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1 **Title**

2 **Large portion of essential genes is missed by screening either fly or beetle**
3 **indicating unexpected diversity of insect gene function**

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34 **Abstract**

35 Most gene functions were detected by screens in very few model organisms but it has remained
36 unclear how comprehensive these data are. Here, we expanded our RNAi screen in the red flour
37 beetle *Tribolium castaneum* to cover more than half of the protein-coding genes and we compared
38 the gene sets involved in several processes between beetle and fly.

39 We find that around 50 % of the gene functions are detected in both species while the rest was
40 found only in fly (~10%) or beetle (~40%) reflecting both technical and biological differences. We
41 conclude that work in complementary model systems is required to gain a comprehensive picture on
42 gene functions documented by the annotation of novel GO terms for 96 genes studied here. The
43 RNAi screening resources developed in this project, the expanding transgenic tool-kit and our large-
44 scale functional data make *T. castaneum* an excellent model system in that endeavor.

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45 Introduction

46 Only in a very small number of genetic model species like the mouse *Mus musculus*, the zebrafish
47 *Danio rerio*, the nematode *Caenorhabditis elegans* and the vinegar fly *Drosophila melanogaster* have
48 the functions of most genes been assayed in systematic screens. This restriction to few model
49 systems is a consequence of the necessity for an elaborate genetic and molecular tool kit, which is
50 extremely laborious to establish (Jorgensen and Mango, 2002; Kile and Hilton, 2005; Patton and Zon,
51 2001; St Johnston, 2002). Unfortunately, it has remained unclear how representative findings in
52 these model species actually are for their clade or in other words, how quickly and profoundly gene
53 function diverges in evolution. Knowing the degrees of gene function divergence is relevant not only
54 for understanding the evolution of biodiversity but also for applied research, e.g. for transferring
55 knowledge from model systems to species relevant for medical applications or pest control.

56 Recently, the study of gene function has been extended to non-traditional model organisms.
57 Predominantly, candidate genes known for their function in the classical model systems have been
58 tested in other organisms. Subsequent comparisons revealed both, conservation and divergence of
59 gene functions. For example, axis formation in *D. melanogaster* has turned out to be a rather
60 diverged process partially based on different genes compared to other insects. The key anterior
61 morphogen of *D. melanogaster*, *bicoid*, is not present in most insects (Brown et al., 2001). Instead,
62 repression of Wnt signaling plays a central role in the red flour beetle *Tribolium castaneum* (Fu et al.,
63 2012) as it does in many animals including other insects, flatworms and vertebrates (Glinka et al.,
64 1998; Gurley et al., 2008; Klomp et al., 2015; Yoon et al., 2019) - but not in *D. melanogaster*. The
65 functions of genes of the Hox cluster, in contrast, appear conserved over very large phylogenetic
66 distances - although some functional divergence has been linked to the evolution of arthropod
67 morphology (Averof, 2002). Likewise, the gene regulatory network of dorso-ventral patterning and
68 head specification show the involvement of similar gene sets, although a few components appear to
69 be involved in only some clades (Kittelman et al., 2013; Kitzmann et al., 2017; Lynch and Roth, 2011;
70 Stappert et al., 2016).

71 Notably, the differences in gene functions documented so far may be an underestimation of the real
72 divergence, because the prevailing candidate gene approach leads to a systematic bias towards
73 conservation. The genes to be tested are usually chosen based on the knowledge of their ortholog's
74 involvement in other species. As a consequence, unrelated genes are rarely tested and the
75 involvement of unexpected genes in a given process is underestimated. Hence, approaches are
76 needed to overcome this bias and to gain a realistic view on the degree of gene function divergence.
77 To that end, genes required for certain biological processes need to be identified in an unbiased and
78 genome-wide manner also in non-traditional organisms, even though this has remained technically
79 challenging.

80 The red flour beetle *T. castaneum* has recently been established as the only arthropod model
81 organism apart from *D. melanogaster* where genome-wide unbiased RNAi screens are feasible. Based
82 on the robust and systemic RNAi response of this species, the *iBeetle* large scale screen was
83 performed where random genes were knocked down and the resulting animals were scored for a
84 number of developmental phenotypes (Bucher et al., 2002; Schmitt-Engel et al., 2015; Tomoyasu and
85 Denell, 2004). Apart from its particularly strong and robust RNAi response, *T. castaneum* offers a
86 comparably large tool kit for analyzing gene function including transgenic and genome editing
87 approaches (Berghammer et al., 1999; Gilles et al., 2015; Schinko et al., 2010).

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88 In this paper, we used an expanded dataset to assess the degree of divergence of the gene sets
89 involved in selected developmental processes between fly and beetle such as head, muscle and ovary
90 development, and dorso-ventral patterning. First, we determined genes that were essential in the
91 beetle for these processes but which had so far not been connected to them in *D. melanogaster*.
92 These a priori unexpected genes sum up to about 37% of the total genes identified to be involved in
93 either one or both species. For 30% of these genes, no functional annotation had been available at
94 FlyBase at all such that we provide the first functional Gene Ontology (GO) assignment for the
95 respective ortholog group in insects. Only two genes essential in *T. castaneum* did not have an
96 ortholog in *D. melanogaster*, i.e. these processes seem not much affected by gene gain or loss. We
97 conclude that restricting genetic screens to one model system only, falls short of identifying a
98 comprehensive set of essential genes. Further, our data reveals an unexpected degree of divergence
99 of gene function between two holometabolous insect species. We also present here an update of the
100 dataset gained in the genome wide iBeetle screen in *T. castaneum*. Our analysis is based on both, a
101 dataset previously published comprising 5.300 genes (Schmitt-Engel et al., 2015) and an additional
102 3.200 genes screened as part of this project. In addition to those, we also make accessible (at iBeetle-
103 Base) the phenotypes for an additional 4,520 genes which were screened while the analysis
104 presented here was ongoing. Hence, with this paper, the coverage of genes tested and annotated at
105 iBeetle-Base sums up to 13.020 Tribolium genes (78 % of the predicted gene set).

106

107 Results

108 Continuation of the large scale *iBeetle* screen

109 We added 3,200 genes to the previously published 5,300 genes of our large scale *iBeetle* screen
110 (Schmitt-Engel et al., 2015), reaching a coverage of 51% of the *T. castaneum* gene set of total 16,593
111 currently annotated genes (Herndon et al., 2020). We followed the previously described procedure
112 for the pupal injection screen (Schmitt-Engel et al., 2015) with minor modifications (see methods). In
113 short, we injected 10 female pupae per gene with dsRNAs (concentration 1ug/ul). We annotated the
114 phenotypes of the injected animals and the first instar cuticle of their offspring using the EQM
115 system (Mungall et al., 2010), the *T. castaneum* morphological ontology *Tron* (Dönitz et al., 2013)
116 and a controlled vocabulary (see Schmitt-Engel et al. 2015). The data is available at the online
117 database *iBeetle-Base* (<http://ibeetle-base.uni-goettingen.de/>) (Dönitz et al., 2015; Dönitz et al.,
118 2018). Our controls revealed a similar portion of false negative and false positive annotations as in
119 the first part of the screen (Fig. 1 and Table S1). The detailed analysis presented below was based on
120 this set of genes covering approximately 50% of the genome. In parallel, we continued the screen
121 and have in the meanwhile reached a coverage of 78 % (13,020 genes). We publish these additional
122 phenotypic data (accessible online at *iBeetle-Base*) with this article, but they were not included in the
123 detailed analysis presented here because both analyses ran in parallel.

124

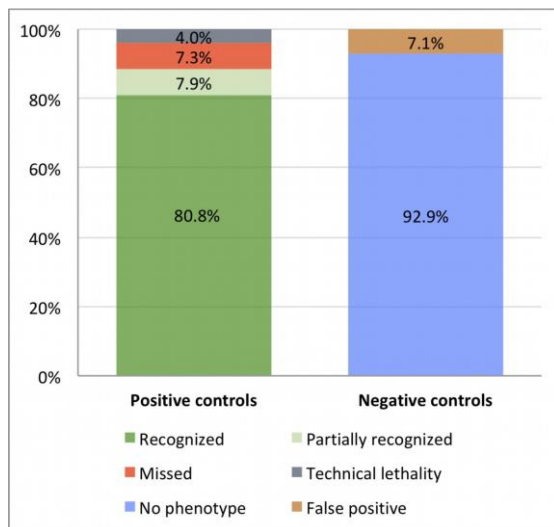


Figure 1 Quality controls of the primary screen

178 positive controls using 35 different genes were included. More than 88% of the positive controls were fully or partially recognized (left bar) while 7.3% were missed. 4% could not be analyzed due to technical lethality before the production of offspring. 7.1% of the negative controls were false positively annotated (right bar). These figures are similar to the first screening phase (Schmitt-Engel et al., 2015).

125

126

127 Unexpected gene functions in developmental processes

128 We wanted to use our large-scale phenotypic dataset to systematically compare the gene sets
129 involved in the same biological processes in *T. castaneum* and *D. melanogaster*. To that end, we first
130 identified in an unbiased way all genes involved in a number of biological processes by searching
131 *iBeetle-Base*. Specifically, we scored for phenotypes indicative of functions in dorso-ventral
132 patterning, head and muscle development, in oogenesis, and epithelial adhesion in wings (wing
133 blister phenotypes). For all these processes, we found a number of gene functions that were
134 expected based on *D. melanogaster* knowledge (see below). This confirmed that the screen design
135 allowed detection of respective phenotypes. Importantly, we also found functions for genes so far
136 not connected to those processes (based on FlyBase information, PubMed searches and scientist
137 expertise). The *iBeetle* screen is a first pass screen with a focus on minimizing false negative results

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138 with the trade-off of allowing for false positive annotations (Schmitt-Engel et al., 2015). The
139 likelihood for this type of error is further increased by off-target effects and/or by strain specific
140 differences in the phenotype (Kitzmann et al., 2013). Hence, we aimed at excluding false positive
141 annotations for the unexpected gene functions. First, we based our analyses only on genes for which
142 phenotypes had been annotated with a penetrance of >50% in the primary screen. Further, we only
143 used phenotypes that were reproduced by RNAi experiments with non-overlapping dsRNA fragments
144 targeting the same gene. In order to exclude genetic background effects, we used another lab strain
145 (our standard lab strain *San Bernardino*, *SB*) except for the muscle project where we needed to use
146 the *pBA19* strain, which has EGFP marked muscles (Lorenzen et al., 2007). This re-screening
147 procedure resulted in a set of genes for which we can claim with high confidence that they are
148 indeed involved in these processes in *T. castaneum* - but which previously were not assigned to these
149 in *D. melanogaster* (Supplementary Table S2).

150 Assigning the first function to a gene versus extending previous annotations

151 One reason for a lack of respective functional data in FlyBase could be that the knocked-down beetle
152 gene does not have an ortholog in the fly. In order to test this hypothesis, we searched for the fly
153 orthologs in orthoDB and by manually generating phylogenetic trees based on searching *T.*
154 *castaneum*, *D. melanogaster* and *M. musculus* genomes for orthologs and paralogs. This analysis
155 revealed that only three genes with a novel function (appr. 3%) did not have a *D. melanogaster*
156 ortholog (yellow in Fig. 2). Evidently, lineage-specific gene loss or gain explains only a minor part of
157 the functional divergence of homologous developmental processes.

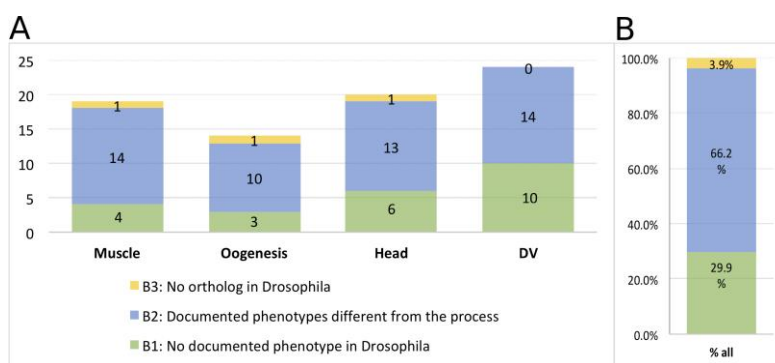


Figure 2 Analysis of genes with unexpected gene functions

A) Numbers of genes with unexpected function in the respective process. B) Combined numbers for all four processes. Only a small portion of genes with novel gene functions did not have orthologs in *Drosophila* (yellow). About two-thirds of the genes had previous phenotypic annotations relating to other biological processes (blue). For one third of those genes, we had detected the first phenotype for this gene within insects (green).

158 Next, we asked whether the respective *D. melanogaster* orthologs were known to be involved in
159 other biological processes or lacked any phenotype information. To that end, we looked up
160 phenotype information of the respective *D. melanogaster* orthologs on FlyBase (analysis done with
161 OrthoDB v9). Among the fly orthologs whose functional annotations did not match with those from
162 the *iBeetle* screen or published record, around two thirds (64.6 %) had annotations that were related
163 to other processes than the ones studied in *T. castaneum* (Fig. 2). Importantly, one third of the genes
164 (32.3 %) did not have any functional annotation in FlyBase. Hence, for those genes, the *iBeetle*-screen
165 had detected the first documented function of that ortholog group in insects. Importantly, due to the

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166 lack of previous phenotypic information, these genes likely would not have been included in a
167 classical candidate gene approach.

168

169 *A quarter of Drosophila gene function annotations were not confirmed for T.* 170 *castaneum*

171 In a complementary approach, we asked how many genes known to be involved in a given process in
172 *D. melanogaster* had been assigned related functions in the *iBeetle* screen. To that end, we first
173 collected lists of genes involved in those processes based on *D. melanogaster* knowledge (expert
174 knowledge, literature and FlyBase) (Table S3). Then we mined *iBeetle-Base* to see how many of the
175 beetle orthologs had an annotation related to that process (Fig. 3A). About two-thirds of those genes
176 had actually been screened in *T. castaneum* (Fig. S1) and all following numbers are based on the
177 analysis of this subset.

178 A surprisingly large portion of genes (26.4%) known to be involved in these processes in *D.*
179 *melanogaster* did not show the expected phenotype in *T. castaneum* (Figure 3B).

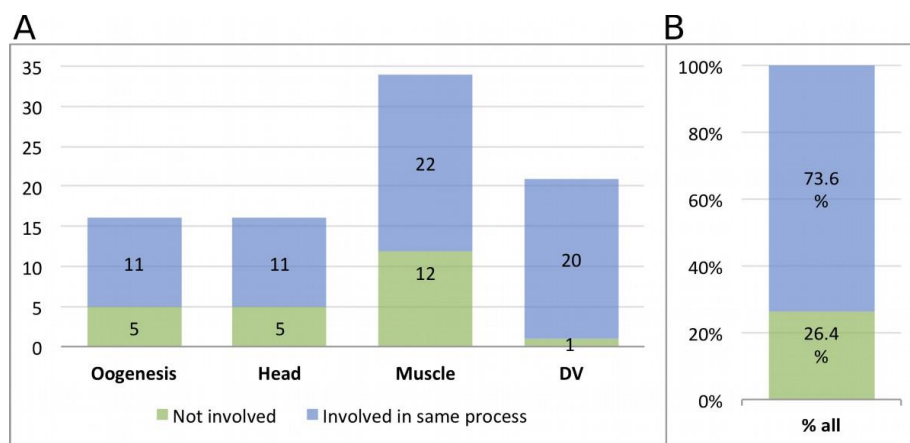


Figure 3 Beetle genes showing phenotypes expected from *Drosophila*

A) Gene sets known to be involved in given processes in *Drosophila* were compared to *iBeetle* data. Many showed related phenotypes (blue) while others had no or different types of phenotypes (green). B) Approximately one quarter of the genes known to be involved in certain *Drosophila* processes were not required in that process in *Tribolium*. This analysis is based on the subset of genes which already had been screened in *Tribolium* (66%).

180

181 *Enriching the GO information with data from Tribolium*

182 Gene ontology (GO) assignment is a powerful tool to establish hypotheses on the function of given
183 gene sets (Carbon et al., 2009). So far, there were no GO terms associated based on *T. castaneum*
184 data. The work presented here revealed that a surprisingly high portion of orthologous genes has
185 diverging functions in different organisms. To enrich the GO database, we submitted GO terms with
186 respect to the biological process for all 96 re-screened genes with functions in dorso-ventral

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187 patterning (GO:0010084), oogenesis (GO:0048477), the development of embryonic muscles
188 (GO:0060538) and head (GO:0048568).

189 [[the new GO terms are submitted but not yet accepted. This part will only be included in the final
190 version of the paper if the terms have been accepted by the GO consortium]]

191 **Materials and Methods**

192 **Screen**

193 We followed the tested and published procedures apart from some minor changes (please find an
194 extensive description of the procedure in Schmitt-Engel et al. 2015). In particular, we used the same
195 strains, injection procedures, and incubation temperatures and incubation times. dsRNAs were
196 produced by Eupheria Biotech Dresden, Germany. Different from the published procedure, the stink
197 gland analysis was performed 21 days after pupal injection (in the first screening phase, this analysis
198 had been performed after larval injection).

199 **Controls of the screen**

200 To assess the sensitivity and reliability of the screen, and also to test the accuracy of each screener,
201 we included approximately 5% positive controls from a set of 35 different genes. By and large, we
202 used the same positive controls as in the first screening phase (see Table Table_S1_controls). *Tc-zen-*
203 *1* was excluded since the phenotypes were much weaker than in the previous screen, probably due
204 to degradation of the dsRNA. We added new positive controls to score for muscle and stink-gland
205 phenotypes, which we took from novel genes detected in the first screening phase. Muscle
206 phenotypes iB_06061, iB_05796, iB_03227, iB_01705; stink gland and ovary phenotypes: iB_02517.
207 Head defects: iB_05442 (that gene was not scored for its stink gland phenotype because it turned out
208 to be too mild to be identified reliably in high throughput). In 143 cases (80.8%, n=177), the
209 phenotypes of positive controls were fully recognized (for comparison: in the first screening phase
210 the respective numbers were: 90%, n=201). In 14 cases (7.9%; phase 1: 4%) the phenotype was
211 partially recognized. This category includes complex phenotypes where half (one of two aspects:
212 *knirps*, *piwi*, *SCR*, *cta*, *cnc*, *iB_01705*, *iB_05442*) or two of three aspects (*aristalless*) of all phenotypic
213 aspects were correctly identified. 13 phenotypes were missed completely (7.3%, phase 1: 4%). *Tc-*
214 *metoprene tolerant* (*Tc-met*) was missed most frequently, probably due to the fact that the
215 embryonic leg phenotype was very subtle and in addition, the penetrance of the phenotype
216 appeared to be lower than in the first screen (penetrance: less than 30%). Seven positive controls
217 (4%, phase 1: 1%) could not be analyzed due to prior technical lethality, i.e. the premature death of
218 the injected pupae prevented the detection of the phenotype. In three cases wrong aspects were
219 annotated (false positive: 1.7%). Depending on the other annotations these positive controls were
220 valued as partially recognized (SCR) or missed (met, CTA). Find more details in Table
221 Table_S1_controls.

222 Negative controls (buffer injections) were mainly annotated correctly (no phenotype in 92.9%; phase
223 1: 96%) and just in 7 cases led to false positive annotations (7.1%; phase 1: 2%) (Table
224 Table_S1_controls; sheet 2).

225 **Re-Screen**

226 Re-screening of selected iBeetle candidates involved in a number of biological processes was
227 performed in order to probe for off-target and strain-specific effects. For that purpose, two

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228 independent dsRNA fragments (original iB-fragments and one non-overlapping fragment, both at
229 concentration 1 µg/µl) of the same gene were injected separately into a different genetic background
230 (*San Bernardino*, *SB* strain), except for the muscle project where it is required to use the pBA19 strain
231 with EGFP marked muscles. The rest of the injection procedures and analyses were as in the first
232 pupal injection experiment (see details in Materials and Methods).

233 **Phylogenetic analysis**

234 The *Tribolium* protein sequences from gene set ([http://ibeetle-base.uni-](http://ibeetle-base.uni-goettingen.de/downloads/OGS3_proteins.fasta.gz)
235 [goettingen.de/downloads/OGS3_proteins.fasta.gz](http://ibeetle-base.uni-goettingen.de/downloads/OGS3_proteins.fasta.gz) - including changes from 2016/02/15) were used
236 to retrieve the most similar proteins of *T. castaneum*, *D. melanogaster* and *M. musculus* excluding
237 isoforms. Multiple alignments were done with the ClustalOmega plugin as implemented in the
238 Geneious 10.1.3 software (Biomatters, Auckland, New Zealand) using standard settings. Alignments
239 were trimmed to remove poorly aligned sequence stretches. Phylogenetic trees were calculated
240 using the FastTree 2.1.5 plugin implemented in Geneious.

241 **Generation of *Unc-76* mutations via CRISPR/Cas9**

242 The procedure used to generate *Unc-76* mutations was described by Bassett et al., 2013 (Bassett et al.,
243 2013). For making the template for the guide RNAs, the *Unc-76* target sequence between the T7
244 promoter and the gRNA core sequence in the forward primer, gRNA_F, was chosen as
245 GGTTC AACGATCTGACCA GTG, and after annealing gRNA_F with SGRNAR the template was PCR
246 amplified with Q5 polymerase (NEB). Guide RNAs were transcribed with Ampliscribe T7 Flash
247 (epicentre), isolated with the MEGAClear kit (Ambion), and injected together with Cas9 mRNA into
248 *w[1118] sn[3] P{ry+t7.2=neoFRT}19A* embryos. Single lines established from the offspring were
249 tested as heterozygotes over *FM7c* with the T7 endonuclease assay for sequence alterations near the
250 target site (Kondo and Ueda, 2013). The lethal *Unc-76*[CR007] allele carries a 16 nucleotide deletion
251 near the target site in the sequence ..TAT CCA CAC ACc aac ggt ttg gga tcc GGA TCC GGA TCC.. of the
252 second exon (X: 2091152... 2091167, r6.32; see lower case letters) that creates a frameshift in the
253 ORF in all known isoforms (after T246 in *Unc-76* RA to -C and after T61 in *Unc-76* RD).

254 **Discussion**

255 **Investigating one species falls short of a comprehensive view on gene function**

256 Large scale screens in the leading insect model organism *D. melanogaster* have revealed gene sets
257 involved in certain biological processes. As consequence, insect-related GO term annotations are
258 almost exclusively based on work in flies. However, there are several reasons to believe that the
259 picture has remained incomplete. On one hand, species-specific or technical limitations may have
260 prohibited identification of an involved gene in *D. melanogaster*. On the other hand, evolution may
261 have led to functional changes such as the loss of ancestral gene functions or the integration of genes
262 into a novel process. Unfortunately, it has remained unclear to what extent the gene sets determined
263 exclusively in flies would be representative of insects as a whole.

264 Our systematic screening in a complementary model organism has revealed that the identified gene
265 sets show an astonishing degree of divergence. Based on our calculations (see details below) we
266 estimate that only half of the gene functions are similarly detected in both species (52%, column 4 of
267 Fig. 4A) while the remaining gene functions were revealed either only in *D. melanogaster* (11%,
268 column 4 of Fig. 4A) or only in *T. castaneum* (37%, column 4 of Fig. 4A). Hence, our current
269 knowledge based on screening in one species appears to be much less comprehensive than

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270 previously thought. We believe that the different proportions of genes detected in only one species
 271 (11% vs. 37%) may reflect both, biological and technical differences (see below).

272 In summary, despite some uncertainties with respect to the exact numbers (see discussion below),
 273 our findings provide a compelling argument that focusing on single model species falls short of
 274 comprehensively revealing the genetic basis of biological processes in any given clade. Further, it
 275 shows that *T. castaneum* is an extremely useful screening system for insect biology, able to reveal
 276 novel gene functions even in processes that have been studied intensely in *D. melanogaster*.

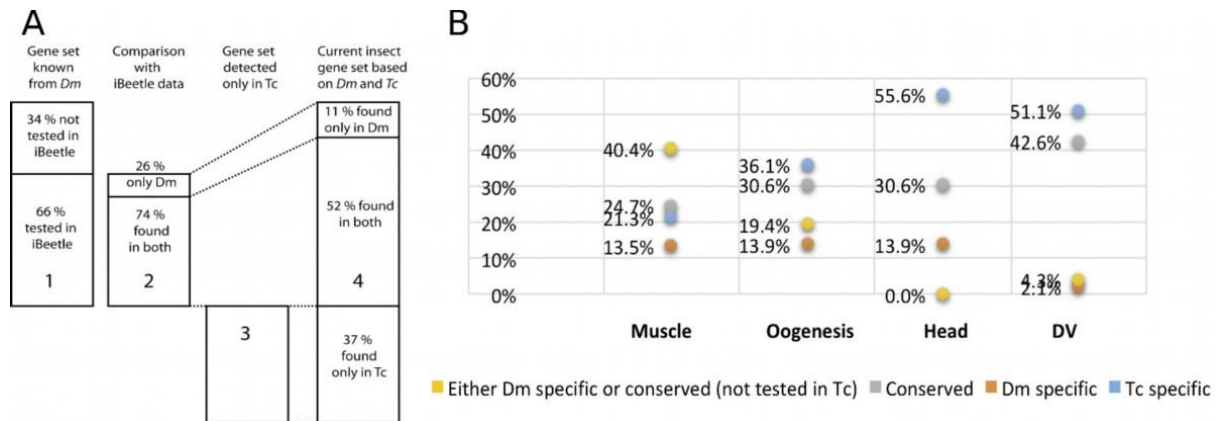


Figure 4 Many genes are detected only in one of the species in the same processes

Combining genes found in fly (column 1) and/or beetle (column 3) leads to the currently known insect gene set for the processes analysed here. Portions shown in column 1 and 2 are based on Fig. S1, Fig.2 and Fig. 3. We calculate the portions of genes of the combined insect gene set (column 4), which were detected only in *Drosophila* (11%), only in *Tribolium* (37%) or in both (52%). See text for details and discussion of potential systematic biases. B) Respective values for the single processes show that the minimum contribution of the *Tribolium* screening platform amounted to 20% genes not detected in *Drosophila*. See table S4 for calculations. Neither model species is able by itself to detect “the insect gene set”.

277

278 Estimating the portions of gene functions revealed in fly versus beetle

279 Our beetle data are based on both, our systematic screening of 51% of the *T. castaneum* gene set
 280 and on previous candidate gene work. With respect to fly data, we rely on information available on
 281 FlyBase and our expert knowledge of the processes under scrutiny. Given these different kinds of
 282 sources and approaches, the data are prone to various types of uncertainties. Therefore, we discuss
 283 the way we combined the numbers to calculate our estimation. Subsequently, we will discuss some
 284 uncertainties and in how far they influence the estimation.

285 Of the genes known from *D. melanogaster* to be involved in the processes investigated here ($n = 132$;
 286 see Table S4), we could compare 66% to iBeetle data (column 1 in Fig. 4A; based on Fig. S1; $n = 87$).
 287 Of those genes, 26% ($n = 23$) were not involved in that process in *T. castaneum* (column 2 in Fig. 4A;
 288 based on Fig. 3). For our overall estimation, we extrapolated this share to the total number of genes
 289 involved in the fly (hatched lines from column 2 to column 4). A number of gene functions detected
 290 in the iBeetle screen had not been assigned such functions in *D. melanogaster* before (column 3 in

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291 Fig. 4A; based on Fig. 2). When combining these numbers, we aimed at providing a minimum
292 estimation for divergence of detected gene functions (Column 4 in Fig. 4A). To be conservative, we
293 assumed that all gene functions known from *D. melanogaster* but not yet tested in the iBeetle screen
294 would fall into the class of genes being involved in both species (see numbers in green square in
295 Table S4). Further, we scored each signaling pathways as one case (finding mostly conservation) even
296 if single components of these pathways had not divergent phenotypes. This conservative assumption
297 leads to the abovementioned minimum estimation of divergence in these gene sets (Column 4 in Fig.
298 4A; calculation in Table S4). Of all genes currently known to be involved in one of the processes we
299 studied, the portion of genes detected exclusively in the fly (11%; n = 23) is much smaller than the
300 one detected only in the beetle (37%; n = 76) while the analogous function of half of the genes (52%;
301 n = 109) is detected in both species.

302 With this work, we present the first and a quite extensive dataset to estimate this kind of numbers.
303 Still, some confounding issues need to be considered. The first uncertainty stems from the fact that
304 the beetle data is based on testing about 50 % of the genes. In the second part of the screen, we had
305 prioritized genes that were e.g. highly expressed, showed sequence conservation and had GO
306 annotations. The prioritization apparently was successful as 66% of the gene functions known from
307 *D. melanogaster* had been covered in the iBeetle screen (Fig. 4A), which is much more than the 40%
308 expected for an unbiased selection (Schmitt-Engel et al., 2015). Hence, our figures might be biased
309 towards conserved gene function. As a consequence, the overall portion of beetle specific genes
310 without conserved functions likely is even higher than reflected in Fig. 4A.

311 Second, we found quite different numbers for the four processes under scrutiny (Fig. 4B). However,
312 even in the process with the lowest portion of genes detected exclusively in *T. castaneum* (muscle
313 development), this portion was 21%, which still indicates a significant degree of unexplored biology.

314 Third, the *D. melanogaster* numbers could be influenced by false negative data. The data on FlyBase
315 has not been gathered in one or few standardized screens where all data were published – it is
316 mainly based on published results of single gene analyses. However, not all genetic screens have
317 reached saturation and not all genes detected in large-scale screens may have been further analyzed
318 and published. Hence, the number of genes in principle detectable in *D. melanogaster* might actually
319 be larger than the numbers extracted from FlyBase. In the iBeetle screen, in contrast, negative data
320 was systematically documented, such that this type of uncertainty is restricted to technical false
321 negative data, which we found to be around 15% in this first pass screen (Fig. 1). This uncertainty
322 could potentially increase the portion of *D. melanogaster* specific or conserved genes. Fourth,
323 theoretically there may be false positive data albeit restricted to the set of genes detected in both
324 species. The reason is that iBeetle was a first pass screen, where we aimed at reducing false negative
325 data with the tradeoff that false positive data are enriched (Schmitt-Engel et al., 2015). Although
326 finding similar phenotypes in two different species will not in many cases be false positive, we tried
327 to minimize this error by manually checking the annotations of the respective genes, excluding those
328 that showed a phenotype with low penetrance or in combination with many other defects indicating
329 a non-specific effect. Of note, the issue of false positives is restricted to the genes detected in both
330 species (column 2; based on Fig. 3). It does not apply to those genes detected only in the beetle but
331 not the fly (column 3; based on Fig. 2) because in this case, all phenotypes were confirmed by
332 independent experiments with non-overlapping dsRNA fragments in different genetic backgrounds
333 such that false positive results are excluded. In summary, while there are a number of uncertainties

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334 that we could not clarify with available data or methods, most of these uncertainties hint at
335 underestimation rather than overestimation of functional divergence between fly and beetle.

336

337 **Technical characteristics contribute to the detection of unequal gene sets**

338 Our numbers reveal that functionally comparable gene sets in two quite closely related model
339 systems are far from identical. A question of obvious biological relevance but not easily resolved is:
340 to which degree do these differences reflect biologically meaningful divergence of gene functions, or
341 alternatively, simply result from technical problems, i.e. reflect different strengths and weaknesses of
342 the respective screening methods and model systems?

343 As discussed above, some degree of false negative data may be expected in both model systems. In
344 case of the iBeetle screen, this will be restricted to technical false negative data. In the *D.*
345 *melanogaster* field, there may be additional false negative data due to the lack of saturation of
346 screens and/or lack of reporting of genes that were not studied in detail. However, given the extent
347 and comprehensiveness of work in the *D. melanogaster* field we feel that this might not be of high
348 relevance. As to different strengths of screening procedures, it is certainly true that the way screens
349 are performed influences what sets of genes can be detected. For instance, our parental RNAi
350 approach knocked down both, maternal and zygotic contributions while some classic *D.*
351 *melanogaster* screens affected only the zygotic contribution. Hence, genes where maternal
352 contribution rescues the embryonic phenotype are easily missed in the fly but not the beetle. For
353 instance, parental RNAi knocking down components of the aPKC complex leads to severe early
354 disruption of embryogenesis in *T. castaneum* while in respective *D. melanogaster* mutants almost no
355 defects are seen on the cuticle level (A. Wodarz, unpublished observation). Conversely, our RNAi
356 screen depended on the accuracy of gene annotations and our approach of screening for several
357 processes in parallel may have reduced detection sensitivity. One striking example for the different
358 strengths of screening designs is provided by wing blister phenotypes. In the first part of the iBeetle
359 screen we detected 34 genes showing wing blister phenotypes where 14 did not have related GO
360 term annotation at FlyBase and 5 did not have any GO annotation at all. Seven of these genes were
361 subsequently tested by RNAi lines in *D. melanogaster* where four of them indeed showed a related
362 phenotype. Likewise, some wing blister genes from *D. melanogaster* were not annotated in the
363 iBeetle screen. When we checked more specifically, this was often due to lethality of the animal
364 before the formation of wings (Schmitt-Engel et al., 2015). When we varied the timing of injection,
365 two of those knock-downs elicited wing blister phenotypes also in *T. castaneum* (Schmitt-Engel et al.,
366 2015). These data show that details of the screening procedure influence the subset of genes that is
367 detected.

368 **Evolutionary divergence of gene function and derivedness of *Drosophila* 369 biology may be larger than appreciated**

370 Most relevant for the field of functional genetics is our conclusion that the degree of divergence of
371 gene functions is larger than previously assumed. Therefore, some genes are detected only in one
372 species because the gene's function is not required for that process in the other. Indeed, there is
373 evidence supporting this view. In a recent study, a number of muscle genes identified in the iBeetle
374 screen were more closely investigated in *D. melanogaster* (Schultheis et al., 2019a; Schultheis et al.,
375 2019b). Despite some efforts, the negative data for fly orthologs appeared to be real negative. For
376 example, null mutations of one of the genes found in our beetle, *nostrin*, did not elicit a phenotype in

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377 *D. melanogaster* unless combined with a mutation of a related F-bar protein *Cip4*. Likewise, *Rbm24*
378 displays strong RNAi and mutant phenotypes in *T. castaneum* and vertebrates, respectively, but *D.*
379 *melanogaster* is lacking an *Rbm24* ortholog, and functional compensation by paralogs was suggested
380 to occur during *D. melanogaster* muscle development. Other genes including *kahuli* and *unc-76* are
381 expressed in the *D. melanogaster* mesoderm but only showed very subtle somatic muscle
382 phenotypes, if any, in Mef2-GAL4 driven RNAi experiments or with CRISPR/Cas9 induced mutations,
383 respectively (see Materials & Methods). By contrast, their beetle counterparts had strong and
384 penetrant phenotypes in single knock-downs (Schultheis, 2016; Schultheis et al., 2019a; Schultheis et
385 al., 2019b). These data suggest that the function of genes or their relative contribution to this
386 biological process have changed significantly. They also indicate that the single gene view may be
387 limited. Phenotypes depend on networks of interacting genes and this may allow for changes and
388 replacements of individual components while the overall network structure is maintained. There are
389 more striking examples of gene function changes. The gene *germ cell-less* was detected in the iBeetle
390 screen to govern anterior-posterior axis formation in the beetle while in *D. melanogaster* it is
391 required for the formation of the posterior germ-cells (Ansari et al., 2018). Also, the *D. melanogaster*
392 textbook example of a developmental morphogen *bicoid* does not even exist in *T. castaneum* (Brown
393 et al., 2001) and yet other genes were found to act as anterior determinants in other flies (Klomp et
394 al., 2015; Yoon et al., 2019). Along the same lines, the genes *forkhead* and *buttonhead* do not appear
395 to be required for anterior patterning in *T. castaneum* but are essential in flies (Kittelman et al.,
396 2013; Schinko et al., 2008; Weigel et al., 1989; Wimmer et al., 1997).

397 These findings with respect to specific genes add to a number of observations arguing for a
398 comparatively high degree of derivedness of fly biology. The number of genes is much smaller in *D.*
399 *melanogaster* (appr. 14,000) compared to *T. castaneum* (appr. 16,500). Further, a number of
400 developmental processes are represented in a more insect-typical way in *T. castaneum* like for
401 instance segmentation (Tautz et al., 1994), head (Posnien et al., 2010) and leg development, brain
402 development (Farnworth et al., 2019), extraembryonic tissue movements (Panfilio, 2008) and mode
403 of metamorphosis (Snodgrass, 1954). In most cases, the situation in the fly is simplified and
404 streamlined for faster development.

405 We think that these biological difference lead to divergence in gene function, which we just started
406 to uncover. Given the large divergence of gene sets found in different screening systems, and the
407 documented cases of biological divergence of gene function, we propose that a more systematic
408 investigation on the divergence of gene function is needed and that hypothesis independent
409 screening now possible in *T. castaneum* may be helpful in that endeavor.

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