Gene expression in *Tribolium castaneum* life stages: Identifying a species-specific target for pest control applications

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The red flour beetle, *Tribolium castaneum*, is a major agricultural pest of post-harvest products and stored grain. Control of *T. castaneum* in stored products and grain is primarily by fumigants and sprays, but insecticide resistance is a major problem, and new control strategies are needed. *T. castaneum* is a genetic model for coleopterans, and the reference genome can be used for discovery of candidate gene targets for molecular-based control, such as RNA interference. Gene targets need to be pest specific, and ideally, they are expressed at low levels for successful control. Therefore, we sequenced the transcriptome of four major life stages of *T. castaneum*, sorted data into groups based on high or low expression levels, and compared relative gene expression among all life stages. We narrowed our candidate gene list to a cuticle protein gene (CPG) for further analysis. We found that the CPG sequence was unique to *T. castaneum* and expressed only in the larval stage. RNA interference targeting CPG in newly-emerged larvae caused a significant (p<0.05) decrease in CPG expression (1,491-fold) compared to control larvae and 64% mortality over 18 d. RNA-Seq of survivors after 18 d identified changes in the expression of other genes as well, including 52 long noncoding RNAs. Expression of three additional cuticle protein genes were increased and two chitinase genes were decreased in response to injection of CPG dsRNA. The data demonstrate that RNA-Seq can identify genes important for insect survival and thus may be used to develop novel biologically-based insect control products.

1 **Gene expression in *Tribolium castaneum* life stages: Identifying a species-specific target for**

2 **pest control applications**

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19 **Abstract**

20 The red flour beetle, *Tribolium castaneum*, is a major agricultural pest of post-harvest products

21 and stored grain. Control of *T. castaneum* in stored products and grain is primarily by fumigants

22 and sprays, but insecticide resistance is a major problem, and new control strategies are needed.

23 *T. castaneum* is a genetic model for coleopterans, and the reference genome can be used for

24 discovery of candidate gene targets for molecular-based control, such as RNA interference.

25 Gene targets need to be pest specific, and ideally, they are expressed at low levels for successful

26 control. Therefore, we sequenced the transcriptome of four major life stages of *T. castaneum*,

27 sorted data into groups based on high or low expression levels, and compared relative gene

28 expression among all life stages. We narrowed our candidate gene list to a cuticle protein gene

29 (CPG) for further analysis. We found that the CPG sequence was unique to *T. castaneum* and

30 expressed only in the larval stage. RNA interference targeting CPG in newly-emerged larvae

31 caused a significant ($p < 0.05$) decrease in CPG expression (1,491-fold) compared to control

32 larvae and 64% mortality over 18 d. RNA-Seq of survivors after 18 d identified changes in the

33 expression of other genes as well, including 52 long noncoding RNAs. Injection of CPG dsRNA

34 also resulted in increased expression of three additional cuticle protein genes and reduced

35 expression of two chitinase genes in *T. castaneum* larvae. The data demonstrate that RNA-Seq

36 can identify genes important for insect survival and thus may be used to develop novel

37 biologically-based insect control products.

40 **Introduction**

41 The red flour beetle, *Tribolium castaneum,* is a pest of stored grain commodities. Traditional

42 control methods for *T. castaneum* and other stored product beetles are rapidly becoming less

43 effective, mostly because insect populations are developing resistance to pesticide treatments

44 (Boyer *et al.* 2012). For example, storage pests around the globe are developing high resistance

45 levels to one of the most common grain fumigants, phosphine (Opit *et al.*, 2012; Pimentel *et al.*,

46 2010). Thus, there is a need for new pest control strategies, and we are evaluating genetic-based

47 treatments with target specificity and less damage to the environment, including the application

48 of RNA interference (RNAi; Baum *et al.*, 2007; Noh *et al.*, 2012).

49

50 *T. castaneum* is a useful model to identify candidate genes because it has a sequenced genome

51 (*Tribolium* Genome Sequencing Consortium, 2008) and responds well to RNAi when dsRNA is

52 injected (RNAi; Brown *et al.*, 1999; Tomoyasu & Denell, 2004; Aronstein *et al.*, 2011; Miller *et*

53 *al.*, 2012). Oral RNAi was successful in *T. castaneum* larvae against genes encoding vATPase

54 (Whyard *et al.*, 2009), inhibitor of apoptosis (Cao et al., 2018), and a voltage-gated sodium ion

55 channel (Haim et al., 2016). However, we and others have not had success with oral RNAi in *T.*

56 *castaneum* (unpublished data, Palli, 2014). Many factors may influence RNAi efficacy in

57 insects, such as target sequence specificity, concentration and length of dsRNA, persistence of

58 silencing effect in the target pest, and nucleases counteracting the effect of dsRNA (Huvenne &

59 Smagghe 2010; Allen & Walker, 2012; Lomate & Bonning, 2016; Guan *et al.*, 2018, Cao et al.,

60 2018). In the meantime, we have focused on the identification of gene targets with low

61 expression requiring lower doses of dsRNA, and those that are expressed in critical feeding

62 stages (larvae and adults) to improve the efficacy of oral RNAi.

63

64   The iBeetle project (http://ibeetle-base.uni-goettingen.de) conducted a large-scale RNAi screen

65   in *T. castaneum* larvae and pupae with injected dsRNA, and various phenotypes were observed,

66   including mortality and developmental abnormalities (Schmitt-Engel *et al.*, 2015). From this

67   screen, eleven genes were identified as potential pest control targets (Donitz *et al.*, 2015; Ulrich

68   *et al.*, 2015). These genes encode mostly products with GO terms related to the proteasome, and

69   mortality was observed after injection of larvae, pupae and adults. Some of these genes have

70   orthologs in other species, such as *Drosophila melanogaster, Aedes aegypti, Apis mellifera,* and

71   *Acyrthosiphon pisum*, and therefore RNAi or other molecular strategies based on these genes

72   may not be species-specific and may not be practical for insect control. Knorr et al. (2018)

73   selected 50 genes from the iBeetle study that resulted in ≥ 90% mortality to evaluate for RNAi in

74   *Diabrotica vigifera vigifera* and found that feeding adults dsRNA targeting 20 genes resulted in

75   mortality, and 36 retarded growth. While this work is important to highlight genes that have

76   application across species, the usefulness in the field may be limited due to off-target effects.

77

78   Previously, we applied RNA-Seq to the major developmental stages of *T. castaneum* to identify

79   highly expressed cysteine peptidase genes in feeding stages (larvae and adults) with likely

80   digestive functions (Perkin *et al.*, 2016). A similar approach was used across the life stages of *D.*

81   *melanogaster* to reveal new transcribed regions and small non-coding RNAs, demonstrating that

82   transcriptome sequencing can discover new elements and help to annotate genomes of even the

83   most well studied insects (Graveley *et al.*, 2011). Transcriptome sequencing of developmental

84   stages of the non-model oriental fruit fly, *Bactocera dorsalis*, and the harlequin bug, *Murgantia*

85   *histrionica,* led to a list of candidate developmental and insecticide resistance genes for

86   downstream studies (Shen *et al.*, 2011; Sparks *et al.*, 2017).

87

88   In the present study, we examined gene expression patterns in *T. castaneum* life stages (adults,

89   eggs, larvae, and pupae), and used statistical analysis to sort expression patterns into groups of

90   low and high expression. Our hypothesis was that genes with low expression can be more

91   successfully targeted with RNAi or other applied control methods because the dose of dsRNA

92   required to knockdown transcript expression will be lower, and highly expressed genes and gene

93   expression patterns may be useful in understanding the physiology of the insect, or to identify

94   promoters for transgenic applications. Previously, we found that some genes with high

95   expression (i.e. those encoding cysteine peptidases) were important to insect physiology and

96   were duplicated in the genome, and complete knockdown of these genes induced a compensation

97   response and upregulation of other closely related genes (Perkin et al., 2016). Through screening

98   of potential targets in the present study, we identified a unique cuticle protein gene (CPG)

99   expressed in larvae, and we provide functional evidence for its potential as a molecular target for

100   pest control. We further evaluated the effect of CPG RNAi on the expression of other genes,

101   defining new potential metabolic connections between CPG and other genes.

102

103   **Materials & Methods**

104   *Insects*

105   Insect strains and collection protocols were as previously described (Perkin *et al.*, 2016).

106   Briefly, the *T. castaneum* lab colony used in this study was originally collected from a grain bin

107   in Kansas and has been maintained on 95% wheat flour and 5% brewer's yeast at 28°C, 75%

108 R.H., 0L:24D. Insects were subcultured from the laboratory colony, and specific life stages were

109 removed. Adults were collected at 3-7 days post-eclosion and were mixed sex. Eggs were sifted

110 from diet 24-48 hours after oviposition and were separated from the fine particulate with a brush.

111 Larvae were collected at a late instar stage that was actively feeding (approximately 14 days post

112 hatch). Pupae included those with pigmentation in the eye, but not the elytra.

113

114 *Library preparation and RNA sequencing of life stages*

115 RNA was collected as three independent biological replicates from mixed sex groups of each life

116 stage (10 adults, approximately 500 eggs, 10 larvae, and 10 pupae per replicate). Tissue was

117 pulverized in TRIZOL (BulletBlender, Next Advance Inc., Averill Park, NY, USA) at speed 8

118 for 2 min with RNAse-free ziroconium oxide beads. RNA extraction and purification were with

119 a Zymo mini prep kit (Irvine, CA, USA). From the total RNA, DIRECTbeads (Agilient, Santa

120 Clara, CA, USA) were used to isolate polyA mRNA, and libraries were made with a 200 bp

121 RNA-Seq v2 kit (Life Technologies, Grand Island, NY, USA). Samples were sequenced on

122 318v2 chips on the Ion Torrent Personal Genome Machine (PGM, Life Technologies). Each run

123 provided approximately 1-5 million reads, with a total of 5-12 million reads per life stage (Perkin

124 *et al.*, 2016). Life stage sequences were deposited at NCBI SRA as part of BioProject

125 PRJNA299695.

126

127 *Data analysis*

128 Relative gene expression was analyzed using ArrayStar (Lasergene Genomics Suite v14,

129 DNASTAR, Madison, WI, USA) by mapping reads to the NCBI Tcas5.2 genome build. Read

130 counts were normalized by Reads Per Kilobase of template per Million mapped reads (RPKM,

131   Mortazavi *et al.*, 2008). All data were filtered to groups with low expression (RPKM greater

132   than 2 less than 8 log$_2$; i.e., the log$_2$ of the RPKM value was between 2 and 8, not inclusive) and

133   high expression (RPKM greater than 8 log$_2$) for comparisons and biological screening. We use a

134   cutoff of RPKM greater than 2 to avoid underrepresented genes that can skew data.

135

136   Gene ontology (GO) terms were obtained and enrichment of GO terms was analyzed in

137   BLAST2GO PRO (version 4.1.5, Valencia, Spain, Götz *et al.*, 2008). Over-representation tests

138   were performed with GO terms associated with genes in low and high categories in each life

139   stage, using the program g:Profiler and the g:GOSt function, which provides statistical

140   enrichment analysis (http://biit.cs.ut.ee/gprofiler/index.cgi; (Reimand *et al.*, 2011). In g:Profiler,

141   results were limited to *T. castaneum* and the main GO categories: biological process (BP),

142   molecular function (MF) and cellular component (CC). Significance was determined using the

143   False Discovery Rate (FDR) multiple-testing correction threshold of 0.05 (Benjamini and

144   Hochberg 1995). Enriched GO terms also were obtained from the pairwise comparison of gene

145   expression in larvae injected with cuticle protein gene (CPG, LOC103313766) dsRNA compared

146   to mock-injected, where the test set was the significant (p<0.05) differentially expressed genes,

147   and the reference set was expression from all genes. GO terms were limited to p<0.05 (after

148   FDR correction) and collapsed to the most specific description of each term.

149

150   *Candidate gene selection*

151   Genes were first filtered to only include those with expression levels between RPKM 2 and 8

152   (log$_2$). The resulting gene list included 181 genes. We then filtered to only include genes

153   expressed at low levels in larvae because a field application would rely on genes expressed

154 during the most active feeding stage, reducing the list to 139 genes. Our next filter was a

155 manually curated gene set and was based on GO analysis and gene annotations, selecting genes

156 with a function potentially necessary for survival.

157

158 The final filter submitted potential genes to the program OfftargetFinder (Good *et al.*, 2016).

159 This program checks gene specificity by searching 101 arthropod transcriptomes, 21mers at a

160 time, to the 1000 Insect Transcriptome Evolution (1KITE) dataset (http://www.1kite.org) and is

161 specifically designed to identify potential problems in designing dsRNA for insect control. The

162 algorithm provides visualization of specific regions of the target gene that may cause off-target

163 effects and allows for dsRNA to be designed to regions with increased specificity to the target

164 pest. This filter limited our gene list to only three genes. We selected CPG as our primary target

165 because the 3' region of CPG was unique to *T. castaneum*, and this region was used to design

166 primers for dsRNA.

167

168 *RNAi*

169 Primers for dsRNA targeting CPG were designed similar to Perkin *et al.* (2017a) via Primer-

170 BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using default parameters, and primer

171 specificity was evaluated against the *T. castaneum* genome. A primary set of primers was

172 designed to amplify CPG, and secondary primers were designed to amplify the 3' region to

173 minimize off-target effects..

174

175 The primary set of primers (Forward: ATAATCAAGCCCGTTTCCAACA, Reverse:

176 AATCACGACTACAAACATTCTTAGG) amplified CPG in 25 µl PCR reactions using

177   genomic DNA template from the lab strain (2.5 µl 10X buffer, 2.0 µl 2.5 mM dNTP, 0.5 µl 10

178   µM forward primer, 0.5 µl 10 µM reverse primer, 0.125 µl JumpStart AccuTaq DNA

179   polymerase (Sigma-Aldrich, Saint Louis, MO, USA), 2 µl genomic DNA, 17.375 µl nuclease-

180   free water) and thermal cycle conditions as specified by the JumpStart AccuTaq DNA

181   polymerase product information guide (denaturation for 30 sec at 95°C, 30 cycles of 95°C for 30

182   sec, primer annealing at T_m 55.5°C for 30 sec, and extension at 68°C for 30 sec, final extension

183   at 68°C for 5 min, and hold at 4°C). The product was assessed on a 1% agarose E-gel

184   (ThermoFisher, Waltham, MA, USA) to ensure the amplified region was the correct size.

185

186   A second round of PCR was done with the secondary set of primers (Forward:

187   TATTCGTCTGTCGTCGCTCC, Reverse: AATCACGACTACAAACATTCTTAGG) for

188   specific targeting of CPG. The secondary PCR reaction was 100 µl and the product from the

189   primary PCR reaction was used as the template (10 µl 10X buffer, 8 µl 2.5 mM dNTP, 2 µl T7

190   forward primer, 2 µl T7 reverse primer, 0.5 µl JumpStart AccuTaq DNA polymerase, 10 µl

191   primary PCR product, 67.5 µl nuclease-free water). The thermal reaction profile was as follows:

192   denaturation for 30 sec at 95°C, 5 cycles of 95°C for 30 sec, primer T_m for 30 sec, 72°C for 30

193   sec, 29 cycles of 95°C for 30 sec, primer T_m plus 5°C for 30 sec, and 72°C for 30 sec, extension

194   for 5 min at 68°C and hold at 4°C. The secondary PCR primers had a T7 construct attached to

195   the 5' end (TAATACGACTCACTATAGGG), and thus 5°C was added to the second round of

196   amplification as suggested in the kit manual. The secondary PCR products were checked again

197   via 1% agarose E-gel for appropriate length (150 bp) and sufficient amplification.

198

199 PCR products from the secondary amplification were used to make dsRNA via MEGAscript T7

200 kit (ThermoFisher) according to the kit protocol (8 µl dNTP mix, 2 µl 10X reaction buffer, 8 µl

201 secondary PCR products with T7 construct, 2 µl enzyme mix), and after a 6 h incubation period

202 at 37°C with constant mixing, products were purified via MEGAclear kit (ThermoFisher). Size

203 and quantity were verified on a digital nanophotometer (Implen, Westlake Village, CA, USA)

204 and TapeStation (Agilent).

205

206 *Micro-injected dsRNA*

207 Immediately before injection, dsRNA solution was mixed with blue dye (1:20) to aid in

208 visualization of the injected liquid as in Perkin *et al.* (2017a). Larvae used in injections were

209 briefly put on ice, transferred to a microscope slide with double-sided tape for stabilization, and

210 the entire slide was placed on top of a small ice block. Thirty individuals were injected with 200

211 ng of dsRNA in triplicate. CPG-dsRNA was injected into larvae weighing approximately 1 mg.

212 Each treatment injection had a corresponding control injection of dye only (Mock) and a non-

213 injection control (Control). Injections were with a Drummond Nanoject (Drummond Scientific

214 Co., Broomall, PA, USA) set at 69 nl and fast injecting speed with a "bee-stinger" needle.

215 Needles were made with 3.5 Drummond glass capillary tubes (3-000-203-G) and a micropipette

216 puller (Sutter Instrument Co. Model P-97, Novato, CA, USA) using the program: heat 750, pull

217 100, velocity 8, time 250, and pressure 500. After injection, each group was allowed to recover

218 for 2 h at room temperature, and then were covered with diet (95% wheat flour, 5% brewer's

219 yeast) and kept at 28°C, 75% R.H., 0L:24D. All treatments and controls were followed for 18

220 days to monitor mortality and/or developmental abnormalities.

221

222 *Library preparation and RNA sequencing and data analysis after RNAi*

223 We validated knockdown of CPG with RNA-Seq as previously (Perkin et al., 2017b). Eight

224 individuals from each treatment and control were flash frozen in liquid nitrogen on day 18 post

225 injection. Total RNA was extracted by Trizol (Thermo Fisher Scientific, Waltham, MA USA)

226 and Quick-RNA Mini Prep kit (Zymo Research, Irvine, CA USA). Libraries were made on the

227 NeoPrep (200 bp insert) and sequenced on a Mi-Seq (2x300, Illumina, San Diego, CA USA).

228 Three independent biological replicates of all groups and 3-4 technical replicates of CPG

229 dsRNA-injected larvae were sequenced (metrics are found in Supplemental File 5). Sequence

230 reads were deposited at NCBI SRA as PRJNA520884.

231 Arraystar (DNAStar, Madison, WI USA) was used to calculate RPKM and fold change between

232 treatment and control groups by mapping reads to the Tcas5.2 genome. We also used Arraystar

233 for statistical analysis, student t-tests between treatments and ANOVA among treatments. Genes

234 were filtered to only include those that had >8-fold difference between treatments, and

235 significant at p<0.01 after FDR correction. To classify ncRNA, sequences were submitted to

236 RNAcon (website: http://crdd.osdd.net/raghava/rnacon/submit.html) for prediction using SVM

237 scores (default of 0.0; Panwar *et al*., 2014).

238

239 *Cuticle protein tree*

240 NCBI RefseqRNA was searched with term "cuticle protein" and limited to *T. castaneum*, which

241 returned a list of 160 genes which we then manually curated to only include relevant genes with

242 a chitin domain (pfam00379). This resulted in a total of 125 sequences in the final data set.

243 Protein sequences from the selected genes were used as input in MEGA X (version 10.0.5;

244 Kumar et al., 2018). Protein sequences were aligned using ClustalW. The evolutionary history

245   of cuticle proteins was inferred using the Maximum Likelihood method, JTT matrix-based

246   model, and 500 bootstrap iterations (Jones et al., 1992), using the tree with the highest log

247   likelihood.

248

249   **Results**

250   In the following sections, transcriptome datasets of gene expression in four developmental stages

251   of *T. castaneum* were analyzed by sorting genes into categories of high and low expression

252   levels. Gene Ontology (GO) terms were associated with each group, and overrepresented terms

253   were identified by enrichment analysis. A cuticle protein gene was selected as a gene expressed

254   at low levels only in larvae and was evaluated via RNAi as a gene with critical function for

255   insect survival.

256

257   *Life stage expression analysis – genes expressed at high levels*

258   Analysis of *T. castaneum* genes with high expression levels (RPKM >8, log$_2$) in all life stages

259   indicated there were 101, 290, 238, and 54 genes uniquely expressed in adults, eggs, larvae, and

260   pupae, respectively (Fig. 1, Supplemental File 1). Eggs and larvae stages shared the most

261   commonly expressed genes (162), followed by adults and larvae (48), and adults and eggs (29).

262   Genes common to eggs and larvae included 44 ribosomal proteins (large and small subunits),

263   indicative of the high degree of protein production occurring in these stages. Adults and larvae

264   feeding stages shared two digestive cathepsin L genes (LOC659441 and LOC659502; Martynov

265   *et al.*, 2015; Perkin *et al.*, 2016) and two chitinase precursor genes (*Cht8* and *Cht11*). Only

266   LOC663906 encoding a single-stranded DNA-binding protein was commonly expressed in eggs

267   and pupae. Twelve genes were highly expressed in all stages, including those encoding two

268   elongation factors, three ribosomal proteins, a cathepsin L (*26-29-p*), glyceraldehyde-3-

269   phosphase dehydrogenase 2*, NADH dehydrogenase subunit 2, ATP synthase, cytochrome c and

270   cytochrome b, and I-rRNA (Table 1).

271

272   In the highly-expressed gene group, the top Molecular Function (MF) GO terms for each life

273   stage varied, but adults and larvae had shared term "nucleotide binding", whereas eggs and

274   larvae shared "hydrolase activity" (Fig. 2). "Oxidoreductase activity" was common to larvae,

275   pupae and adults. An enrichment analysis found 16 MF terms belonging to "structural

276   constituent of the ribosome" ($p=1.07 \times 10^{-11}$) and 62 with "catalytic activity" ($p=0.028$) enriched

277   only in adults.

278

279   We also examined the most highly expressed genes in each developmental stage. The top 10

280   most highly expressed genes in adults included those encoding an odorant binding protein,

281   protamine, and cytochrome c oxidase subunit II (RPKM 7,694-20,300), and the highest

282   expressed genes in eggs had similar function, including chemosensory protein 2, I-rRNA and

283   cytochrome c oxidase subunit I and II (RPKM 7,960-26,611; Supplemental File 1). These genes

284   also were highly expressed in larvae, along with cathepsin L genes involved in digestion of

285   cereal proteins (RPKM 13,248-43,202; Martynov *et al.*, 2015; Perkin *et al.*, 2016). Highly

286   expressed genes in pupae encoded two cuticular proteins and a conserved cathepsin L, 26-29-p

287   (RPKM 3,576-13,818).

288

289   *Life stage expression analysis – genes expressed at low levels*

290   A comparison of genes with low expression levels (RPKM between 2-8 $\log_2$) revealed

291   expression of 273 genes unique to adults, 167 to eggs, 139 to larvae, and 14 to pupae (Fig. 3;

292   Supplemental File 2). Adults and larvae shared 138 genes including chitin deacetylase 7 (*Cda7*)

293   and two chitinase precursors (*Cht4* and *Cht9*). Larvae and pupae shared a single gene,

294   LOC664054 encoding pathogenesis-related protein 5, which also was expressed in adults but at

295   higher levels and thus was not part of the low expression group. Nine genes expressed at low

296   levels were common across all life stages, four of which had uncharacterized functions (Table 1).

297   The other five included genes encoding maternal protein tudor (*Tctud*), poly(A) polymerase type

298   3, THO complex subunit 2, transmembrane protein 35, and sentrin-specific protease.

299

300   A GO analysis of each group of unique genes indicated differences in top level functions among

301   stages, with more similar expression in the adults and larval stages, sharing MF terms "serine-

302   type endopeptidase activity", "oxidoreductase activity", and "transition metal ion binding" (Fig.

303   4). However, an enrichment analysis indicated that serine-type endopeptidases were

304   overrepresented only in the adults stage (p=0.005), and substrate-specific transporter activity was

305   overrepresented in the larval stage (p=0.04). Still, these two life stages shared 138 genes

306   expressed at low levels, not unexpected since both stages are active and feeding. Pupae had the

307   least number of genes and GO terms and included those with MF term "serine-type

308   endopeptidase activity", and two unique terms: "NADH dehydrogenase activity", and "structural

309   constituent of cuticle". Enrichment analysis for pupae indicated overrepresentation of negative

310   regulation of programmed cell death (BP; p=0.002), which was due to reads mapping to the gene

311   LOC663274 that encodes fas apoptotic inhibitory molecule 1, consistent with cell division and

312   tissue rearrangement occurring during this stage. Genes expressed in eggs were associated with

313   terms "sequence-specific DNA binding" and "transcription factor activity sequence-specific

314   DNA binding". The most enriched categories in eggs were BP anatomical structure development

315   (p=0.002), sex determination (p=0.011), and cell differentiation (p=0.006), consistent with

316   highly complex developmental processes during embryogenesis.

317

318   *Selection of candidate gene*

319   To find candidate genes for RNAi, we manually curated genes unique to each life stage and

320   expressed at low levels (Supplemental File 2). Six cuticle genes were expressed only in larvae,

321   but unfortunately many cuticle genes have orthologs in other species. However, a BLAST of

322   CPG (LOC103313766) indicated no hits to other species (data not shown). In examining

323   previous data (Morris *et al.*, 2009), CPG was found to be moderately expressed in the larval gut

324   (relative to other gut-expressed genes). Unfortunately, this gene was not analyzed by the iBeetle

325   project (Schmitt-Engel *et al.*, 2015). We then submitted the sequence to OffTargetFinder and

326   found hits to only four other insect species which were focused to the 5' and middle regions,

327   leaving the 3' as a unique portion of the sequence (Supplemental Fig. 1). So, we chose

328   LOC103313766 CPG to evaluate as a potential candidate, since it met our criteria of uniquely

329   expressed in *T. castaneum* larvae, contained regions where dsRNA would minimize off target

330   effects, and was expressed in the larval gut.

331

332   *RNAi validation of CPG*

333   To demonstrate that this was a biological target, we used RNAi to knockdown expression of

334   CPG in *T. castaneum* larvae. We injected dsRNA into early stage larvae (2nd-3rd instar), which

335   we hypothesized would have the most impact on transcript abundance. Knockdown and off

336   target effects were evaluated by another round of RNA-Seq on RNA isolated from the injected

337   larvae and controls (mock-injected "Mock" and noninjected "Control") 18 d post injection. The

338   RNAi treatment significantly reduced the expression of CPG target transcript 1,491-fold

339   compared to Control ($p=2.17\times10^{-7}$) and 284-fold compared to Mock ($p=7.14\times10^{-7}$). An ANOVA

340   comparing the RPKM values of Mock, Control, and CPG treatments also was significantly

341   different (Fig. 5A; $p=2.64\times10^{-5}$). The large decrease in expression was biologically significant

342   because 64% mortality was observed in the CPG dsRNA treatment group compared to the non-

343   injected control at day 18 post-injection (Fig. 5B; $p=0.057$). We also observed 29% mortality in

344   Mock, but it was not significant when compared to Control mortality (19%; $p=0.79$).

345

346   RNA-Seq analysis of larvae injected with CPG dsRNA compared to Mock resulted in 449

347   significantly differentially expressed genes ($p<0.01$ and at least 8-fold change; Supplemental Fig.

348   2 and Supplemental File 3). Of those genes, 100 were uncharacterized, and 20 were annotated as

349   hypothetical proteins. Most (62%) had decreased expression, and the 10 most highly repressed

350   genes were uncharacterized except the target gene and LOC100142553 adenylate kinase. Of

351   those with increased expression, the most highly increased was *Jtb*, which encodes an orphan

352   receptor with unknown function, LOC662244 F-box SPRY that contributes to ubiquitin-protein

353   transferase activity, and *BBIP1* which is a chromosome associated protein. Other up regulated

354   genes related to transcription/translation included LOC107398339 (splicing factor for mRNA),

355   LOC103313738 (a repressor of developmentally regulated gene expression), and LOC664045,

356   involved in chromatin silencing. In response to knockdown of CPG, three other cuticle protein

357   genes were significantly increased: LOC100141875 cuticle 19-like, LOC103313752 cuticle

358   protein 70, and LOC655183 cuticle protein.

359

360   There were 52 genes in this dataset that were annotated as noncoding RNA (ncRNA), 25 with

361   increased and 27 with decreased expression (Supplemental File 4). All were preliminarily

362   characterized as long ncRNAs since they were greater than 200 nt. All ncRNAs were screened

363   through RNAcon (Panwar *et al*., 2014) to predict the classification of each. There were 10 SSU-

364   rRNA5, three Intron-GP-1, and one IRESe; the remaining sequences were below the default

365   Support Vector Machines (SVM) threshold (0.0) and were classified as coding mRNA.

366   However, these sequences were characterized by NCBI as ncRNA, probably due to unusual

367   structure. Regardless, our transcriptome data suggests that ncRNA are actively transcribed in

368   response to RNAi, as has been described previously (Ji *et al*., 2015).

369

370   A GO term enrichment analysis of significantly decreased gene expression in response to CPG

371   RNAi identified functions involved in chitin breakdown (BP GO:0006032 and MF GO:0004568;

372   p=0.04 and 0.04, respectively). LOC107398196 and *Cht13,* both encoding chitinases, were

373   responsible for these enriched GO terms. In contrast, the GO term aspartic-type endopeptidase

374   activity ($p=8.28 \times 10^{-5}$) was significantly enriched in up regulated genes.

375

376   *Characterization of CPG*

377   To gain insight into the function of the CPG target gene, a maximum likelihood tree was

378   constructed with *T. castaneum* protein sequences annotated as 'cuticle protein' (Fig. 6). CPG

379   predicted protein (XP_008196095, highlighted with a yellow box) was most similar to cuticle

380   proteins 16.5 (XP_015833062) and 19.8 (XP_976285). Interestingly, the target CPG protein also

381   was found in the same major clade as the up regulated cuticle protein 70 gene LOC103313752

382   (XP_008196069, highlighted in red)*,* and found in close proximity on LG3 (Table 2). Another

383   cuticle protein gene with increased expression, LOC655183 (XP_008190752) also on LG3, was

384   related to those encoding resilin proteins. The protein product of the cuticle gene with highest

385   increased expression, LOC100141875 (XP_001809825), was related to cuticle protein 7

386   (XP_008193006) encoded by LOC656347, and both genes were found on LG5.

387

388   **Discussion**

389   Current control methods for *T. castaneum* and other stored product pests are becoming less

390   effective due to development of insecticide resistance (Collins, 1990; Jagadeesan *et al.*, 2012;

391   Opit *et al.*, 2012). Alternative management strategies are limited, and new molecular-based

392   controls are needed because they offer increased efficacy and selectivity, and targeted

393   approaches to combatting resistance. There are major hurdles in developing molecular-based

394   strategies in *T. castaneum.* The first step is to identify a target gene that can be effectively

395   knocked-down. The target gene should be specific to the pest or a narrow range of pests. The

396   most difficult challenge is delivery, whether it is topical or oral RNAi, or another mechanism. In

397   this study, we evaluated molecular targets by injection of dsRNA prior to attempts of topical or

398   oral delivery.

399

400   An RNA-Seq analysis of the four major life stages of *T. castaneum* identified twelve genes that

401   were highly expressed in all developmental stages. One gene was a cathepsin L named *26-29-p*.

402   Cathepsin L genes in *T. castaneum* are part of a gene expansion group that are expressed at high

403   levels in adults and larvae for the digestion of cereal proteins (Goptar *et al.*, 2012; Oppert *et al.*,

404   2010; Oppert *et al.*, 2003; Oppert *et al.*, 1993; Perkin *et al*., 2016; Perkin *et al.*, 2017a;

405   Vinokurov *et al.*, 2009). Based on phylogenetic analysis, *26-29-p* is conserved across insects

406   and may be the ancestral cathepsin L gene, but it most likely functions in development and

407   immunity (Perkin *et al.*, 2016). While this gene may not be useful for species-specific pest

408   control because of the conservation of sequence among insects, its function in *T. castaneum* and

409   other insects is of biological interest, as it appears to provide critical function(s) throughout

410   development.

411

412   Nine genes were commonly expressed at low levels in all developmental stages, including *Tctud*

413   (maternal protein tudor). *Tctud* has been found in many eukaryotes and has been implicated in

414   protein-protein interactions, where methylated substrates bind to the tudor domain. In *D.*

415   *melanogaster,* tudor proteins are found in chromatin, small nuclear RNA assembly, RNA-

416   induced silencing complex, and germ granules (Ying & Chen, 2012). Similar to *Tctud*, temporal

417   expression data shows low to moderately high expression of *Dmtud* in early fly embryos through

418   30-day old adults in both males and females (flybase.org, modENCODE temporal expression

419   data, Gramates *et al.,* 2017). Additionally, mutants of various alleles caused lethality of early

420   embryos through maternal effects, and both male and female sterility (Thomson & Lasko, 2004;

421   flybase.org, Summary of Phenotypes, Gramates *et al.,* 2017). We submitted *Tctud* nucleotide

422   sequence to OffTargetFinder and found multiple 21mer hits to 35 other species (data not shown),

423   and therefore the gene was not selected for RNAi.

424

425   We have found that validation of RNAi knockdown through RNA-Seq can provide unique

426   insights into function. Previously, we used RNA-Seq to validate knockdown of aspartate 1-

427   decarboxylase (*ADC*) in *T. castaneum* larvae, resulting in the identification of additional gene

428   interconnectivity (Perkin *et al.*, 2017b). Not only was the significant knockdown of *ADC*

429   confirmed through RNA-Seq, a previously unknown interaction between *ADC* and dopamine

430   receptor 2 also was discovered. This information led to biological assays that determined that

431   the reduction of *ADC* transcripts via RNAi resulted in adults with decreased mobility. Therefore,

432   we used the same approach in the present study to evaluate knock down of a target gene and also

433   understand the overall impact to the transcriptome of reduced gene expression.

434

435   To isolate a candidate gene that could be used as a molecular-based pest control target, we

436   sought genes unique to a feeding stage (i.e. larvae or adults) and expressed at low levels.

437   Through manual curation of life stage transcriptome data, we found CPG was uniquely expressed

438   in larvae at low levels and lacked an ortholog in other species at the 3' end of the gene; in fact,

439   BLAST did not return any hits at the mRNA level. We were encouraged that this target also has

440   potential as an oral RNAi product, since the gene was moderately expressed in the larval gut.

441   The protein product of this gene, XP_008196095, is a peptide of 61 amino acids, with 41%

442   identity to a neuropeptide-like precursor from the flesh fly, *Sacrophaga crassipalpis* (Li *et al.*,

443   2009). Additionally, Bhatia and Bhattacharya (2018) found that knockdown of a related cuticle

444   protein gene in the green peach aphid (*Myzus persicae*) through oral delivery of dsRNA

445   expressed in *Arabidopsis thaliana* resulted in reduced fecundity. We did not investigate whether

446   the *T. castaneum* target cuticle protein also had neuropeptide properties or whether reduced gene

447   expression caused a reduction in fecundity, but our functional test with RNAi demonstrated that

448   decreased gene expression in early stage larvae resulted in significant mortality compared to

449   Control and Mock. Thus, this gene is a candidate target for oral delivery of dsRNA.

450

451   RNA-Seq analysis of the transcriptome after RNAi knockdown of CPG identified three other

452   cuticle genes with increased expression and two chitinase genes with decreased expression

453   compared to Mock. The cuticle genes with increased expression included one encoding cuticle

454   protein 70 (XP_008196069), clustering in the same major clade as the target cuticle protein gene,

455   suggesting similar function and perhaps an indication of redundancy in function as has

456   previously been noted with gene expansion groups. In fact, this redundancy may explain

457   survivors in the dsRNA-injected larvae, albeit a low percentage (32%), and it may complicate

458   oral RNAi if there is compensation response similar to that previously identified in attempting to

459   target cysteine protease genes via RNAi (Perkin et al., 2017a). Another cuticle protein gene

460   LOC655183 also was up regulated after RNAi treatment, and this gene product (XP_008190752)

461   clustered with resilin proteins. Resilin is a specialized cuticle protein that is found in soft parts

462   of the adult insect allowing for movement of wings (Andersen & Weis-Fogh, 1964).

463   LOC655183 is an ortholog to a gene in *Drosophila melanogaster* encoding cuticular protein 56F,

464   which is expressed in the imaginal discs of late larvae (flybase.org, Gramates *et al.,* 2017). The

465   protein product of another cuticle gene with increased expression, LOC100141875

466   (XP_001809825), clustered with cuticle protein 7 (XP_008193006), an ortholog to one found in

467   the carapace cuticle of juvenile horseshoe crab, *Limulus Polyphemus* with a high tyrosine content

468   (Ditzel *et al.*, 2003).

469

470   Opposite in response to the increased expression of cuticle protein genes, *Cht13,* a chitinase, was

471   decreased in response to the injection of CPG dsRNA. *Cht13* is predicted to be part of group IV

472   chitinases expressed in the gut or fat body and expressed in response to feeding in larval and

473   adult stages (Zhu *et al.*, 2008). All differentially expressed chitin-related genes are apparently

474   interconnected through a regulatory pathway that responds to the loss of function of CPG by

475   RNAi.

476

477   We were surprised at the number of long ncRNAs (52) that were differentially expressed at a

478   significant level (p<0.01) in surviving *T. castaneum* larvae that were injected with CPG dsRNA.

479   Most ncRNA were not classified as typical ncRNAs and may represent new classes, or

480   alternatively may be miss-annotated. However, differentially expressed long ncRNA have been

481   implicated in human disease states, particularly cancer (Ma *et al.*, 2015). Of relevance to this

482   study, differentially-expressed long ncRNAs can impose epigenetic changes that alter the

483   transcription of other genes, including silencing (Tufarelli *et al.*, 2003). Therefore, we can

484   speculate that the large number of differentially expressed genes in this study (449) may have

485   been regulated in part by ncRNAs. While not to the same degree, ncRNAs were implicated in a

486   previous RNAi study, in which LOC107398253 ncRNA (decreased 12.3-fold, p=0.001, in the

487   current study) was decreased 593-fold in response to RNAi of *ADC* (Perkin *et al.*, 2017b). This

488   particular ncRNA was annotated as an U3 snoRNA, predicted to be involved in site-specific

489   cleavage of ribosomal RNA (rRNA) during pre-rRNA processing (Clery *et al.,* 2007). More

490   work is needed to understand the relationship of ncRNAs and RNAi in insects.

491

492   **Conclusions**

493   In summary, these data add to the sparse stage-specific studies in *T. castaneum* and other beetles.

494   We demonstrate that by using this approach, we were able to identify a candidate gene, CPG,

495   that may be useful in developing an insect control product based on dsRNA. Because the gene

496   was expressed at low levels overall and moderate levels in the larval gut, oral delivery methods

497   for CPG dsRNA have the potential to control damage by *T. castaneum* larvae.

498

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Genes that were expressed at high and low levels in all life stages.

List of genes that were expressed at high (top) and low (bottom) levels in all life stages of *T. castaneum*.

1 **Tables**

2 Table 1. Genes that were expressed at high (top) and low (bottom) levels in all life stages of *T. castaneum*.

Name	Description	Average RPKM			
		Adult	Egg	Larvae	Pupae
LOC656235	Elongation factor 1-gamma	1,560	1,334	657	6,278
RpS3	Ribosomal protein S3	3,953	2,663	1,475	263
26-29-p	Cathepsin L	369	281	261	5,710
LOC659992	40S ribosomal protein S29	1,135	1,443	626	3,238
LOC663023	Glyceraldehyde-3-phosphate dehydrogenase 2	2,405	812	2,135	2,375
LOC660435	60S ribosomal protein L11	2,673	2,134	1,096	6,999
Efa1	Elongation factor 1-alpha	2,253	2,541	1,762	3,576
ND2	NADH dehydrogenase subunit 2	1,081	1,457	1,000	401
ATP6	ATP synthase F0 subunit 6	5,442	4,960	4,555	270
COX3	Cytochrome c oxidase subunit III	4,267	3,923	6,559	857
CYTB	Cytochrome b	3,181	4,829	7,123	558
I-rRNA	I-rRNA	16,635	11,824	34,216	11,020
LOC103315067	Uncharacterized	5.91	57.1	7.30	18.7
LOC103312214	Uncharacterized	10.3	89.0	8.11	24.6
LOC103315070	Uncharacterized	17.5	94.1	16.9	26.9
LOC107399196	Uncharacterized	25.5	181	9.69	22.7
LOC103312419	Maternal protein tudor	5.25	15.3	5.34	5.45
LOC103312455	poly(A) polymerase type 3	9.11	65.5	13.0	15.0
LOC663288	THO complex subunit 2	7.55	61.8	8.69	10.6
LOC659780	Transmembrane protein 35	240	7.72	241	179
LOC103313244	Sentrin-specific protease	10.5	74.6	35.0	9.45

Differentially expressed cuticle genes.

Differentially expressed cuticle genes in *T. castaneum* larvae injected with CPG dsRNA.

Shaded row indicates target gene.

1 Table 2. Differentially expressed cuticle genes in *T. castaneum* larvae injected with LOC103313766 cuticle protein dsRNA. Shaded
2 row indicates target gene.
3

Gene	LG	Coordinate Start	Coordinate Stop	Protein	Annotation	Fold Change	Expression
LOC103313766	3	4099231	4115167	XP_008196095	Cuticle protein	1,491	↓
LOC658110	3	4097672	4112513	XP_015833062	Cuticle protein 16.5	2.51	↓
LOC658489	3	4077823	4109423	XP_976285	Cuticle protein 19.8	1.44	↓
LOC103313752	3	4114536	4126952	XP_008196069	Cuticle protein 70	10.8	↑
LOC655183	3	2446367	2620651	XP_008190752	Cuticle protein	4.01	↑
LOC100141875	5	3608978	3616499	XP_001809825	Cuticle protein	16.9	↑
LOC656347	5	3612511	3613210	XP_008193006	Cuticle protein 7	4.89	↑
LOC107399215	2	6796409	6962942	XP_015840495	Larval cuticle protein A2B-like	25.1	↓

Figure 1

Venn diagram comparing genes expressed in *T. castaneum* life stages: adult, egg, larvae, and pupae with high expression values (>8 log$_2$ RPKM).

Shades of grey indicate the relative number of genes in each comparison. Dark grey indicates a higher number of genes and lighter shades represent comparisons with fewer genes.

Figure 2

Top GO terms for genes with high expression in each life stage.

Each pie chart identifies the top GO terms for each life stage; (A) Egg, (B) Larvae, (C) Pupae, (D) Adult.
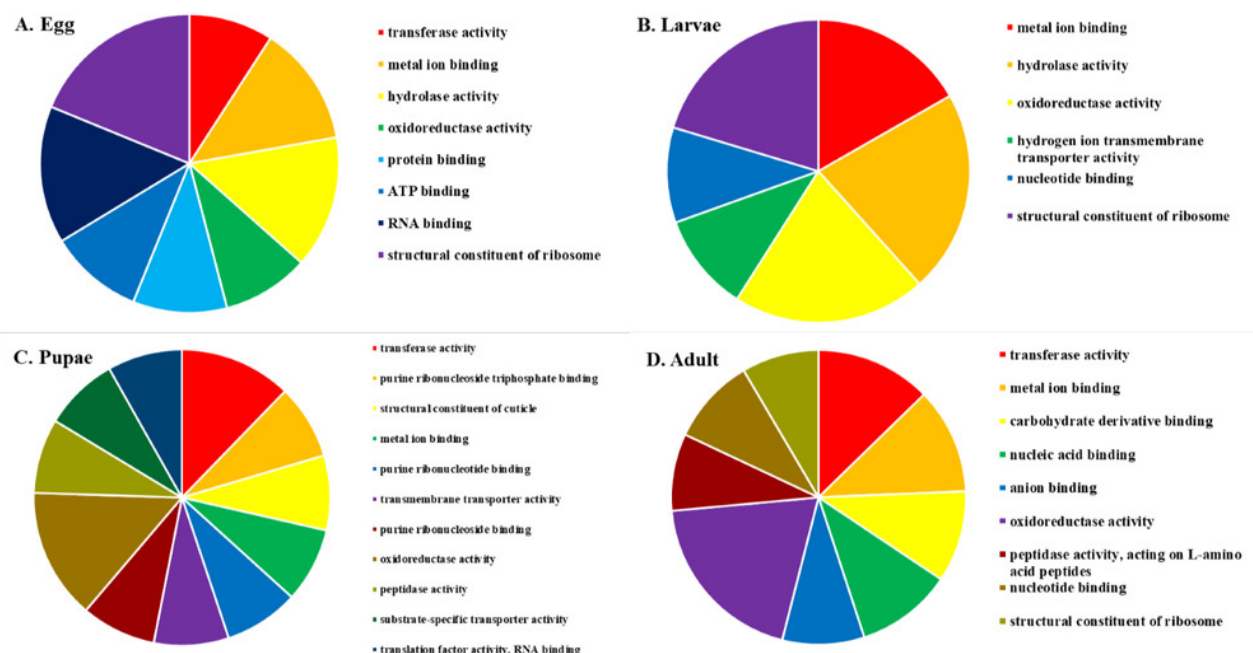
Figure 3

Venn diagram comparing genes between all *T. castaneum* life stages: adult, egg, larvae, and pupae with low expression values (2-8 log$_2$ RPKM).

Shades of grey indicate the relative number of genes in each comparison. Dark grey indicates a higher number of genes and lighter shades represent comparisons with fewer genes.
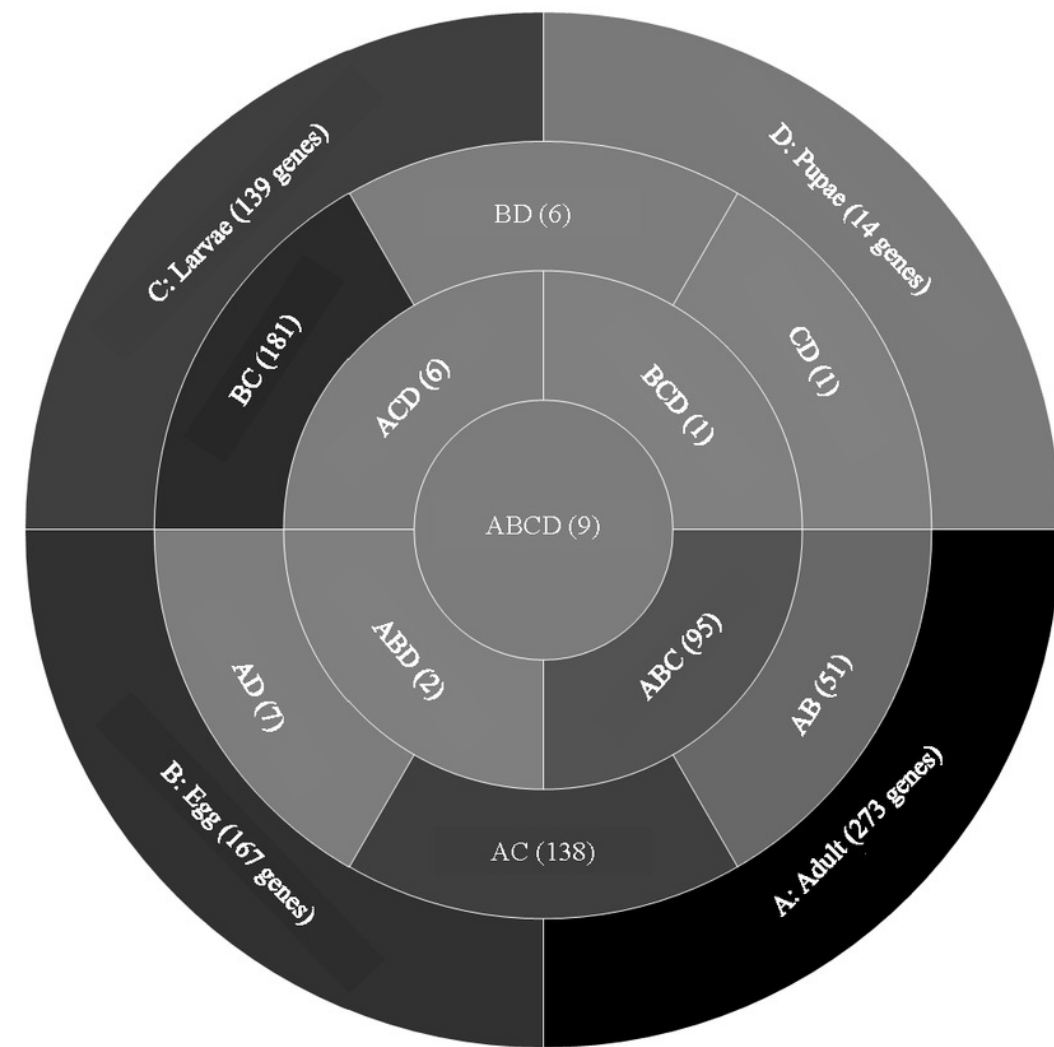
Figure 4

Top GO terms for genes with low expression in each *T. castaneum* life stage.

Each pie chart identifies the top GO terms for each life stage; (A) Egg, (B) Larvae, (C) Pupae, (D) Adult.
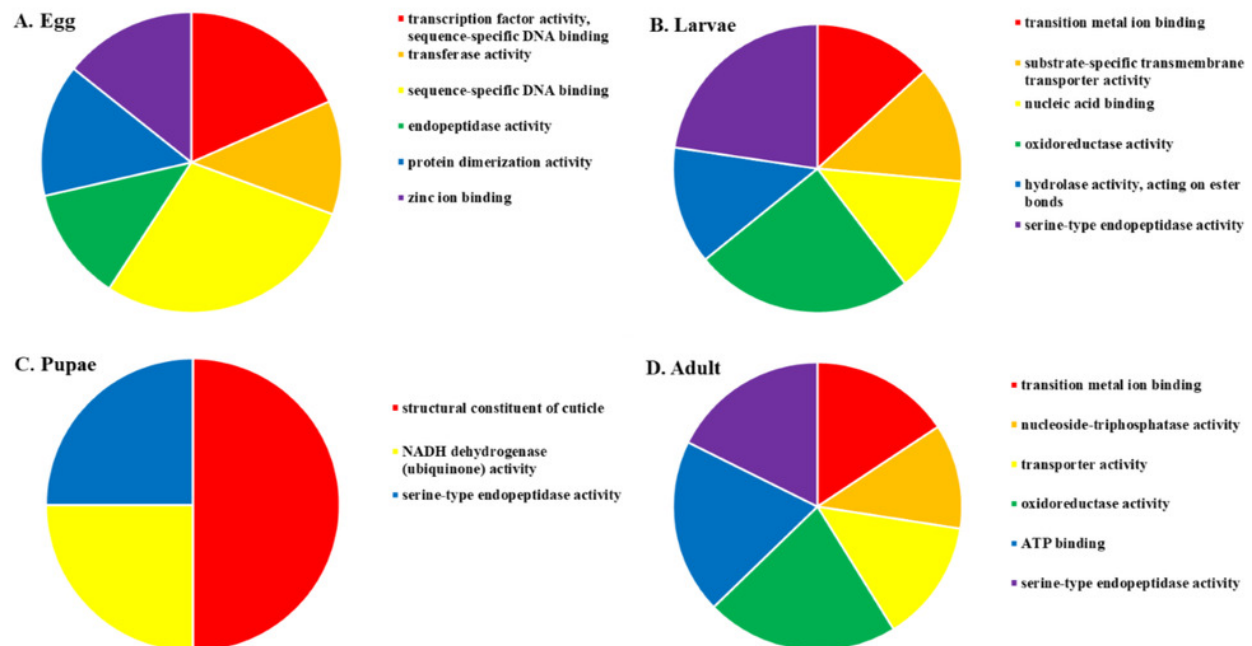
Figure 5

Effects of injection of CPG dsRNA in *T. castaneum* larvae.

(A) Average expression of CPG in control, mock, and dsRNA-injected *T. castaneum* larvae 18 d following RNAi application. (B) Percent survival of *T. castaneum* larvae following RNAi treatment.
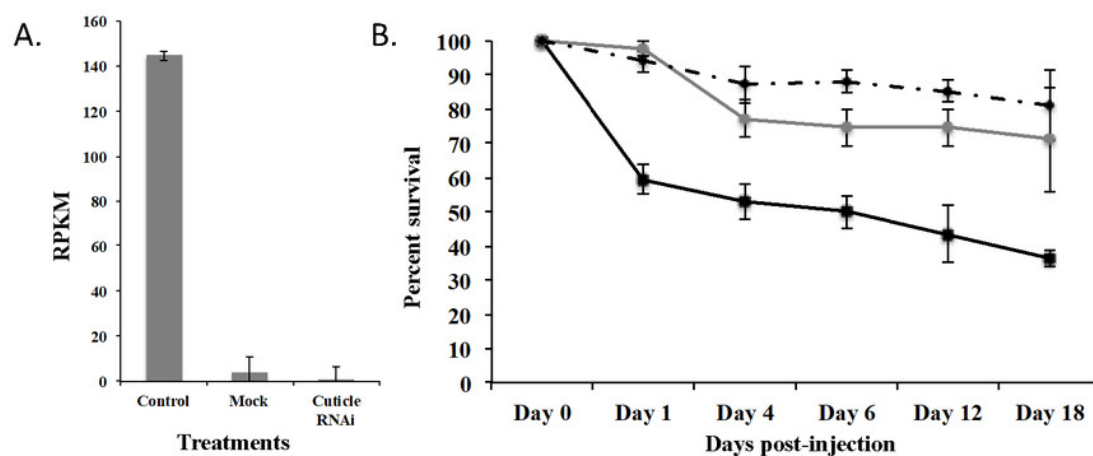
Figure 6

T. castaneum cuticle protein maximum likelihood tree.

CPG (XP_008196095) is highlighted with a yellow box, and proteins from the three cuticle genes with increased expression (XP_008196069, XP_001809825, XP_008190752) are highlighted with a red box.