed
66 simultaneously across multiple tissues after speciation, leading to correlated patterns of gene
67 expression evolution and causing the genes to group by species in hierarchical clustering
68 (Liang et al. 2018). Studying constraints in the transcriptomes of stages beyond nematode
69 embryogenesis is of tremendous interest, but remains challenging as developments in each
70 stage can be vastly different across intraspecific generations and interspecific morphologies.
71 One of the first experiments comparing transcriptomes of nematode developmental stages
72 beyond embryogenesis was performed by comparing synchronized transitions from embryo
73 to adult stages in *C. elegans* and *C. briggsae* (Grün et al. 2014). It measured fluctuating
74 mRNA and protein expressions across the life stages, and showed that transcript fold changes
75 were conserved during embryo-to-larva transitions. Over the past few years, insights gained
76 from transcriptomic comparisons between developmental stages during and before infection
77 have increased our understanding of parasitism. The recent availability of transcriptomic data
78 from parasitic nematodes (Choi et al. 2011; Stoltzfus et al. 2012; Laing et al. 2013; Hunt et al.
79 2018; Tanaka et al. 2019) is an exciting resource for identifying the evolution of gene
80 expression throughout development.

81 The life cycle of nematodes usually consists of one embryo, four to five larval, and one
82 adult stages, which are separated by moulting (Lee 2002; Sommer and Streit 2011). The body
83 size of larvae increases after every moult, eventually reaching sexually mature adult size.
84 Several nematodes have evolved specialised developmental stages, such as a dauer stage
85 whereby the larva undergoes developmental arrest to survive unsuitable conditions, such as a
86 food shortage or high population density. The occurrences of dauer and diapause stages have
87 been associated with gene expression changes in several invertebrates (Flannagan et al. 1998;
88 Bao and Xu 2011; Hand et al. 2016). Unique developmental stages with morphological traits
89 specialized for parasitism also frequently occurred during the evolution of nematode species.
90 These include the sedentary and swollen females in plant-parasitic nematodes and the
91 ensheathed larvae in animal-parasitic nematodes (Lee 2002). In addition, the microfilariae of
92 *Brugia malayi*, which have a morphology very different from any general life stage of
93 nematodes, migrate to and develop in the mosquito, making *Brugia malayi* the intermediate

94 host and transitional insect vector. The transcriptomes of these specialised developmental
95 stages are often distinct to those of previous stages, and these differences in expression
96 mainly come from members of expanded gene families that arose from lineage-specific
97 duplications (Stoltzfus et al. 2012; Baskaran et al. 2015; Hunt et al. 2018).

98 We hypothesize that, although there is vast phenotypic diversity across the life cycles of
99 different nematodes, these life cycles can be compared and therefore the levels of
100 conservation between gene expression across developmental stages can be quantified. Hence,
101 high throughput sequencing data across nematodes that previously focused on species-
102 specific differences may be further utilized to reveal the conservation of transcriptomes
103 associated with the life cycles. In this study, we compared the transcriptomes of different
104 developmental stages across several nematode species and profiled the conservation of gene
105 expression in whole worms, particular tissues, and biological processes. To investigate if
106 there was conservation at each developmental stage, we estimated the similarities between
107 transcriptomes across developmental stages and clustered based on their similarities. We
108 further quantified these similarities to assess whether specialisation occurred at that
109 developmental stage, and categorised genes into different expression profiles. The frequent
110 changes in orthologue switching profiles between species were revealed and discussed. This
111 study provides the first investigation of developmental conservation across the evolutionary
112 trajectory of multiple nematode species beyond the embryonic stage.

113

114 **Results**

115 **Data collection and clustering among intraspecies transcriptomes**

116 We collected independently published transcriptome datasets from five to seven
117 developmental stages in eight nematodes: *C. elegans* and *C. briggsae* (Grün et al. 2014),
118 *Pristionchus pacificus* (Baskaran et al. 2015), *S. stercoralis* (Stoltzfus et al. 2012), *S.*
119 *venezuelensis* (Hunt et al. 2018), *Haemonchus contortus* (Laing et al. 2013), *B. malayi* (Choi
120 et al. 2011), and *Bursaphelenchus xylophilus* (Tanaka et al. 2019). A summary of the data is
121 shown on Figure 1. Grün et al. (2014) profiled the transcriptomes of two *Caenorhabditis*
122 species during development under the same conditions. The combined dataset consisted of 13
123 stages with one to six biological replicates per stage and 1.5 to 75 million reads per sample.

124 On average, 82.4% of reads per sample were aligned to corresponding nematode genomes
125 using HISAT2 (Kim et al. 2015) under the same parameters (Supplementary Table S1).

126 Rather than re-compiling a list of differentially expressed genes between adjacent
127 development stages as done in previous studies (Choi et al. 2011; Stoltzfus et al. 2012; Laing
128 et al. 2013; Grün et al. 2014; Baskaran et al. 2015; Hunt et al. 2018; Tanaka et al. 2019), we
129 were primarily interested in the genome-scale similarities of mRNA expression between
130 development stages. Correlation coefficients between different developmental stages within
131 each species were computed and hierarchically clustered. In general, the transcriptome of the
132 embryo was the most distinct, while those of larval stages were similar to those of their
133 adjacent stages (FIG. 2). In the case of *C. elegans*, each clustered stage—embryo, adult, and
134 larval—were further clustered into early (L1 and L2) and late stages (L3, L4 and LL4) (FIG.
135 2A). Clustering patterns of larval stages in *P. pacificus* (FIG. 2B) and *B. xylophilus* (FIG. 2C)
136 also supported the early-late partition. This partitioning pattern was not observed for
137 *Haemonchus contortus* (FIG. 2D) or *C. briggsae* (FIG. 2E), possibly due to intra-species
138 variation in the speed of development, which results in worm cultures consisting of
139 individuals with different development stages (Baskaran et al. 2015; Perez et al. 2017).
140 Consistent with previous findings (Baskaran et al. 2015), we also found that specialised
141 phenotypic stages, such as dauer (FIG. 2B), were assigned to its adjacent exit stage (larval
142 L4). In addition, the larval stages were divided into two branches: presence and absence of
143 infectious ability in *S. stercoralis* (FIG. 2F). Similar patterns were found in parasitic larval
144 stages of *S. venezuelensis* (FIG. 2G) and the insect transition stages of *B. malayi* (FIG. 2H).
145 These data suggested that factors associated with parasitism are not as important as factors
146 associated developmental stages in determining similarities of transcriptomes that were
147 analysed. To compare transcriptomes among stages and different species, orthology between
148 genes was first assigned into a total of 15,835 orthogroups, including 2,548-6,736 single copy
149 orthologues across 28 species-pair comparisons.

150

151 **Transcriptomes across nematode development were clustered into embryo, larval, and** 152 **adult stages**

153 We first computed the correlation between *C. elegans*-*C. briggsae* single copy
154 orthologues across development stages. The development stages were clustered into embryo,

155 larva, and adult stages (FIG. 3A). Interestingly, expressions across five larval stages were
156 grouped by species, i.e., all *C. elegans* larval stages were grouped together before clustering
157 with the *C. briggsae* larval stages. As this particular dataset was performed under
158 synchronized conditions (Grün et al. 2014), we included additional published data from
159 modEncode (Celniker et al. 2009), which contains transcriptomes from multiple
160 developmental stages (Supplementary Table S3). The same clustering was even observed
161 (Supplementary FIG. S1), even when gene families were also included (Supplementary FIG.
162 S2), demonstrating that such clustering was robust beyond potential batch effects from
163 different studies.

164 Similar clustering patterns were also observed in the soil-transmitted gastrointestinal
165 parasitic nematode *Strongyloides* (FIG. 3B). These nematodes are particularly interesting
166 because they alternate between free-living and parasitic generations (Hunt et al. 2016; Hunt
167 et al. 2018). The transcriptomes of *S. stercoralis* (which parasitizes humans) and *S.*
168 *venezuelensis* (which parasitizes rats) were divided into larva and adult stages, but differences
169 were observed in each cluster. Free-living and parasitic female adult stages of each species
170 were grouped together before clustering into a major ‘adult’ group. Transcriptome clusters in
171 the larval branch were instead separated by lifestyles, consistent with the observation that
172 infection ability was already available in their common ancestor (Hunt et al. 2016) and was
173 conserved despite having different hosts.

174 We applied the same analysis to all species pairs to systematically compare gene
175 expressions across nematodes (Supplementary FIG. S3). Of the 28 possible combinations,
176 clustering of developmental stages in 13 species pairs again revealed three groups
177 corresponding to three broad developmental processes from embryo to adulthood. We found
178 a lower number of orthologue pairs inferred from species of different genera (2,548-4,295)
179 than pairs from the same genus (5,394-6,736), and such comparisons partitioned the larval
180 stages further into early and late phases. This was the case for the *C. elegans*-*B.*
181 *xylophilus* and *C. elegans*-*H. contortus* comparisons, but not *C. elegans*-*C. briggsae*. We
182 were concerned that different pairs of orthologue sets would influence the analysis results,
183 and therefore repeated the *C. elegans* and *C. briggsae* clustering with different one-to-one
184 orthologue lists (*C. elegans*-*B. xylophilus* and *C. elegans*-*H. contortus*). The clustering results
185 remained identical (Supplementary FIG. S4, FIG. 3A), suggesting transcriptome
186 independence between *C. elegans*-*C. briggsae* larval stages.

187 Similar environmental pressures lead to recurrent cases of convergence in common
188 genomic adaptations of nematodes of different ancestries (Coghlan et al. 2019). We found
189 one case of transcriptome convergence in the *B. malayi*-*B. xylophilus* comparison (FIG. 3C).
190 Both species are obligate parasites dispersed by insect vectors. The microfilaria of all stages
191 shared by *B. malayi*-*B. xylophilus* followed the three-theme pattern of embryo, adult and
192 larval stages—except adults of *B. xylophilus*, which were more similar to the late larvae. The
193 late larval stages tended to be grouped by species. In addition, the immature microfilaria and
194 microfilaria congregated with the L2 and D3 larval stages of *B. xylophilus*. First observed by
195 Patrick Manson in the nineteenth century, the microfilaria of *B. malayi* is a unique larval
196 form that allows it to move from the human host’s bloodstream to the intestine of the
197 intermediate vector mosquito. Conversely, *B. xylophilus* is a plant parasitic nematode
198 responsible for pine wilt disease. When faced with an environment high in nematode density
199 and low in food supplies, the *B. xylophilus* L2 larva moults to form an alternative third stage
200 dispersal juvenile (D3), which is taken up by longhorn beetles and transmitted to another
201 healthy host tree (Tanaka et al. 2019). These distinct larval forms adapted to the insect vector
202 were derived independently in their evolution, suggesting transcriptome convergence.

203 The proportion of different cell types composing the worm body changed dynamically
204 during development. To determine if a similar class of orthologues was also overexpressed in
205 specific tissues throughout development, we dissected the entire worm transcriptome to focus
206 on gene expression in the hypodermis and oocytes. We first obtained 209 and 172 genes
207 involved in these two processes in *C. elegans* based on one previous study of mutants that
208 cause defects in germ cell proliferation (Reinke et al. 2004) and another on cell type- specific
209 RNAseq (Spencer et al. 2011), respectively. We then obtained their orthologues and
210 corresponding expression values in other species. Finally, proportions of the transcriptome
211 from orthologues of *C. elegans* genes in each stage were found to range from 0.2-18% (FIG.
212 4A-4B). We identified significant differences in transcriptome proportions across
213 developmental stages (embryo, early larva, late larva, and adult; Wilcoxon ranked sum test;
214 FIG. 4C-D). Except for the post parasitic L1 and post free-living L1 stages, expression in the
215 hypodermis was highest during the late larval and embryo stages, followed by the early larval
216 and lowest during the adult stages (FIG. 4A). This is consistent with the finding that mutants
217 with defects in genes involved in hypodermis development produced arrested embryos or
218 larvae (Riddle et al. 1997). Orthologues of *C. elegans* genes that participated in oogenesis
219 had the opposite expression trend, and were expressed the least in the larval stages (FIG. 4C).

220 Interestingly, the immature and mature microfilaria are distinct larval forms in *B. malayi* and
221 also had high expression proportions, which makes sense as this stage comes immediately
222 after the embryo stage and the sheath originates in the envelope of the embryo.

223 **Differential levels of correlated transcription evolution (LCE) in nematodes**

224 To further quantify the similarities in the transcriptome across developmental stages, we
225 estimated the level of correlated transcriptome evolution (LCE) (Liang et al. 2018) in stage
226 pairs across species. LCE is a statistical model developed by Liang *et al.* that measures the
227 correlation between transcriptomes by estimating the average correlation across all genes
228 (Liang et al. 2018). High LCE (0.32-0.75) was observed between different larval stages in
229 two *Caenorhabditis* species. In contrast, LCE was 0.01-0.27 for the embryo stage, which
230 overlaps with the LCE bound of 0.235 retrieved from the simulated stage independence
231 (Liang et al. 2018; FIG. 5A). These observations were consistent with the aforementioned
232 finding that different larval stages clustered by species, suggesting that gene expressions in
233 larval stages are correlated. In addition, the finding that LCE was lowest between the embryo
234 and any other stage indicated that the gene expression during embryonic development was
235 distinct from those during larval development and adulthood. In *Strongyloides*, the LCE
236 values for the six comparisons between larva and adult stages were lower than those for all
237 the other comparisons. LCE values in the comparison between parasitic and free-living
238 females, and the infectious L3 and L3+ (L3 collected from host), were 0.64 and 0.36,
239 respectively (FIG. 5B). Consistent with the clustering analysis, LCE results suggested that
240 different lifestyles in adults seemed to have evolved concertedly and not individually.

241 Next, we sought to determine whether concerted evolution occurred across nematodes
242 of different genera (FIG. 5C). Species-specific developmental stages, such as microfilaria
243 in *B. malayi* and D3 in *B. xylophilus*, were excluded because they lacked equivalent stages in
244 the other species. Four of the five comparisons between larval stages showed high LCEs
245 (0.27 to 0.53) whereas six of the eight comparisons between larva and embryo or adult had
246 low LCEs (-0.15 to -0.11). Co-evolution of larval stages within a species seemed to be a
247 general phenomenon in nematodes. Of the larval stages, L3 had on average the lowest LCE,
248 while the other stages have average LCEs of 0.38 to 0.51. This suggested that L3 was the
249 most individualized stage. In parasitic nematodes, infectious ability was frequently observed
250 in the third larval stage. This preference for gaining parasitic capabilities provoked the
251 question of whether L3 in parasitic nematodes has evolved independently since early

252 evolutionary history. Our findings on the LCE of parasites suggested that the answer is no, as
253 L3 was not individualized and instead evolved in concert with the adjacent stages after L3
254 (Supplementary FIG. S5).

255 **Orthologues are frequently expressed in different developmental stages**

256 To further quantify the differences in transcriptomes among species, we ranked the
257 genes based on relative expression using the Z-score angle-sorted value index for temporal
258 sorting (ZAVIT) (Levin et al. 2016). Next, each gene in each species was categorized based
259 on its ranking and differential expression analysis across developmental stages (FIG. 6A,
260 Supplementary Table S4-5). The expression profiles for *C. elegans* and *C. briggsae* were
261 similar, both showing a stage-like pattern in the embryo, all larval, and adult stages
262 (Supplementary FIG. S6A). The majority of genes were expressed in multiple stages (72.5%
263 and 63.4% in *C. elegans* and *C. briggsae*, respectively). To examine whether there is an
264 evolutionary preference for any particular category of genes, we assessed the differences in
265 expression categories between species. Interestingly, only just half (50.03%) of *C. elegans*
266 orthologues were expressed in the same category in *C. briggsae* (FIG. 6A); the proportion of
267 genes that shifted across developmental profiles varied from 20.6% (in embryo + larva) to
268 86.1% (in larval + adult). Interestingly, genes of the orthologue pairs expressed in the latter
269 category in both *Caenorhabditis* species exhibited ratios of the nonsynonymous substitution
270 rate to the synonymous substitution rate (d_N/d_S) significantly lower than four other expression
271 profiles (FIG. 6C), implying stronger purifying selection on coding sequences of genes that
272 maintained the same role, despite being in the category with the highest switching.
273 Enrichment of Gene Ontology revealed the significant terms including the “small molecule
274 metabolic process” and “purine nucleoside monophosphate metabolism” (Supplementary
275 Table S6). Small-molecular signalling has been extensively studied in *C. elegans* for its
276 important roles across multiple aspects of development and behaviour (Ludewig 2013)(ref,
277 while purine homeostasis was recently revealed to be necessary for developmental timing in
278 *C. elegans* (Marsac et al. 2019). Both conservation of expression category and higher
279 purifying selection of these genes further imply their functional importance across
280 *Caenorhabditis* genera.

281 Expressions patterns in free-living and parasitic life cycles were complex and included
282 multiple possible combinations, so we first empirically assigned 88.0% and 91.4% of genes
283 in *S. stercoralis* and *S. venezuelensis*, respectively, into four categories based on when they

284 were expressed (FIG. 6B, Supplementary FIG. S6B): in free-living generations, throughout
285 adulthood, parasitic larval stage only, and throughout adult stage. Interestingly, the rest of
286 genes were assigned to expressed in parasitic larval + free living adult or parasitic larval +
287 parasitic adult in *S. venezuelensis* and *S. stercoralis*, respectively. Strikingly, the majority of
288 genes (87.0% in *S. stercoralis* and 82.4% in *S. venezuelensis*) were expressed in more than
289 one developmental phase, while over half (54.8%) of *Strongyloides* orthologues were
290 assigned to different developmental categories. Higher number of *Strongyloides*' genes were
291 expressed at multiple stages than *Caenorhabditis*, but similar levels of developmental
292 switching. The d_N/d_S ratios were calculated for each category, and none exhibited a
293 significantly higher ratio than genes that exhibited a different expression category (FIG. 6D).

294

295 Discussion

296 Very little is known about transcriptome conservation between nematodes beyond the
297 embryonic stage. In this study, we compared the developmental transcriptomes of eight
298 nematodes species with similar developmental stages. These species have many
299 morphological and developmental differences and a diversity of living environments,
300 lifespans, and host types. The transcriptomes used in this study came from datasets from
301 multiple sources, but all clustered into three broad stages (embryonic, larval, and adult)
302 across nematodes' entire life cycles. One major concern was that batch effects would lead to
303 systematic differences between datasets (Fei et al. 2018) that could not be separated from
304 species effects (Leek et al. 2010). Attempts to treat batch effects would also remove
305 biological signals from species. Despite this caveat, the same pattern was observed when we
306 incorporated transcriptomes of the same stage from multiple sources of *C. elegans*,
307 suggesting that the biological signals were robust enough to make up for the technical
308 variations across different studies. Another challenge presented in our study was that the
309 development stages were incomplete in some nematodes, which led to reduced resolution in
310 our analyses. Imperfect synchronization of worm cultures was observed during the staging
311 protocol in *P. pacificus*, and such issue may be applicable to all non-model species. In
312 addition, the definition of developmental stages beyond embryogenesis to adulthood in
313 nematodes was only loosely defined by moulting. Even in the model *Caenorhabditis*, we still
314 have concerns related to perfect synchronization of developmental stages. For instance, the

315 culturing environment may have been more stressful for one species than the other. Further
316 experimental work across all nematode genera is needed to characterize patterns of
317 conservation across their entire life cycles.

318 Cell-specific information is critical for deciphering how molecular mechanisms control
319 the phenotypes of multicellular animals. Large scale research on gene expression in
320 mammalian organ development suggests that organs become increasingly more distinct and
321 the breadth of gene expression gradually decreases during development (Cardoso-Moreira et
322 al. 2019). This evidence supports the theory of von Baer, that morphological differences
323 between species increase as development advances (Abzhanov 2013). So far, gene expression
324 has been determined in specific cell types of *C. elegans* by dissecting different worm tissues
325 (Spencer et al. 2011) and machine learning-based predictions (Kaletsky et al. 2018). Across
326 nematodes, we revealed that orthologues of oogenesis and hypodermis specific genes in *C.*
327 *elegans* also displayed a shared pattern of transcriptome proportions across developmental
328 stages. Identifying tissue-specific genes in other nematodes using data from *C. elegans* alone
329 will inevitably underestimate the proportions of tissue expression contributing to whole-
330 worm transcriptomes. We attempted to minimise such bias by normalising total gene
331 expression to only one-to-one orthologues. Although one-to-one orthology may capitulate a
332 subset of developmentally conserved genes, we have shown that they provided initial insights
333 into gene expressions during tissue development across nematodes.

334 We examined the stage clustering further by inferring the level of correlated
335 transcriptome evolution (LCE), a statistical method originally intended to quantify correlation
336 between tissue transcriptomes (Liang et al. 2018). Although the LCE estimation revealed
337 strong concerted evolution between individual larval stages, an alternative explanation may
338 be the imperfect synchronization of worm culture in non-model organisms. For instance, the
339 staging protocol in *P. pacificus* was observed in major developmental transcriptome
340 clustering with a mixture of early larvae, late larvae, and adult (Baskaran et al. 2015).
341 Nevertheless, this may not be the case, at least in the *Caenorhabditis* dataset, as the largest
342 proportion of expression profiles were non-adjacent developmental stages (FIG. 6A). At least
343 half of the genes in nematodes were expressed at multiple stages, and half of their
344 orthologues were found to be expressed at multiple other stages; this suggests that a change
345 in a gene's expression during development may rapidly lead to transcriptome divergence
346 after speciation.

347 Using *Strongyloides*, a unique model to investigate the parasitism based on the
348 fascinating features of both free-living and parasitic generation, allowed us to systematically
349 examine the differences between the same developmental stages in different life style
350 strategies. We found that the transcriptomes of *Strongyloides* can be categorized based on
351 developmental stage instead of lifestyle, which is in contrast to the observation that up to
352 20% of the genes are differentially expressed between parasitic and free-living females (Hunt
353 et al. 2018). The reason behind the two different observations is that that the majority of these
354 differentially expressed genes were duplicated in the *Strongyloides* lineage (Hunt et al. 2018),
355 and we focused on one-to-one orthologues. We have to a certain extent identified a theme of
356 developmental conservation across nematodes, and shown that the specialisation into
357 parasitic stages was the result of duplication events in gene families, as is evident in many
358 nematode genomes (Hunt et al. 2016; Coghlan et al. 2019). We speculate that altering the
359 expression of a gene to adapt to a new environmental niche may take place before genomic
360 innovation without reducing much fitness.

361 The third larval stage is thought to be a hot spot for obtaining infectious ability (Lee
362 2002). Gene expressions in the third larval stages were clustered in parasites, but not with all
363 corresponding stages, especially the free-living L3 and diapause ones. This suggests that the
364 similarities in transcriptomes among parasitic stages were not inherited from their common
365 ancestors but through convergent evolution of having similar selection pressures to tolerate
366 the host environment. The results of the expression divergence analysis show that genes tend
367 to be expressed multiple times over the course of the developmental process. We propose that
368 the life cycle of the nematode common ancestor consisted of an embryo stage and an adult
369 stage, with several larval stages in between. The specialised larval stages—such as the dauer,
370 filarial, and sheathed larva stages—may have independently evolved in response to biological
371 requirements over evolutionary time.

372 Our study adds to recent efforts to sequence and compare genomes across many
373 nematodes (Coghlan et al. 2019) by providing a first step towards revealing life cycle
374 conservation and convergence at the transcriptome level. The most striking pattern was
375 perhaps the finding that some patterns are conserved in species that diverged many millions
376 of years ago and have drastically different lifestyles. Our results also provide initial insights
377 into how ancestral life strategies such as parasitism evolved to become specialised. Future

378 large-scale synchronised experiments across life cycles as well as tissue specific or single cell
379 transcriptomes between nematodes may further elucidate life cycle evolution in nematodes.

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384 **Methods**

385 **RNA-seq mapping and normalization**

386 A description of locations where RNA sequencing (RNA-seq) reads were downloaded
387 is presented in Supplementary Table S1. RNA-seq reads were first trimmed using
388 Trimmomatic (v0.36; parameter: LEADING:5 TRAILING:5 SLIDINGWINDOW:3:15
389 MINLEN:34; Bolger et al. 2014) to remove the adaptor and leading, tailing, and low quality
390 sequences. Trimmed reads from each species were mapped to a corresponding genome
391 assembly downloaded from Wormbase (ver. WS269; Lee et al., 2018) using HISAT2 (ver.
392 2.2.1; Kim et al. 2015). Raw gene counts were assigned using featureCounts (v.1.6.3; Liao et
393 al. 2014). The raw counts of orthologous genes in all samples were transformed into TPM
394 (transcripts per million), and the median of replicates were calculated to represent the raw
395 gene expressions of developmental stages in each species. To normalise the data, we initially
396 removed the 25% lowest-expressed genes in each species using the sum of samples. Next, we
397 performed the ‘withinLaneNormalization’ function in EDASeq (v2.18.0) (Risso et al. 2011)
398 to remove GC bias for each gene, and transformed the expressions by log₂. Considering that
399 our data were collected from multiple studies, we accounted for study design batch effects
400 using the ‘ComBat’ function from the sva (v3.32.1) (Leek et al. 2016). In the case of species-
401 paired comparisons, both orthologues below the 25% expression category were removed for
402 further analyses. Pearson correlation coefficient of normalised transcriptomes in different
403 developmental stages within and between species were determined using the ‘corr’ function
404 in R (v3.6.0; R Core Development Team 2019). The heat maps of correlation matrices were
405 hierarchically clustered with the average agglomeration method.

406 **Phylogenetic and evolutionary analysis**

407 Orthology of proteomes from species investigated in this study was inferred using
408 OrthoFinder (v2.2.7; Emms and Kelly 2015). If multiple isoforms exist for a given gene, only
409 the longest or major isoform was chosen for analyses. A maximum likelihood phylogeny was
410 constructed by the concatenated amino acid alignments of 2,205 single copy orthologues
411 across eight nematodes using RAxML (v8.2.11; -m PROTGAMMAILGF -f -a; (Stamatakis
412 2014) with 500 bootstrap replicates. To calculate sequence-based metrics, sequences of single
413 copy orthologues were retrieved and aligned using TranslatorX (version 1.1; Abascal et al.
414 2010). We identified the synonymous (dS) and nonsynonymous (dN) substitution rates using
415 Codeml in PAML (v4.9; parameter: runmode=-2, seqtype=1, CodonFreq=3, fix_omega=0;
416 Yang 2007). The 209 oogenesis-enriched genes were defined by (Reinke et al. 2004). The
417 172 hypodermis-specific genes in *C. elegans* were defined by (Spencer et al. 2011). One-to-
418 one orthologues of these *C. elegans* genes in other species were retrieved. To deal with the
419 differences in orthologue numbers, expression levels of selected genes were normalised to
420 those of one-to-one orthologues with *C. elegans*, generating a relative proportion to represent
421 the expression of specific gene sets.

422

423 **Comparative transcriptomic analysis**

424 Levels of correlated evolution (LCE) between transcriptome datasets was calculated
425 according to Liang *et al.*, (<https://github.com/loverliang/LCE>). We applied the Z-score
426 angle-sorted value index for temporal sorting (ZAVIT) method (Levin et al. 2016) to
427 organize gene expressions across the developmental process. ZAVIT sorted the standardized
428 gene expressions by the relative order of the first two principal components. The standardized
429 expression profiles were obtained by subtracting the mean and dividing by the standard
430 deviation of the orthologous gene expression for all developmental stages. Principal
431 component analysis was performed to standardize gene expression profiles into ordered genes
432 in a circle, and the angle computed by an invert tangent from the origin represented the
433 temporal expression profiles during development. The first gene in the sorted standardized
434 gene expression profile plot was defined according to the sliding window below.

435 All developmental stages were first categorized into embryo, larva, and adult stages.
436 Additionally, free-living or parasitic stages were considered separated in the *Strongyloides*
437 comparison. This yielded three and four stages in the *Caenorhabditis* and *Strongyloides*

438 comparisons, respectively. Differential expression analyses between developmental stages
439 were performed using DESeq2 (v1.24.0, $\text{padj} < 0.05$; Love et al. 2014). 81.3-82.2% and 63.1-
440 70.2% of genes were first unambiguously sorted into seven and nine expression categories in
441 the *Caenorhabditis* and *Strongyloides* comparisons, respectively, based solely on DESeq2
442 results. These results were used to further split the ZAVIT-sorted genes into five major
443 expression categories. The boundaries were set by choosing the 5% and 10% quantile of the
444 DESeq2 categories in the *Caenorhabditis* and *Strongyloides* comparisons, respectively. We
445 were interested in the relative order of each gene's position within the transcriptome, and this
446 approach allowed for the placement of certain genes—e.g., the housekeeping genes, which
447 were expressed throughout development, were sorted relative to the rest of the transcriptome.
448 The starting position of the parasitic larva + parasitic adult expression category in *S.*
449 *stercoralis* and *S. venezuelensis* were determined solely by the 95% quantile of the adjacent
450 free-living adult + parasitic adult DEseq category, as genes positioned around this region tend
451 to exhibit no significantly different expressions in DEseq2 analyses. Gene Ontology
452 enrichment was performed using topGO (v2.36.0; Alexa and Rahnenfuhrer 2019) with GO
453 annotations downloaded from WormBase.

454

455 **Figure legends**

456

457 **FIG. 1. Nematode phylogeny and life cycles.**

458 To the left are the phylogenetic relationships among eight nematodes. The abbreviation for
459 each species is shown after the species name, and is used throughout the study. To the right
460 are the life cycles for each species. Different colours correspond to the different nematode
461 lifestyles. The available transcriptome data used in this study are denoted with yellow stripes.
462 Nematode host types are labelled above their life cycles. In total, three free-living, four
463 animal parasitic, and one plant parasitic nematodes were included in this study. (*¹ L3 plus:
464 Infectious third-stage larva (isolated from host), *² PFemale: Parasitic female, *³ PP: Post
465 parasitic, *⁴ PFL: Post free-living, *⁵ L3i: Infectious third-stage larva (isolated from
466 environment), *⁶ FLFemale: Free-living female, *⁷ D3: Dispersal third-stage juvenile (D3),
467 *⁸ D4: Dispersal forth-stage juvenile).

468

469 **FIG 2. Transcriptome correlation among nematode developmental stages.**

470 Results of hierarchical clustering of Pearson correlations at different stages in single species.
471 The number of genes included in the analysis for each species are as follows: *C. elegans*
472 15,156; *C. briggsae* 16,606; *P. pacificus* 19,137; *S. stercoralis* 9,823; *S. venezuelensis* 12,678;
473 *B. xylophilus* 12,259; *B. malayi* 8,899; *H. contortus* 14,572.

474

475 **FIG. 3. Correlation clustering across nematodes.**

476 Hierarchical clustering of Pearson correlation of different stages between (A.) two
477 *Caenorhabditis* species, (B.) two *Strongyloides* species and (C) *B. xylophilus* and *B. malayi*.
478 6,736, 5,394 and 3,109 one to one orthologues were included in the analysis, respectively.
479 Parasitic larva of *Strongyloides* and the other larva stages were labelled separately.

480

481 **FIG. 4. Gene expression in specific gene sets.**

482 Proportion of gene expression specifically expressed in (A) hypodermis or (B) during
483 oogenesis compared to the rest of transcriptome. The relative expression level on the y-axis
484 was calculated from the proportion of subset gene expressions in all of the one-to-one
485 orthologues' expression. The stages across nematodes were assigned to five developmental
486 categories: embryo (green), early larva (yellow), late larva (red), adult (blue), and other(grey).
487 Stages in the 'other' group were species-specific stages and excluded in the following
488 analysis. The upper-right figures showed the proportional differences among four different
489 developmental categories in (C) hypodermis or (D) during oogenesis. Wilcoxon rank sum test
490 was performed between each category (p value * < 0.05, ** < 0.01, *** < 0.001).

491

492 **FIG. 5. Estimates of levels of correlated evolution (LCE)**

493 (A.) LCE between *C. elegans* and *C. briggsae* in 7 developmental stages. (B.) LCE between
494 *S. stercoralis* and *S. venezuelensis*. Free-living L3 (PPL3) and L1 (PFL1) in *S. stercoralis*
495 were excluded. The PPL1 stage in *S. stercoralis* was assigned as the L1 stage in the
496 comparison because it has the same post parasitic features as the L1 stage in *S. venezuelensis*.
497 (C.) LCE among all the species. The parasitic and free-living generations in *Strongyloides*
498 were separated to compare them to developmental stages in other species. Stage comparisons
499 with fewer than three species pairs were excluded. The lowest LCE value with theoretical p-
500 value < 0.05 was 0.235. Colours indicate the different categories of stage comparison. All
501 larval stages were classified into larva categories to test the level of co-evolution.

502

503 **FIG. 6. Expression profiles and sequence divergences during development in species of**
504 ***Caenorhabditis* and *Strongyloides*.**

505 Expression profiles of (A.) *C. elegans* and *C. briggsae*; and (B) *S. stercoralis* and *S.*
506 *venezuelensis*. Expression profiles were categorized into five developmental categories in

507 each species. Sequence divergence of different expression categories in (C.) *C. elegans* and *C.*
508 *briggsae*; (D.) *S. stercoralis* and *S. venezuelensis*. The five boxes underneath include the
509 genes with the same expression profiles in both species. Wilcoxon test was performed to test
510 the difference in dN/dS between categories. Asterisks were used to represent the p-value (* <
511 0.05, ** < 0.01, *** < 0.001). Comparisons with p-value > 0.05 were not labelled.

512

513

514 **Authors contribution**

515 I.J.T conceived the study. M.R.L carried out the majority of analysis with help from C.K.L
516 and B.Y.L. M.R.L and I.J.T wrote the manuscript.

517

518 **Competing interests**

519 The authors declare no competing interests.

520

521

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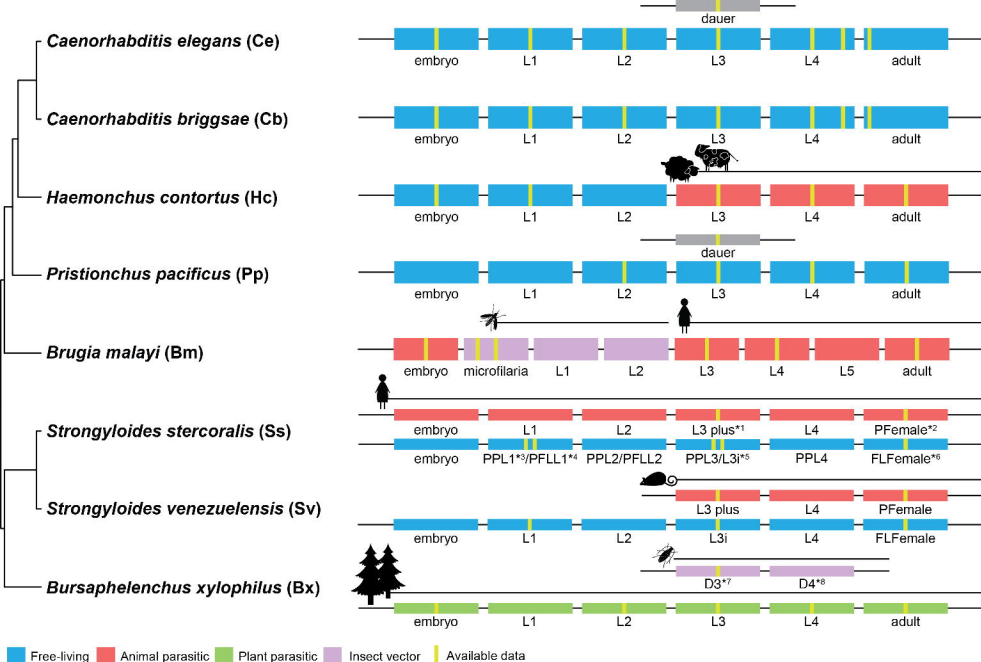
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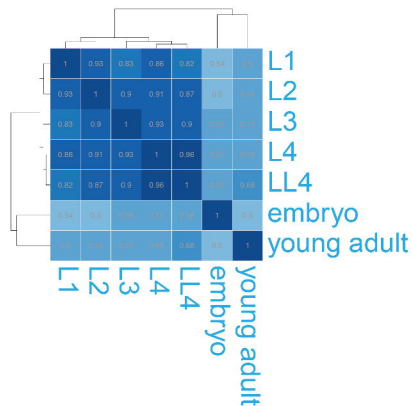
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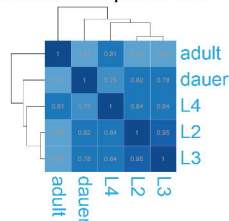
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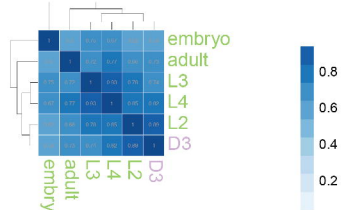
A. *Caenorhabditis elegans*



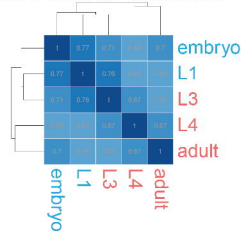
B. *Pristionchus pacificus*



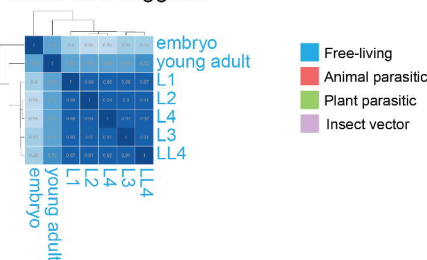
C. *Bursaphelenchus xylophilus*



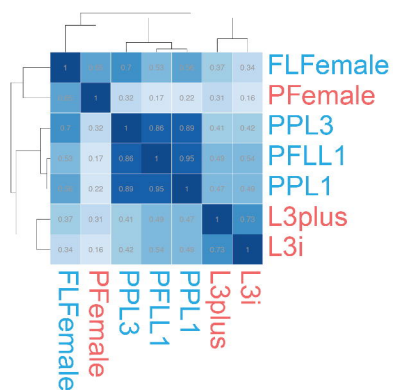
D. *Haemonchus contortus*



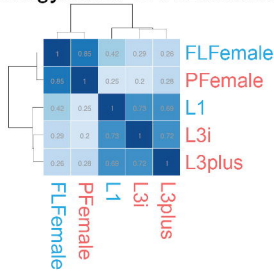
E. *Caenorhabditis briggsae*



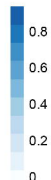
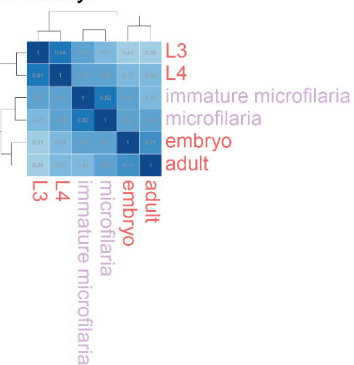
F. *Strongyloides stercoralis*



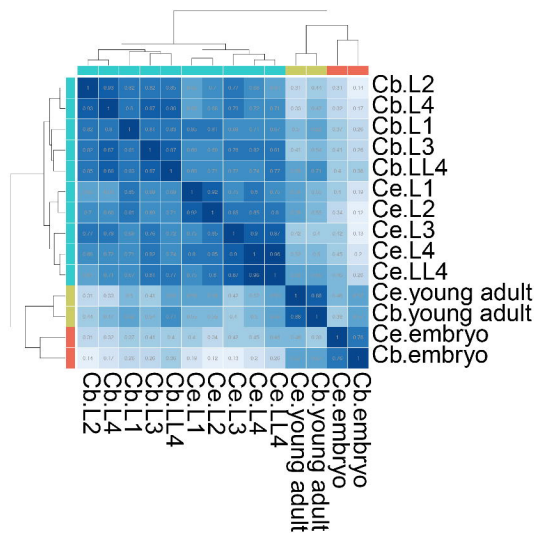
G. *Strongyloides venezuelensis*



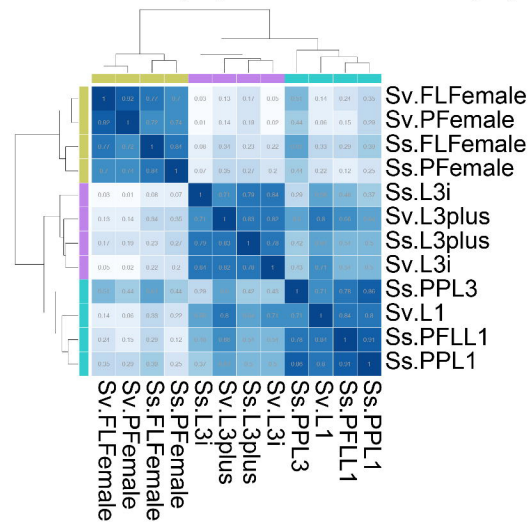
H. *Brugia malayi*



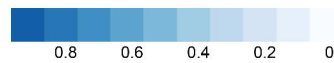
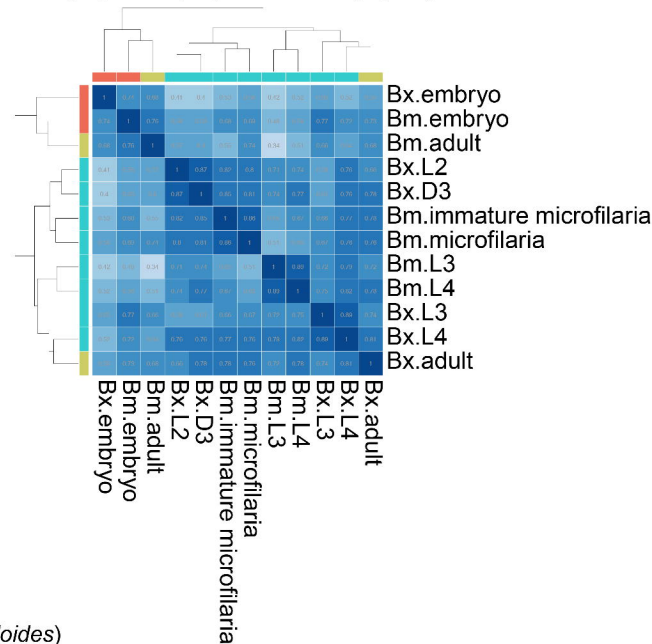
A. *C. elegans* (Ce) vs. *C. briggsae* (Cb)



B. *S. stercoralis* (Ss) vs. *S. venezuelensis* (Sv)



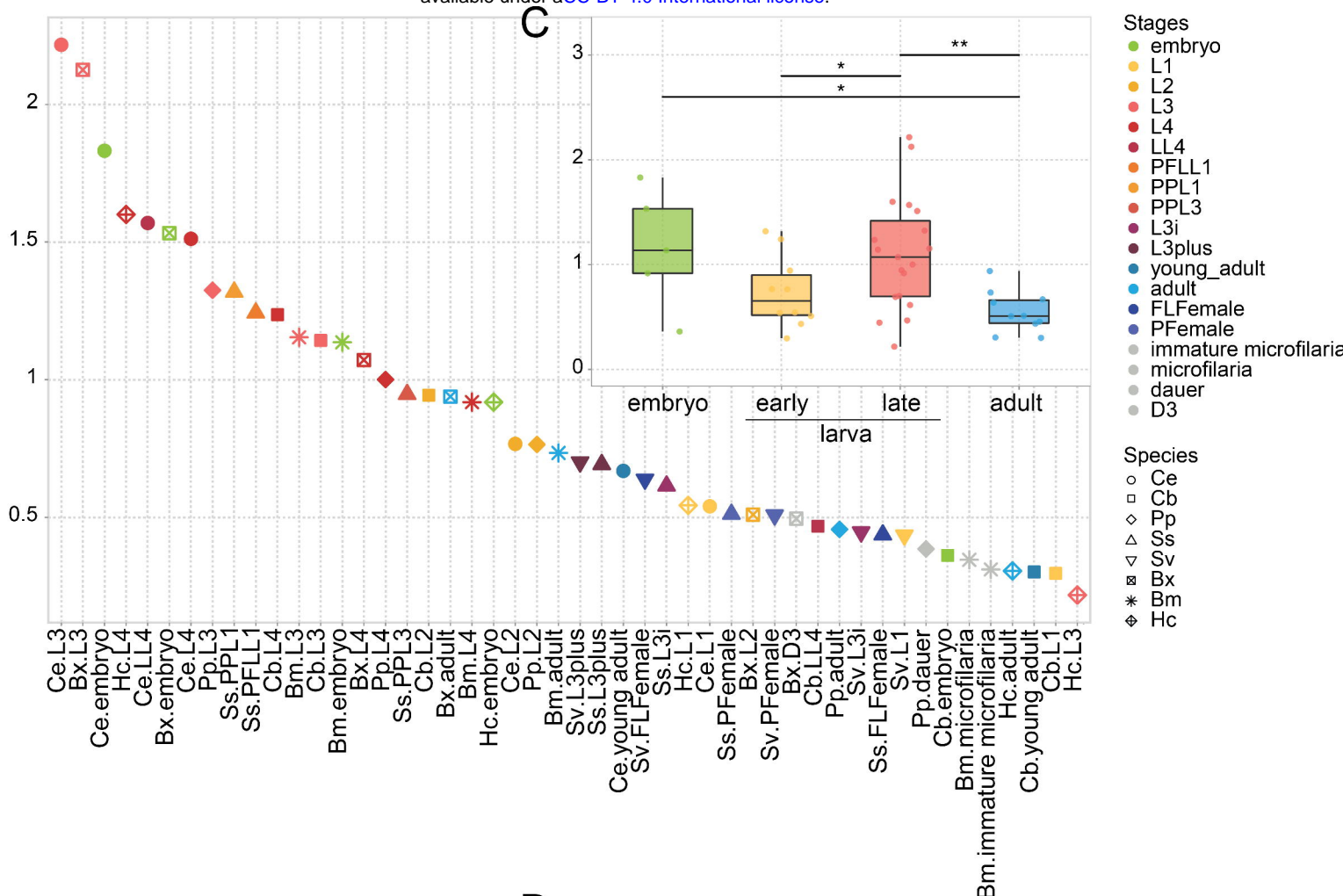
C. *B. xylophilus* (Bx) vs. *B. malayi* (Bm)



Adult Embryo Free-living larva Parasitic larva (*Strongyloides*)

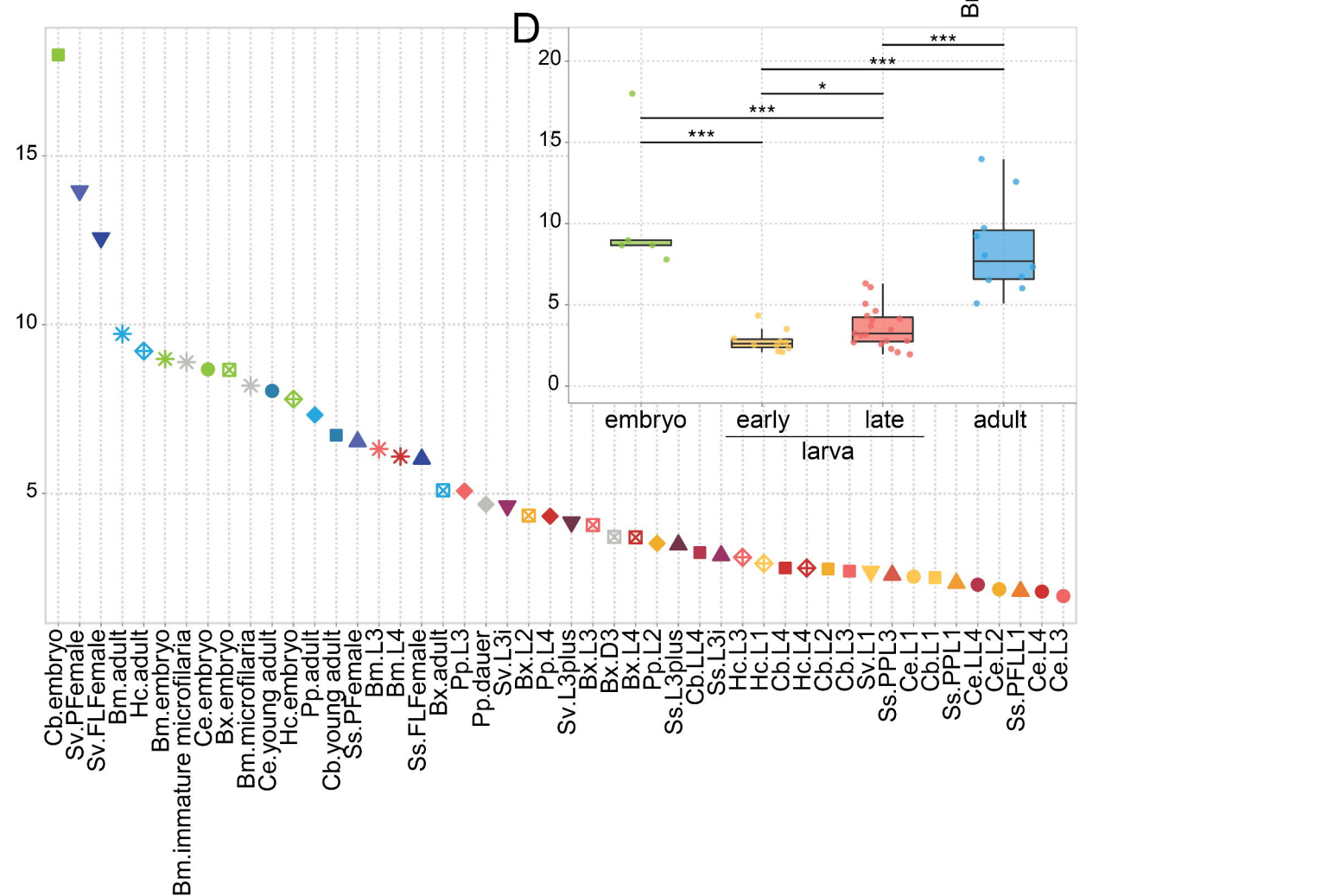
A

Hypodermis (expression percentage)



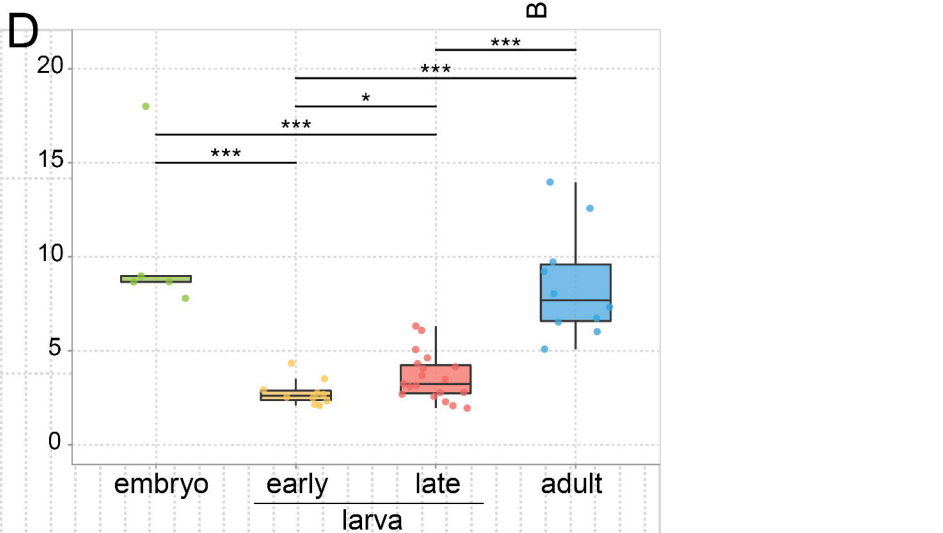
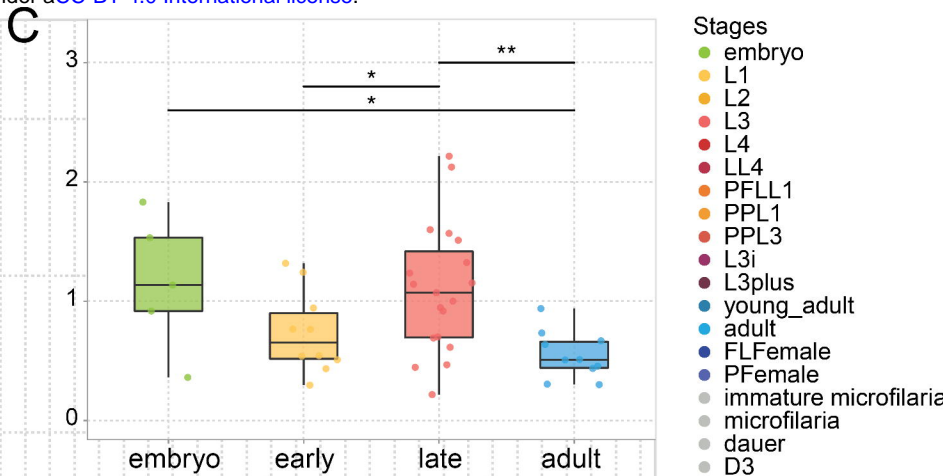
B

Oogenesis (expression percentage)

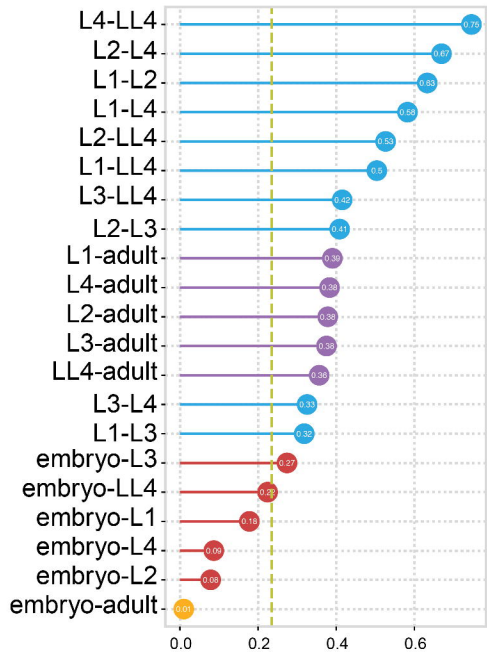


C

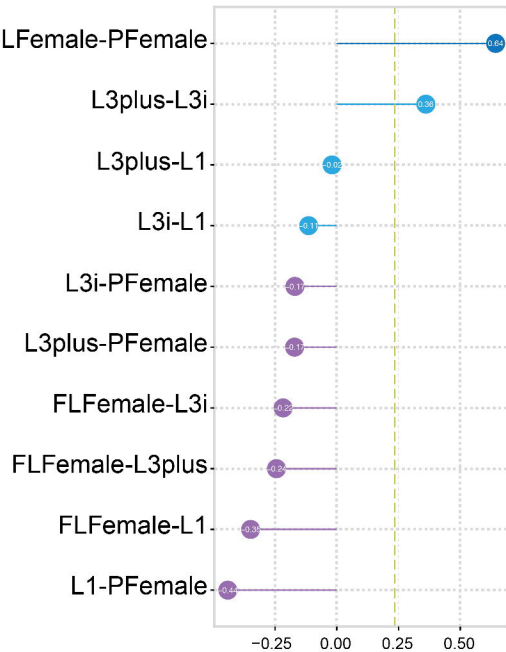
D



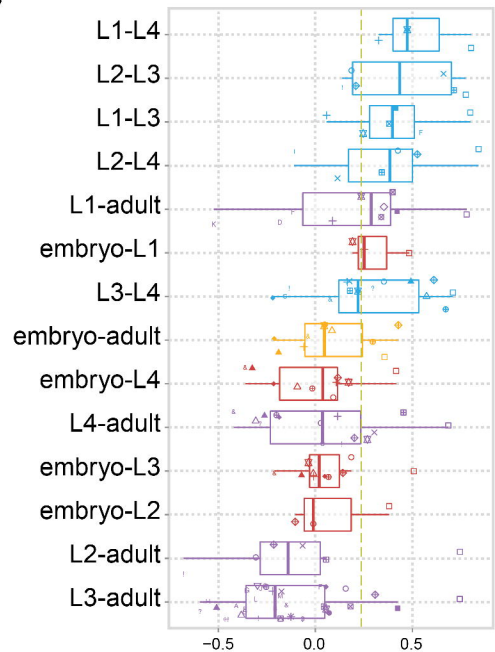
A



B



C



● embryo-larva ● embryo-adult ● larva-larva ● larva-adult ● adult-adult

