Interactions between evolved pesticide resistance and pesticide exposure influence immunity against pathogens

Running Title: Pesticide and pathogen interactions

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Abstract

Pesticide resistance readily evolves in natural insect populations and often coopts the same stress, detoxification, and immune pathways involved in physiological responses against primary pathogen and pesticide exposure. As a result, resistance evolution can alter antagonism or facilitation among chemical and biological pest control strategies in directions that remain difficult to predict. To investigate the interactive effects of chemical pesticide resistance, exposure, and bacterial infection on insect phenotypes, we experimentally evolved resistance to two different classes of pesticides (organophosphates and pyrethroids) in the red flour beetle, *Tribolium castaneum*. We exposed pesticide susceptible and resistant lines to pesticides, the entomopathogen and biocontrol agent *Bacillus thuringiensis* (Bt), or both. Pesticide resistance and Bt exposure were individually associated with slower development, indicating sub-lethal fitness costs of resistance and infection, respectively. After organophosphate exposure, however, beetles developed more quickly and were more likely to survive if also exposed to Bt. We used RNAseq to examine the interactive effects of pesticide resistance, pesticide exposure, and Bt exposure on gene expression. Pyrethroid-resistant insects exhibited dampened immune responses to Bt infection relative to susceptible ones. In a similar vein, simultaneous exposure to organophosphates and Bt resulted in muted stress-associated transcriptional responses compared to exposure with only one factor. Our results suggest that direct and host-mediated indirect interactions among pathogens and pesticides may buffer the cost of exposure to host fitness-associated traits within generations but exacerbate trade-offs over evolutionary time.

Introduction

Control of insect agricultural pests and disease vectors remains a central problem worldwide as widespread use of chemical pesticides has led to the rise of pesticide resistance amongst important insect species (Bass & Jones, 2018). Insect resistance management (IRM) and integrative pest management (IPM) strategies strive to overcome problems of evolved resistance by alternating or implementing the use of both chemical pesticides and biological control measures (Sappington & Miller, 2017; Zhu et al., 2016). Entomopathogens, including bacteria, fungi, and viruses, can complement or even replace the use of chemical pesticides by increasing the mortality rate and slowing development and reproduction in pest and vector populations (Cory, 2017; Liu et al., 2017). The physiological effects of chemical and microbial control agents are not completely independent, however, as both can activate stress, detoxification, and immune responses (James & Xu, 2012). Since the evolution of pesticide resistance also often arises through these mechanisms (Minetti et al., 2020), host responses to chemical and microbial control agents could mediate facilitation or antagonism among control strategies on both proximate and evolutionary time scales (Booton et al., 2019; Hite & Cressler, 2018; Tompkins et al., 2001; Wolz et al., 2021). A better understanding of the molecular basis and phenotypic outcomes of these interactions would improve the design and predictive power of pest and vector control strategies (Bass & Jones, 2018; Sappington & Miller, 2017) while also providing fundamental insight into the integration and evolution of organismal stress and immune responses.

Pesticides target a diverse set of physiological functions in insects ranging from neurotoxic activity to the regulation of growth and development. The mechanisms of pesticide resistance show a similarly diverse set of solutions within and among pesticide classes, including

44 target-site modifications, increased metabolic detoxification, and cuticular modifications (Boyer
45 et al., 2012; Rösner et al., 2020). Organophosphates (OP), which inhibit acetylcholinesterase
46 (AChE) to overexcite cholinergic synapses (Casida & Durkin, 2013), and pyrethroids (Pyr),
47 which disrupt voltage-gated sodium channel (vgsc) function (Davies et al., 2007), are two
48 pesticide classes widely used in agricultural systems and against disease vectors. Target-site
49 mutations and AChE gene duplications have been described for several OP-resistant insects
50 (Siegfried & Scharf, 2001), while Pyr resistance has been associated with mutations in the
51 voltage-gated sodium channel (Soderlund, 2011). Target site mutations are not the only path to
52 resistance, however. OP and Pyr pesticides are mainly detoxified through oxidation and
53 hydrolysis, and resistance associated with differential expression of diverse canonical
54 detoxification genes, including cytochrome P450s, esterases, and glutathione S-transferases
55 (GSTs), has also been described for both pesticide types (Boyer et al., 2012; Meinke et al., 2021;
56 Oakeshott et al., 2013; Pavlidi et al., 2018; Sogorb & Vilanova, 2002). Moreover, recent studies
57 have implicated changes in cuticle and serine endopeptidase gene expression in increased
58 resistance to penetration by OP and Pyr pesticides (David et al., 2014; Lilly et al., 2016;
59 Tandonnet et al., 2020; Zimmer et al., 2017).
60 Recent evidence suggests that the mechanisms of pesticide resistance could also impact
61 immune and physiological responses against parasites (Minetti et al., 2020). For example,
62 esterase-mediated pesticide resistance in *Culex pipiens* is associated with changes in immune
63 gene expression including constitutive upregulation of antimicrobial peptide (AMP) and nitric
64 oxide synthase (NOS) genes (Vézilier et al., 2013). Moreover, resistance-associated constitutive
65 changes in metabolic detoxification mechanisms like cytochrome P450s or GSTs can alter
66 concentrations of damaging reactive oxygen species (ROS) that a pathogen would encounter

67 within the host (Minetti et al., 2020; Pang et al., 2021) and influence the success of pathogen
68 colonization, growth, and transmission (Minetti et al., 2020; Ndo et al., 2019; Wang et al., 2016).
69 For example, changes in cap‘n’collar transcription factor expression have been shown to alter
70 detoxification gene expression and increase ROS levels, thereby conferring pesticide resistance
71 in several species (Kalsi & Palli, 2017; Lu et al., 2020; Misra et al., 2011) while also modifying
72 vector competence in *Aedes aegypti* (Bottino-Rojas et al., 2018). Populations of OP and Pyr
73 resistant mosquito strains in possession of target-site mutant *ace-1* (AChE) and *kdr* (vgsc)
74 alleles, respectively, support a higher prevalence of *Plasmodium falciparum* parasites, but *kdr*
75 mutations were also associated with reduced midgut oocyst burden in infected individuals (Alout
76 et al., 2013).

77 Even in the absence of evolved resistance, host exposure to pesticides may impact
78 pathogen growth directly through contact with toxins or indirectly through the induction of insect
79 detoxification enzymes (Duke, 2018; Minetti et al., 2020). Exposure to pesticides can also have
80 complex effects on components of the cellular, humoral, and oxidative stress responses of host
81 immunity (James & Xu, 2012). For example, exposure to OP pesticides has been associated with
82 increased hemocyte numbers and phenoloxidase (PO) and encapsulation activity in wax moth
83 (*Galleria mellonella*) and Colorado potato beetle (*Leptinotarsa decemlineata*) larvae (Dubovskiy
84 et al., 2014). However, dual exposure to OP and a pathogenic virus in silkworm larvae (*Bombyx*
85 *mori*) resulted in increased mortality and differential expression of oxidative stress and AMP
86 genes (Gu et al., 2016). Exposure to Pyr pesticides, meanwhile, has been associated with
87 increased melanization responses and decreased replication of *Escherichia coli* bacteria (Hauser
88 & Koella, 2020) and decreased *P. falciparum* infection prevalence and intensity in *A. gambiae*
89 (Kristan et al., 2018). Pyr exposure is also hypothesized to impact the production of other

immune responses including serine proteases, lytic enzymes including esterases and lysozymes, and reactive oxidative stress responses (James & Xu, 2012).

Clearly, both pesticide resistance and exposure independently have important effects on insect immunity against pathogens. However, it remains an open question whether resistance and exposure influence host-microbe interactions in the same direction, and whether this influence arises through the same or different mechanisms. To address this gap in knowledge, we experimentally evolved resistance to two different classes of pesticides (OP and Pyr) in the red flour beetle (*Tribolium castaneum*), an emerging model for studies on insect genomics, immunity, and resistance (Herndon et al., 2020; Rösner et al., 2020). As a stored-product pest, *T. castaneum* may be directly or indirectly exposed to the pesticides used to combat a variety of pests that co-inhabit stored grain facilities and that impose selection for resistance.

To investigate the interactive effects of pesticide resistance, exposure, and infection, we exposed our evolved lines to *Bacillus thuringiensis* (Bt), an entomopathogenic Gram-positive bacterium that has been developed into a widely used biopesticide (Raymond, 2017; Burges & Hurst, 1977; Sansinenea, 2012). While much of the research on host-Bt interactions focuses on the lethal biocontrol aspects, natural strains also occur in the environment and vary in lethality, thereby representing a selective force within natural populations (Milutinović et al., 2014; Milutinović et al., 2013). Insect resistance to Bt commonly results from changes in specific toxin-receptor interactions (Banerjee et al., 2017; Pardo-López et al., 2013), providing little expectation of cross-resistance with chemical pesticides (Siegwart et al., 2015). However, Bt-resistance has been associated with increased susceptibility to bacteria-derived pesticides (Xiao et al., 2016), and exposure to Bt has been shown to increase susceptibility to viruses and entomopathogenic nematodes (Carvalho et al., 2021; Mastore et al., 2021; Moltini-Conclois et

113 al., 2018). The immune responses of susceptible *T. castaneum* to oral Bt infection involve
114 significant upregulation of a suite of immune, stress, and developmental genes (Behrens et al.,
115 2014; Ferro et al., 2019; Greenwood et al., 2017; Tate & Graham, 2017; reviewed in Pinos et al.,
116 2021) that potentially overlap with the response to chemical pesticides and the mechanisms of
117 pesticide resistance. However, the mode of infection may have important implications for
118 interactions with pesticide resistance as *T. castaneum* demonstrated contrasting patterns of
119 expression of infection-related genes dependent on the route of infection, *i.e.,* oral compared to
120 septic infection (Behrens et al., 2014).
121 To explore the complex interactions between pesticide resistance and exposure to
122 pesticides and pathogens, we first investigated the main and interactive effects of selection
123 regime (pesticide resistant and susceptible) and pesticide exposure on host fitness-associated
124 phenotypes after Bt infection. Having established these phenotypes, we turned to transcriptional
125 data to identify potential mechanisms that could explain the observed phenotypes. We first asked
126 whether evolution regime, *i.e.*, evolved pesticide resistance, alone influences constitutive gene
127 expression and the transcriptional response to infection in the absence of pesticides. We next
128 included pesticide exposure into the regime-by-infection interaction to investigate whether
129 pesticides facilitate or antagonize the host response to infection, and whether differential gene
130 expression depends on the experimental evolution regime. For both investigations, we compared
131 the results of OP and Pyr treatments to determine whether evolved resistance or exposure to
132 pesticides with different physiological targets exert different effects on host-pathogen
133 interactions. Our study provides a comprehensive window into the physiological and
134 evolutionary processes that shape interactions among two important ecological stressors.
135

136 **Methods and Materials**

137 *T. castaneum Control and Pesticide-selected Colony Maintenance*

138 We used six *T. castaneum* populations (Adairville, Coffee, Dorris, RR, Snavely, WF
139 Ware) collected from stored grain facilities in the southeast USA (Jent et al., 2019). In these
140 environments with other stored product pests, *T. castaneum* are exposed to a variety of parasites,
141 both within and between species in the community, and may be incidentally or intentionally
142 exposed to a range of different pesticides. Beetle colonies had been maintained under laboratory
143 control conditions (standard diet of whole wheat flour + 5% yeast; 30°C; 70% humidity; in the
144 dark) for at least 20 generations prior to the start of the experiment.

145 For each of the six ancestral *T. castaneum* populations, we initially exposed
146 approximately 200 larvae per population to control conditions (no pesticides) or to pesticides
147 (organophosphate [OP] or pyrethroid [Pyr]) (Suppl. Fig. 1; 18 populations total). Initial exposure
148 doses were calculated as one tenth the manufacturer's recommended dose (OP [Malathion 50%
149 EC, Southern Ag]- 0.103 mg/ml; Pyr [Demon WP, 40% cypermethrin, Syngenta]- 0.0251
150 mg/ml). We approximated LC50 pesticide concentrations for ancestral populations using dose
151 response curves (Birnbaum et al., 2021), and adjusted these after the second generation (LC50:
152 5.14 mg/ml OP and 0.188 mg/ml Pyr). To create pesticide exposure diets, we combined 0.15
153 g/ml standard diet with 10 ml of pesticide solutions diluted in DI water (Milutinović et al., 2013).
154 This diet slurry was added to 1 L plastic colony containers and allowed to dry overnight at 55°C.
155 For each pesticide-selected population, we allowed approximately 200 pupae and larvae to
156 develop on the pesticide diets for one week before supplementing colonies with 100ml of fresh
157 standard diet. We transferred individuals to new pesticide exposure containers every four weeks.
158 After six generations of selection, we tested for increased pesticide resistance in F2 larvae

159 (reared for two generations without selection to avoid parental effects) from the pesticide-regime
160 (here, used interchangeably with evolution-regime) populations and found these populations had
161 significantly increased survival against pesticides compared to control-regime populations
162 (Birnbaum et al., 2021). Meanwhile, we continued selection in all populations for another two
163 generations (to generation 8).
164
165 *Bt Spore Culture Preparation*
166 To orally expose beetles to Bt, we created a spore culture supernatant according to
167 previously described protocols (Milutinović et al., 2013). Briefly, we inoculated 5 ml LB liquid
168 media with *Bacillus thuringiensis subsp. morrisoni bv. tenebrionis* (*Btt;* obtained from the
169 *Bacillus* Genetic Stock Center, Columbus, OH) from glycerol stock and incubated overnight at
170 30°C. We streaked liquid *Btt* from the overnight culture onto LB agar plates and incubated the
171 plates overnight at 30°C. The next day, 18 individual colonies were picked and each inoculated
172 into 5 ml of Bt medium (w/V–0.75% bacto peptone (Sigma), 0.1% glucose, 0.34% KH_2PO_4,
173 0.435% K_2HPO_4) with the addition of 25 µL of salt solution (w/V–2.46% $MgSO_4$, 0.04%
174 $MnSO_4$, 0.28% $ZnSO_4$, and 0.40% $FeSO_4$) and 6.25 µL of 1M $CaCl_2$•$2H_2O$; these cultures and a
175 negative control (Bt medium only) were allowed to grow overnight on a bacterial shaker at 30°C,
176 200 rpm. The following day, 1 ml of the liquid overnight culture was inoculated into 200 ml Bt
177 medium with the addition of 1 ml salt solution and 50 µl calcium chloride. These cultures and a
178 negative control were incubated for seven days in darkness on a bacterial shaker at 30°C, 200
179 rpm; additional 1 ml salts solution and 50 µl calcium chloride were added after four days of
180 incubation. Spore cultures were centrifuged at 4,000 rpm for 15 minutes, washed once with

181 insect saline, and resuspended in Millipore H_2O. We calculated the final spore concentration of
182 $8.62*10^9$ cells/ml by counting spores using a Hausser Scientific counting chamber.
183
184 *Pesticide and Bt Spore Oral Diet Preparation*
185 We prepared control diet by mixing 0.15 mg/ml standard diet (described above) in DI
186 H_2O. We prepared pesticide diets by combining approximate LC50 pesticide dilutions in H_2O
187 with 0.15 mg/ml standard diet (5.14 mg/ml OP and 0.188 mg/ml Pyr). *Btt* diet was prepared by
188 mixing the previously described $8.62*10^9$ cells/ml spore culture suspension with 0.15 mg/ml
189 standard diet. Pesticide + *Btt* diet was prepared by diluting 9.75 µl/ml OP or 0.188 mg/ml Pyr in
190 the spore culture suspension with 0.15 mg/ml standard diet. Oral diet plates were prepared by
191 pipetting 50 µl of the appropriate treatment (Suppl. Fig. 1; control, control + *Btt*, OP, OP + *Btt*,
192 Pyr, Pyr + *Btt*) into 96-well cell culture plates. Plates were covered and allowed to dry overnight
193 at 55°C.
194 We investigated the effects of diet disk preparation and pesticide treatments on Bt spore
195 germination by preparing control, *Btt,* and *Btt* + pesticide diet disks as described above and
196 counting the number of formed colonies from each treatment (*Btt*, *Btt* + OP or *Btt* + Pyr n=10;
197 control n=6). We conducted the spore germination on two separate days. We stored dried diet
198 disks for four or five days respectively at 30°C in the dark and then dissolved individual disks in
199 1 mL ultrapure water. To initiate germination and aid in the dissolving of the disks, we heated
200 the samples to 50°C for ten minutes and then immediately plated the samples. For the bacteria
201 culture plates, we spread 100µl of the final dilution onto LB agar, included a negative control
202 with only water, incubated plates over night at 30°C and counted all *Btt* colonies after 15 hours.
203 On the first day, we cultured bacteria from a $1:10^7$ dilution in ultrapure water. On the second day,

204 we used a 1:10^6 dilution, to ensure that sufficient colonies would grow. We analyzed the effect of
205 treatment on the number of colony forming units (CFUs) using a generalized linear mixed model
206 with day as a random factor and a negative binomial distribution.
207
208 *Btt in vitro Growth Curve Assays*
209 To measure *in vitro* effects of pesticides on Bt growth, we performed growth curve
210 analyses for OP and Pyr exposure separately. For each analysis, *Btt* from a glycerol stock was
211 freshly streaked onto Nutrient Broth (NB) agar plates and allowed to grow overnight at 30°C.
212 We grew overnight cultures from single colony inoculates (n = 9) in 5 ml NB liquid media at
213 30°C, 200 rpm; cultures were incubated for only 16 hours to avoid entrance into sporulation
214 phase. The following day, we grew log phase bacterial cultures by inoculating 100 µl of the
215 overnight culture into 2.9 ml NB liquid media and incubated for 3.5 hours at 30°C, 200 rpm.
216 To replicate *in vivo* conditions, each growth curve analysis included control NB, 1:10
217 pesticide NB dilution, and 1:100 NB pesticide dilution treatments based on larval exposure OP
218 and Pyr pesticide concentrations (described above). We added 200 µl of the appropriate
219 treatment solution to a 96-well CYTO-One spectrophotometer plate. An aliquot of log phase
220 bacterial culture (n = 9) was added to each well to achieve an initial OD_{600} = 0.4 (3 technical
221 replicates/ culture). OD_{600} bacterial growth was measured every 15 minutes for 17 hours using a
222 BioTek Epoch 2 spectrophotometer (30°C). Three or four independent growth curve replicates
223 were performed for each pesticide.
224 We obtained growth curve metrics using the R package growthcurver (Sprouffske &
225 Wagner, 2016). For OP and Pyr separately, we analyzed the individual effects of replicate and
226 treatment on growth rate values (r) obtained from growthcurver using non-parametric Kruskal-

227 Wallis tests. We measured the effect of pesticide treatment separately for each replicate if the
228 effect of replicate was significant.
229
230 *Exposure of Control and Pesticide-selected Populations to Pesticide and Bt Oral Diets*
231 After eight generations of selection, we generated same-age F2 larvae from pesticide-
232 selected and susceptible colonies. Approximately 200 adults from Gen. 8 pesticide-selected and
233 susceptible control colonies were allowed to reproduce on control diet for three days. We
234 removed these F0 adults and allowed offspring to develop to F1 adults. To generate F2
235 experimental larvae, we placed 4-5 replicates of 80 adults per selection line in 100 mm petri
236 dishes with control diet and allowed them to reproduce for 24 hours. F2 larvae were allowed to
237 develop for 12 days.
238 We placed twelve day old larvae (~4 mm long) on oral diet plates prepared as described
239 in the previous section (Suppl. Fig. 1; n = 96/ population/ treatment). Half of the plate (n = 48)
240 was monitored daily for three weeks for survival and development. Individuals in the remaining
241 half were sampled after two days of exposure prior to the onset of mortality and used for
242 RNAseq analyses (described below).
243
244 *Statistical Methods for Fitness Experiments*
245 The effects of selection regime (Regime), pesticide exposure (PTx), Bt exposure (BtTx),
246 and interactions between the three factors on beetle survival and development were investigated
247 using population as a random factor in Cox mixed effects survival models with the package
248 coxme (Therneau, 2015) in R. For both time to death and time to develop into adulthood, we
249 evaluated the individual effects of pesticide exposure (PTx), selection regime (Regime), and Bt

250 exposure (BtTx), and the interactions between selection regime and pesticide exposure
251 (Reg:PTx), selection regime and Bt exposure (Reg:BtTx), and co-exposure to pesticides and Bt
252 (PTx:BtTx). The full model for both time to death and adult development time is as follows:
253 Surv(Day, Status) ~ (PTx + BtTx + Regime + Regime:PTx + Regime:BtTx + PTx:BtTx +
254 (1|Pop)). Hazard ratios were calculated from coxme survival models and were plotted using the
255 sjPlot package in R (Lüdecke , 2021).
256
257 *Septic Infection Experiment*
258 Given the impact of infection route on immune gene expression (Behrens et al., 2014),
259 we wanted to clarify whether the interaction between pesticides and infection outcomes might be
260 infection route-dependent. We selected two populations, Coffee and Dorris, as representative
261 populations that may demonstrate interactions with between pesticide resistance and exposure on
262 infection outcomes. Therefore, we exposed 14-day old larvae from control- and OP-regimes to
263 control and OP oral diets (as described above) for three days, and then septically infected
264 individuals with *Btt* and recorded their survival.
265 We produced a mixture of *Btt* cultures of vegetative cells from both the logistic growth
266 and early stationary phase for septic infections as previously described (Jent et al., 2019), which
267 caused around 50% mortality in preliminary experiments. We pricked larvae laterally between
268 the head and second segment with an ultra-fine borosilicate needle dipped in the bacterial
269 suspension and recorded survival after 24 hours. We repeated the experiment three times with 20
270 individuals per population (Coffee or Dorris), selection regime (OP selected and control) and
271 pesticide treatment (OP exposure and control) each (n = 480).

272 Differences in survival between the control group (control selection regime, no pesticide)
273 and the other groups were analyzed in a GLMM with experiment (block) and population as
274 random factors and a binomial error distribution (Bates et al., 2015).
275
276 *RNA Extractions and Sequencing*
277 Samples for 3' TagSeq (Lohman et al., 2016) were collected after two days of exposure
278 to oral diets for three representative populations (Coffee, Dorris, Snavely), prior to the main
279 onset of mortality. For each population, regime, and treatment, we collected nine surviving
280 larvae and immediately stored them at -80°C until processing, and subsequently pooled larvae
281 into three replicates of three larvae each. We extracted total RNA using a Qiagen RNeasy Mini
282 kit according to manufacturer protocols and confirmed nucleic acid concentration and purity
283 using a Nanodrop. We depleted remaining DNA using the Invitrogen RNaqueous Micro DNase
284 treatment according to manufacturer protocols. We confirmed the concentration and quality of
285 these DNA-depleted RNA samples using a Bioanlayzer (RIN > 9). We generated single indexed
286 3' TagSeq libraries from 1 μg total RNA using the Lexogen QuantSeq 3' mRNA-Seq Library
287 Prep Kit according to manufacturer's protocols; libraries were amplified using 15 PCR cycles.
288 Library concentration and quality were confirmed prior to PE100 sequencing on four runs on the
289 NextSeq platform (VANTAGE, Vanderbilt University).
290
291 *RNAseq Analyses*
292 Adapters were trimmed from the raw mRNAseq reads and low-quality reads were
293 removed using fastqc (Andrews 2010); an average of 10,869,280 reads per sample were retained
294 (Suppl. Table 1). Sequence reads are deposited in the NCBI SRA under the BioProject

295 Accessions: PRJNA753142, PRJNA756317. We took advantage of the well-annotated, published
296 genome for *T. castaneum* (Herndon et al., 2020) to align trimmed reads to the most recent *T.*
297 *castaneum* assembly (Tcas5.2) using BWA-MEM (avg. reads mapped = 84.89%, s.d. = 0.023;
298 Suppl. Table 1) (Li, 2013). Gene count files were generated using samtools (Li et al., 2009). We
299 used DESeq2 (Love et al., 2014) to perform differential expression analyses.

300 First, to investigate the effects of Bt exposure and pesticide selection regime on gene
301 expression in the absence of pesticide exposure, we subset only pesticide-free and pesticide-free
302 + Bt libraries and examined the effects of selection regime, Bt exposure, and their interaction on
303 gene expression (no pesticide model form: count matrix ~ Reg + BtTx + Reg:BtTx). Next, we
304 analyzed the main effects of pesticide exposure, pesticide selection regime, and Bt exposure and
305 the interactive effects between selection regime and pesticide exposure, selection regime and Bt
306 exposure, and pesticide and Bt exposure for OP and Pyr separately (OP and Pyr model form:
307 count matrix ~ PTx + Reg + BtTx + Reg:PTx + Reg:BtTx + PTx:BtTx). For each factor, the
308 control selection regime, control pesticide treatment, or control Bt treatment were set as the
309 baseline comparisons.

310 Differentially expressed transcripts were annotated using the Ensembl *T. castaneum*
311 database and the R package 'biomaRt' (Durinck et al., 2009). We manually curated the annotated
312 gene lists to categorize differentially expressed transcripts into groups potentially impacted by
313 pesticide resistance and exposure and Bt treatment such as those involved in detoxification,
314 immunity, development, and cuticle associated transcripts (Suppl. Table 2). Gene ontology (GO)
315 enrichment analyses for the subsets of significantly differentially expressed gene sets were
316 performed using the online Gene Ontology Resource (Ashburner et al., 2000; Gene Ontology
317 Consortium, 2021).

318 To analyze global patterns of gene expression, we used a weighted gene co-expression
319 network analysis with the R package 'wgcna' (Langfelder & Horvath, 2008). This approach
320 allows us to group genes with correlated expression patterns into modules and statistically
321 associate gene module expression with experimental factors. We constructed the initial network
322 using all replicate samples for all treatments (n = 126) with a variance stabilizing transformation.
323 A soft thresholding power of 6 was used to construct the topological overlap matrix, and we then
324 identified modules with highly correlated module expression patterns ($p < 0.05$).
325
326 **Results**
327 To investigate how evolved pesticide resistance and pesticide exposure impact host
328 interactions with pathogens, we exposed control-regime and pesticide-selected populations to
329 control and pesticide oral treatments, with and without Bt. We measured the main and interactive
330 effects of pesticide selection regime (Reg), pesticide treatment (PTx), and Bt treatment (BtTx) on
331 survival, development, and gene expression for OP and Pyr separately. In brief, we found that
332 pesticide selection resulted in increased resistance to pesticide exposure. Exposure to *Btt* did not
333 have an overall effect on survival but did delay development, and dual exposure to OP and *Btt*
334 resulted in improved survival compared to larvae exposed to only OP. Moreover, dual exposure
335 to either OP or Pyr and *Btt* resulted in faster development compared to individuals exposed to
336 only pesticides. Last, we identified antagonistic gene expression interactions between *Btt* and OP
337 exposure and *Btt* and Pyr selection that indicate changes in immune function in infected
338 individuals either exposed or selected with different pesticides.
339
340 *Effects of Pesticide Selection and Exposure to Pesticides and Bt on Survival and Development*

341 Overall, exposure to both pesticide types, OP (OP PTx) and Pyr (Pyr PTx), decreased
342 survival likelihood, while there was no main effect of Bt treatment (BtTx) on survival (Figs. 1A-
343 B; Suppl. Table 3). Compared to control-regime populations, pesticide selection significantly
344 improved survival after pesticide exposure, reinforcing previous observations of evolved
345 pesticide resistance (Figs. 1A-B, compare OP Reg:PTx and Pyr Reg:PTx; Suppl. Table 3;
346 Birnbaum et al., 2021). Interestingly, there was a positive significant interaction between OP and
347 Bt treatment (OP PTx:BtTx) indicating that larvae dually exposed to OP and *Btt* had improved
348 survival compared to larvae exposed to OP only (Fig. 1A; Suppl. Table 3). There was no
349 significant interaction between Pyr exposure and Bt treatment (Pyr PTx:BtTx) or between either
350 pesticide selection regime and Bt treatment (OP Reg:BtTx, Pyr Reg:BtTx) on survival (Figs. 1A-
351 B; Suppl. Table 3). Thus, while pesticide selection resulted in increased pesticide resistance,
352 pesticide selection regime did not interact with *Btt* exposure to influence survival compared to
353 control-regime populations. However, dual exposure to OP and *Btt* improved survival outcomes
354 compared to pesticide treatment alone.
355 Pesticide selection regime (OP Reg, Pyr Reg), pesticide exposure (OP PTx, Pyr PTx),
356 and Bt treatment (BtTx) individually delayed development, indicating a cost from each (Figs.
357 1C-D; Suppl. Table 3). There was no significant interaction between pesticide regime and Bt
358 treatment on development (OP Reg:BtTx, Pyr Reg:BtTx; Suppl. Table 3). However, resistant
359 individuals developed faster than susceptible individuals in the presence of pesticides, as
360 indicated by a significant interaction between pesticide regime and treatment (OP Reg:PTx, Pyr
361 Reg:PTx; Fig. 1C-D; Suppl Table 3). There was also a significant positive interaction effect
362 between both pesticide treatments and *Btt* exposure (OP PTx:BtTx, Pyr PTx:BtTx), indicating
363 that larvae exposed to both *Btt* and pesticide treatments developed faster than those exposed to

364 only pesticide or only *Btt* (Figs. 1C-D; Suppl. Table 3). Overall, exposure to pesticides delayed
365 development but this effect was mitigated in pesticide-selected individuals. *Btt* exposure
366 lengthened development time regardless of evolution regime but concurrent pesticide exposure
367 partially mitigated this effect.
368
369 *Direct Effects of Pesticides on Bt*
370 To investigate direct interactions between pesticides and Bt, we used *in vitro* growth
371 curves and viable spore counts of oral diet disks. *In vitro Btt* growth rate significantly increased
372 upon OP exposure, but there was no effect of Pyr exposure on *in vitro* growth rate (Suppl. Fig. 2;
373 Suppl. Table 4). The germination rates of *Btt* spores on the diet disks exhibited no significant
374 differences among control, OP, or Pyr pesticide diet treatments (Suppl. Fig. 3; Suppl. Table 5).
375
376 *The influence of pesticide exposure on route-specific infection outcomes*
377 While oral infection with *Btt* did not result in immediate excess mortality compared to
378 unexposed individuals, we considered the possibility that infection outcomes could be exposure
379 route specific. To determine whether the interaction between pesticide resistance, exposure to
380 pesticides, and infection outcomes might be route-dependent, we septically infected susceptible
381 and OP-selection regime larvae exposed to control and OP diets. Individuals from the susceptible
382 regime that were exposed to OP had lower survival against septic *Btt* infection compared to the
383 other groups (Fig. 2; Suppl. Table 6) indicating an outsized cost of combined OP exposure and
384 septic *Btt* infection in control regime larvae. This result contrasts with the improved survival
385 observed in the Bt x OP treatment interaction term after oral exposure in that after septic
386 infection, beetles co-exposed to OP do not have higher survival (Fig. 1A, OP PTx:BtTx).

387

388 *Overall Effects of Btt Exposure, Pesticide Selection, and Pesticide Exposure on Gene Expression*

389 Overall, we identified differentially expressed genes important in immunity in beetles
390 exposed to *Btt* compared to unexposed beetles. We also identified differentially expressed genes
391 implicated in detoxification and resistance processes in OP- and Pyr-regime populations
392 compared to control-regime populations. Few genes were significantly differentially expressed in
393 pesticide-regime populations exposed to *Btt* compared to control-regime populations. However,
394 Pyr-regime larvae infected with *Btt* exhibited dampened expression of immune genes. Dual
395 exposure to OP pesticides and *Btt* also resulted in dampened immune, cuticle, and detoxification
396 gene expression compared to expression with *Btt* infection alone. We expand on these main
397 results below.

398

399 *Effects of Btt Exposure and Pesticide Selection on Gene Expression in the Absence of Pesticide*
400 *Exposure*

401 We investigated the interaction between pesticide selection regime and *Btt* exposure on
402 gene expression in the absence of pesticides, as this provides information on the main host
403 response to oral *Btt* infection as well as whether the evolution of pesticide resistance modifies the
404 host response to *Btt*. Using only the data from individuals never exposed to pesticides, we
405 identified 104 genes that were differentially expressed in *Btt*-exposed individuals compared to
406 unexposed individuals (Fig. 3A; Bt Tx +). This gene set largely concurred with previously
407 published results on Bt infection in *T. castaneum* (Behrens et al., 2014; Greenwood et al., 2017;
408 Tate & Graham, 2017), and included the upregulation of AMPs (two attacins and two defensins),
409 a pathogenesis-related protein, and a cytochrome P450 (Fig. 4, no pesticide model BtTx). GO

410 enrichment analyses revealed the importance of immune system processes and defense against
411 bacteria, among other immune related terms, in the genes differentially expressed with *Btt*
412 treatment in the absence of pesticide exposure (Suppl. Fig. 4A, no pesticide model BtTx).
413 Only two transcripts (an elastin and one unannotated transcript) were upregulated upon
414 *Btt* infection in OP-selection regime individuals relative to susceptible ones (Fig. 3A, no
415 pesticide model OP Reg:BtTx interaction), indicating that evolved resistance to OP minimally
416 affects the baseline response to infection in the absence of pesticides. Fourteen transcripts were
417 differentially expressed in Pyr-selected individuals relative to susceptible-regime individuals
418 after *Btt* infection (Fig. 3A; no pesticide model Pyr Reg:BtTx interaction), including the
419 downregulation of two defensins, a pathogenesis-related protein, and a histidine-rich
420 glycoprotein (Fig. 4B, no pesticide model Pyr Reg:BtTx). Notably, these genes were
421 differentially expressed in the opposite direction in Pyr regime-*Btt* infected larvae compared to
422 gene expression induced by the main effect of *Btt* exposure, indicative of dampened expression
423 of these genes (Fig. 4B, no pesticide model Pyr Reg:BtTx vs. BtTx, *e.g.* Def: TC006250). GO
424 enrichment analysis revealed significant enrichment of humoral immune response and defense
425 response among the differentially expressed genes from this interaction term supporting the
426 impact on immune processes associated with the interaction effect of Pyr-selection regime with
427 *Btt* infection (Suppl. Fig. 4A, Pyr Reg:BtTx).
428
429 *Effects of Pesticide Selection and Dual Exposure to Pesticides and Pathogens on Gene*
430 *Expression*
431 Next, we introduced the pesticide-exposed samples into the analysis, still treating OP and
432 Pyr-associated treatments separately, to investigate interactions between pesticide selection

433 regime, pesticide exposure, and *Btt* exposure on gene expression. For both pesticide class
434 analyses, pesticide exposure had the greatest effect on gene expression (Figs. 3B-C; Pesticide Tx
435 OP or Pyr) and induced differential expression of many canonical detoxification genes, including
436 several cytochrome P450s, esterases, GSTs, UDP-glucuronosyltransferases (UGTs), and ABC
437 transporters (Suppl. Fig. 5; OP or Pyr PTx). Genes differentially expressed after both OP and Pyr
438 exposure were commonly enriched for GO processes involved in xenobiotic and detoxification
439 responses including oxidoreductase and serine hydrolase activity (Suppl. Fig. 4B).
440 Genes differentially expressed with respect to pesticide selection regime and to the
441 interaction between selection regime and pesticide treatment can identify genes associated with
442 pesticide resistance. Overall, Pyr-regime had a greater effect on differential gene expression
443 compared to OP-regime (Fig. 3; Regime OP or Pyr). Similarly, a greater number of genes were
444 differentially expressed with the interaction between Pyr regime and Pyr exposure compared to
445 OP regime and OP exposure (Figs. 3B [Regime OP, Pesticide Tx OP], 3C [Regime Pyr,
446 Pesticide Tx Pyr]). Both OP-regime and Pyr-regime induced differential expression of canonical
447 detoxification (e.g., P450s, esterases, GSTs, and ABC transporters) and cuticle-associated genes
448 (Suppl. Figs. 5, 6; OP or Pyr Reg). GO enrichment analyses revealed the importance of structural
449 constituent of cuticle for genes differentially expressed with OP regime and the interaction
450 between OP regime and OP exposure (Suppl. Fig. 4B; OP Reg and OP Reg:PTx). Pyr-regime
451 individuals differentially expressed genes enriched for molecular functions involved in
452 oxidoreductase activity, heme binding, and structural constituent of cuticle, while genes
453 differentially expressed with the interaction between Pyr regime and Pyr exposure were enriched
454 for these functions and also serine hydrolase activity (Suppl. Fig. 4B; Pyr Reg and Pyr Reg:PTx).
455 Thus, GO enrichment analyses revealed the common differential expression of cuticle-related

genes in both OP and Pyr resistance, while Pyr resistance was additionally associated with differential expression of oxidoreductase metabolic detoxification processes.

In the models containing pesticide exposure samples, no genes were differentially expressed with the interaction between OP regime and *Btt* treatment (Fig. 3B; Regime OP, Bt Tx +) providing further evidence that, here, evolved OP resistance had minimal effects on immune responses to *Btt*. In contrast, 61 genes were differentially expressed with the interaction between Pyr selection regime and *Btt* treatment (Fig. 3C; Regime Pyr, Bt Tx +), although there was no significant GO term enrichment. Mirroring patterns observed in the no-pesticide model, the model that includes pesticide exposure samples also indicates that Pyr regime larvae had lower induced expression of a defensin-like transcript compared to control-regime larvae when exposed to *Btt* (Fig. 5B).

When considering the interaction effect between pesticide exposure and *Btt* infection, 175 genes were differentially expressed with dual OP and *Btt* exposure (Fig. 3B; Pesticide Tx OP, Bt Tx +), and only one gene (an upregulated pupal cuticle protein) was differentially expressed with dual exposure to Pyr and *Btt* (Fig. 3C; Pesticide Tx Pyr, Bt Tx +). OP pesticide exposure interacted with *Btt* exposure to induce differential gene expression of immune, detoxification, cuticle, and development genes compared to expression induced by the main effect of *Btt* exposure (Fig. 4A; compare BtTx and OP PTx:BtTx). Further analysis revealed that these interaction effects were mainly driven by significantly dampened upregulation upon combined OP and *Btt* exposure compared to upregulation induced with *Btt* alone; transcripts upregulated with *Btt* alone were not differentially expressed or were downregulated with combined OP and *Btt* exposure (Fig. 4A). For example, while *Btt* exposure alone induced upregulation of two attacins, three cytochrome P450s, and several cuticular proteins, larvae exposed to both OP and

479 *Btt* had more modest differential expression of these same transcripts (e.g. *attacin-1* in Fig. 5A).
480 Genes differentially expressed with the interaction between OP treatment and *Btt* exposure were
481 enriched for GO terms involved in the molecular functions of oxidoreductase activity and
482 structural constituent of cuticle (Suppl. Fig. 4B; OP PTx:BtTx).
483
484 *Correlated patterns of gene expression using WGCNA*
485 Weighted gene correlation network analysis (WGCNA) allows for the identification of
486 genes with expression correlated and significantly associated with experimental factors. We
487 identified 25 modules containing genes with highly correlated expression, and several of these
488 modules significantly correlated with one or more experimental factors (OP Regime, Pyr
489 Regime, OP PTx, Pyr PTx, BtTx; Suppl. Fig. 7). We mapped differentially expressed transcripts
490 to each gene module and identified one that was significantly associated with all five
491 experimental factors (Suppl. Fig. 7; magenta module) and was also associated with a high
492 number of significantly differentially expressed transcripts (Fig. 4; Suppl. Fig. 7; 201 DE
493 transcripts/ 258 total). Thus, genes in this module displayed common expression patterns and
494 were overall upregulated with both OP and Pyr selection regimes and downregulated with OP,
495 Pyr, and *Btt* exposure (Suppl. Fig. 7). GO enrichment analysis of the magenta module transcripts
496 revealed the importance of genes involved in hydrolase activity (GO:0016787; FDR = $6.07*10^{-7}$)
497 and monooxygenase activity (GO:0004497; FDR = $1.81*10^{-6}$).
498
499 **Discussion**
500 Our study experimentally evolved pesticide resistance in flour beetles (*T. castaneum*) to
501 investigate the interactive effects of pesticide resistance and pesticide exposure on immunity and

502 host-pathogen interactions. Pesticide-resistant insects and insects exposed to *Btt* both developed
503 more slowly than their counterparts, indicating sub-lethal fitness costs to resistance and infection,
504 respectively. We found that Pyr-resistant insects exhibited dampened transcriptional immune
505 responses to *Btt* infection relative to their control-regime counterparts, although overall survival
506 and development did not differ among OP- and Pyr-resistant and susceptible insects after *Btt*
507 exposure. While the evolution of resistance did not substantially influence host infection
508 phenotypes, exposure to pesticides did. Specifically, we observed that larvae were more likely to
509 survive OP exposure if they were also orally exposed to *Btt*, with gene expression patterns
510 suggesting dampened transcriptional stress responses in those beetles. Taken together, our data
511 suggest that evolved resistance, pesticide exposure, and biopesticide infection all exert
512 contrasting effects on life history parameters that could ultimately influence the demography and
513 control of pest populations in complex ways. The factorial nature of our study design, moreover,
514 provides a new window into the physiological basis and fitness consequences of host responses
515 to overlapping environmental stressors.

516 Insects, including *T. castaneum*, can rapidly evolve resistance to pesticides through
517 mechanisms that enhance metabolic detoxification and cuticular modifications (Rösner et al.,
518 2020). Here, populations selected for pesticide resistance evolved it within 6-8 generations
519 (Birnbaum et al., 2021), demonstrating increased survival and faster development after pesticide
520 exposure compared to control-selected populations. Differential gene expression between
521 pesticide- and control- selection regimes suggested that OP resistance is associated with
522 constitutive changes in cuticle gene expression while Pyr resistance is associated with
523 oxidoreductase activity, heme binding, and cuticular modifications, consistent with previous
524 studies in OP and Pyr resistant insects (Carvalho et al., 2013; Tandonnet et al., 2020; Zimmer et

525 al., 2017). Thus, while both OP and Pyr resistance involved changes in cuticle gene expression,
526 Pyr resistance was also associated with changes in metabolic detoxification.

527 Oral *Btt* infection did not decrease survival in our study but was associated with slower
528 development in *T. castaneum* larvae, indicating a direct cost of infection or an indirect cost from
529 mounting an immune response, as previously observed in the same system (Tate & Graham,
530 2015) and in other insects challenged with Bt toxins (Hernández-Martínez et al., 2017; Rahman
531 et al., 2011). Previously documented variation in resistance in *T. castaneum* against Cry3
532 proteins effective against other Tenebrionid beetles may explain why *Btt* had negative effects on
533 development but did not instigate high mortality (Milutinović et al., 2013; Oppert et al., 2011).
534 Here, exposure to *Btt* induced the upregulation of AMPs (attacins and defensins) and one
535 pathogenesis-related protein as well as differential expression of detoxification genes, including
536 P450s and a CEH, GST, an ABC transporter, and several cuticle and development related genes.
537 Previous transcriptomic studies of oral Bt infection in *T. castaneum* have identified similar
538 transcripts, including attacin and defensin AMPs, a pathogenesis-related protein, a P450, and a
539 GST (Behrens et al., 2014; Greenwood et al., 2017), supporting the assumption that *Btt* oral
540 exposure in our experiments presented an immune challenge despite the lack of infection-
541 induced mortality.

542 Previous work has suggested that exposure to OP and Pyr pesticides can reduce defenses
543 against pathogens and stimulate an array of changes in humoral, cellular, and oxidative stress
544 components of immunity (Dubovskiy et al., 2014; Gu et al., 2016; Hauser & Koella, 2020). Our
545 results reveal a strong interaction effect between *Btt* and OP exposure that manifests at both
546 phenotype and gene expression levels. For example, dual oral exposure to *Btt* and OP improved
547 larval survival and partially rescued development time relative to OP exposure alone, without

548 exacerbating *Btt*-induced mortality. At the same time, *Btt* co-exposure modified the differential
549 expression of 175 genes relative to OP exposure alone (Fig. 3B). In contrast, Pyr exposure did
550 not influence survival with *Btt* infection, but larvae exposed to both Pyr and *Btt* also partially
551 rescued development time compared to those exposed only to pesticides. However, in contrast to
552 the effects on gene expression observed with dual OP and *Btt* exposure, *Btt* co-exposure with Pyr
553 had negligible effects on gene expression with the overexpression of only one cuticle-related
554 transcript. It is possible that the positive effects on development time seen with pesticide and *Btt*
555 co-exposure compared to single exposure to pesticides are part of an advantageous response to
556 increased risk (Roth & Kurtz, 2008) as has been seen in viral infected caterpillars reared on more
557 toxic diets (Smilanich et al., 2018).

558 There is also the potential for indirect antagonistic interactions between OP and *Btt*, e.g.
559 through induction of cross-protective host defenses. For example, while *Btt* exposure induced
560 upregulation of attacins, cytochrome P450s, and cuticle-related transcripts, combined OP and *Btt*
561 exposure dampened the expression of these transcripts. It is possible that this dampened
562 expression of immune and detoxification genes may underlie reductions in immunopathology or
563 ROS damage corresponding to increased fitness, or the benefits to fitness may be due to
564 differences in resource allocation when faced with dual pressures (Lazzaro & Tate, 2022).
565 Interestingly, single exposure to OP or *Btt* induced downregulation of an ABC transporter but
566 this transcript was upregulated with co-exposure to OP and *Btt*. ABC transporters have been
567 identified as receptors for Bt Cry proteins and aid in membrane pore formation (Güney et al.,
568 2021; Heckel, 2021; Pauchet et al., 2016), and the additional function of ABC transporters as
569 xenobiotic pumps may help to mediate Bt toxicity (Dermauw & Leeuwen, 2014; Wu et al.,
570 2019). It is also possible that OP and *Btt* interact directly, as would be the case if *Btt* actively

571 degrades OP for energy (Kumar et al., 2018) or if OP facilitates *Btt* germination rates. Our *in*
572 *vitro* data suggest that *Btt* vegetative cells in liquid medium replicate faster in the presence of
573 OP, lending credence to a direct interaction. On the other hand, there was no difference in
574 germinated spore counts between control-diet and OP-diet disks, suggesting that direct
575 interactions, if they matter *in vivo*, are not relevant until after the host ingests the diet.

576 Supporting the idea of localized cross-protective mechanisms, a previous study of oral vs.
577 septic *Btt* infection in *T. castaneum* demonstrated that oral infection induced greater changes in
578 cuticle-related gene expression compared to septic infection (Behrens et al., 2014). In our study,
579 OP-exposed individuals were not protected from greater mortality upon septic *Btt* infection,
580 suggesting that infection route has important implications for pesticide-pathogen interactions.
581 We found cuticle-associated gene expression changes induced by OP exposure, and cuticular
582 modifications of gut tissues may provide an additional layer of protection from oral Bt infection
583 (Raymond et al., 2010). Future work dissecting the relative importance of these direct and
584 indirect interactions will be crucial for refining integrative pest management (IPM) strategies
585 employing both chemical and biopesticides.

586 The impact of evolved pesticide resistance on immunity is still poorly understood
587 (Minetti et al., 2020), and in the absence of pesticides we did not observe any significant
588 interactions between pesticide resistance and Bt exposure on survival or development. Few genes
589 were differentially expressed between OP resistant and susceptible beetles after *Btt* exposure, but
590 *Btt* exposure in Pyr-resistant populations was associated with dampened upregulation of several
591 genes, including two defensins, a histidine-rich glycoprotein, and a pathogenesis-related protein,
592 that were more strongly upregulated in Pyr-susceptible populations after *Btt* exposure. It is also
593 possible that Pyr-resistant populations had constitutively higher immune gene expression

594 resulting in reduced immune activation upon Bt infection. Moreover, an apolipophorin-III
595 transcript, previously shown to be important in immunity against coleopteran-specific Bt toxins
596 (Contreras et al., 2013), was upregulated in Pyr-resistant populations. In contrast, cuticle-related
597 protein and neuropeptide transcripts were downregulated in Pyr-regime larvae exposed to *Btt*.
598 These immune gene changes associated with Pyr resistance may have important effects on
599 interactions with other pathogens, and future research into Pyr resistance interactions with other
600 pathogens may yield important insights.

601 While we did not observe any phenotypic differences with pesticide selection regime and
602 *Btt* infection here, we also did not observe mortality from *Btt* alone and it is possible that the
603 immune challenge was not strong enough to elicit an interaction with resistance mechanisms.
604 However, the differential expression of immune and cuticle functions in Pyr-regime larvae
605 exposed to *Btt* could have important implications for insect interactions with other pathogens or
606 xenobiotic pressures by modifying detoxification capabilities or providing an additional barrier
607 against infection or toxins (Erlandson et al., 2019; James & Xu, 2012; Lilly et al., 2016).
608 Furthermore, changes in oxidoreductase activity observed after Pyr selection are likely to alter
609 the status of reactive oxygen species (ROS) within the host and may impact immunity against
610 different pathogens (Minetti et al., 2020; Wang et al., 2016). Finally, it is possible that adaptation
611 to pesticides could constrain host evolutionary responses to pathogens, or vice versa. Future
612 experiments could simultaneously select for dual resistance to both pesticides and pathogens to
613 see if evolutionary trajectories are similar or constrained relative to selection under a single
614 stressor.

615

616 *Conclusions*

617 A better understanding of the complex interactions between pesticide exposure and
618 resistance for host-pathogen outcomes is critical for effective pest and vector control strategies
619 (James & Xu, 2012; Minetti et al., 2020; Orr et al., 2020). Here, we identified antagonistic
620 interactions between Bt and OP that mitigated the effects of pesticides on fitness-related host
621 traits. Our results suggest that both pesticides and Bt individually influence important
622 demographic parameters like development time and mortality rate, but that co-exposed hosts
623 may actually perform better than those exposed to pesticides alone. We also observed dampened
624 expression of important candidate genes upon dual exposure to OP and *Btt* and in Pyr-resistant
625 individuals exposed to *Btt* compared to the main effect of *Btt* exposure. Are these within-host
626 interaction effects sufficient to alter the dynamics of disease and economic damage in pest
627 populations? Mathematical models would be useful to address this question, as the answer will
628 likely depend on feedbacks from ecological variables like intraspecific competition, relative
629 transmission among single- and co-exposed hosts, and the relative costs of pesticides and
630 infection to host reproductive rates (Booton et al., 2019; Booton et al., 2018; Kuniyoshi &
631 Santos, 2017). Moreover, our data suggest that the rise of pesticide resistance within populations
632 could reduce the magnitude of the interaction effects, likely altering population outcomes.
633 Overall, our results shine light on the physiological and evolutionary processes that shape
634 interactions among two important ecological stressors and should prompt further study of their
635 interactions at multiple levels of biological organization on ecological and evolutionary time
636 scales.
637
638 **Data Accessibility:** Sequence reads are deposited in the NCBI SRA under the BioProject
639 Accessions: PRJNA753142, PRJNA756317.

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936 **Figure Legends**

937 **Figure 1.** Overall effects of organophosphate (OP) and pyrethroid (Pyr) factors on survival and
938 development. Hazard ratios depicting the effects of selection regime (OP Reg or Pyr Reg),
939 pesticide exposure (OP PTx or Pyr PTx), Bt treatment (BtTx), the interaction between selection
940 regime and pesticide exposure (OP Reg:PTx or Pyr Reg:PTx), the interaction between selection
941 regime and Bt treatment (OP Reg:BtTx or Pyr Reg:BtTx), and the interaction between pesticide
942 exposure and Bt treatment (OP PTx:BtTx or Pyr PTx:BtTx) on survival (A, B) and development
943 (C, D) are shown in the left panels. Raw data illustrating the proportion of surviving individuals
944 (A, B) or the proportion of adults (C, D) are shown in the right panels. For hazard ratio plots
945 depicting survival and development estimates, positive significant effects (higher survival or
946 faster development) are colored blue, negative significant effects (lower survival or slower
947 development) are red, and nonsignificant effects are black (Suppl. Table 2).

948 **Figure 2.** The influence of pesticide exposure on septic infection outcomes: control-regime
949 larvae exposed to OP are more susceptible to *Btt* septic infection. The proportion of surviving
950 individuals 24h post septic *Btt* infection are shown for control (Regime –) and OP selection
951 regime (Regime OP) larvae exposed to control (Pesticide Tx –) or OP diet (Pesticide Tx OP)
952 treatments. Circles indicate surviving proportions of individuals per block. Significant
953 differences between groups are denoted with asterisks ($p < 0.0005$; Suppl. Table 6).

954 **Figure 3.** Number of significantly differentially expressed (sig. DE) genes (padj < 0.05) for
955 factors within each DESeq2 model (number of sig. DE transcripts are denoted above bars;
956 relative to control regime or no pesticide/ Bt treatments, positive counts are upregulated
957 transcripts and negative counts are downregulated). A. No pesticide model: main and interaction
958 effects of pesticide selection regime and Bt treatment in the absence of pesticide exposure.

959 Hatched bars indicate no pesticide treatment in the model. B. OP model: main and interaction
960 effects of OP selection regime, pesticide exposure, and Bt treatment. C. Pyr model: main and
961 interaction effects of Pyr selection regime, pesticide exposure, and Bt treatment. The table below
962 indicates the differentially expressed factor. Main effect of selection regimes = Regime OP/ Pyr.
963 Main effect of Bt treatment = Bt Tx +. Main effect of pesticide treatments = Pesticide Tx OP/
964 Pyr. Interactions between selection regimes and pesticide exposure = Regime OP/ Pyr &
965 Pesticide Tx OP/ Pyr. Interactions between selection regimes and Bt exposure = Regime OP/ Pyr
966 & Bt Tx +. Interactions between pesticide and Bt exposure = Pesticide Tx OP/ Pyr & Bt Tx +.
967 **Figure 4.** Profiles of differentially expressed transcripts demonstrate opposing responses in Bt-
968 infected individuals that were or were not also exposed to or selected with pesticides. Transcripts
969 significantly differentially expressed (padj < 0.05) with Bt treatment and interactions with **A.** OP
970 exposure, and **B.** Pyr selection regime and exposure. The DE Factor/ Model legend refers to the
971 factor corresponding to the differentially expressed gene set and the DESeq2 model used (no
972 pesticide- hatched bars; OP or Pyr- solid bars; BtTx = main effect of *Btt* treatment; OP PTx =
973 main effect of OP exposure; OP or Pyr PTx:BtTx = interactions between OP or Pyr exposure and
974 *Btt* treatment; Pyr Reg:BtTx: interaction between Pyr selection regime and *Btt* treatment).
975 Colored circles on the left indicate transcripts included in the magenta WGCNA module (Suppl.
976 Fig. 7). Transcript abbreviations are as follows: Stc = shuttle craft-like protein, Def = defensin,
977 Att = attacin, PR = pathogenesis-related protein, HRG = histidine-rich glycoprotein, SP = serine
978 protease, P450 = cytochrome P450, CEH = carboxylic ester hydrolase, GST = glutathione S-
979 transferase, ABC = ABC transporter, Chit = chitinase protein, CP = cuticle-related protein, PM =
980 peritrophic matrix related protein, Vit = vitellogenin, Ins = insulin-like peptide, LSP = larval

981 serum protein, AchE = acetylcholinesterase, KLF = Krüppel-like factor, ApoIII = apolipophorin-
982 III, Neur = neuropeptide.
983 **Figure 5.** Normalized gene counts illustrate dampening effects of Pyr selection regime and OP
984 treatment on immune gene expression induced with Bt expsoure. **A.** OP (OP model- OP PTx)
985 and Bt treatment (OP model- BtTx) effects are shown for TC007737 (attacin1), and **B.** Pyr
986 Regime (Pyr model- Pyr Reg) and Bt treatment (Pyr model- BtTx) effects are shown for
987 TC010517 (defensin-like).
988
989

990 **Supplemental Table Legends**

991 **Suppl. Table 1.** Read counts and alignment statistics for RNAseq libraries.

992 **Suppl. Table 2.** Significantly differentially expressed (DE) transcripts from the no pesticide
993 model (Conly), OP model (allOP), and Pyr model (allPyr) ($p < 0.05$). Fold change differences
994 (log2FC) and adjusted p-values (padj) are listed for each factor within each model (BtTx: main
995 effect of Bt; OPReg, PyrReg: main effect of OP and Pyr selection regime, respectively; OPPTx,
996 PyrPTx: main effect of OP and Pyr pesticide treatment; OPReg:PTx, PyrReg:PTx: interaction
997 effect between pesticide selection regime and exposure; OPReg:BtTx, PyrReg:BtTx: interaction
998 effect between selection regime and Bt expsoure; OPPTx:BtTx, PyrPTx:BtTx: interaction effect
999 between pesticide and Bt exposure. DE transcripts belonging to WGCNA modules are listed
1000 (moduleColor).

1001 **Suppl. Table 3.** Main and interaction effects of pesticide exposure, selection regime, and Bt
1002 exposure on survival and adult development (coxme(Surv(Day, Status) ~ PTx+ Regime + BtTx
1003 + Reg:PTx + Reg:BtTx + PTx:BtTx +(1|Pop)); compared to control regime and treatments).

1004 **Suppl. Table 4.** Effect of pesticide exposure on in vitro Bt growth. The effects of OP and Pyr
1005 treatment were analyzed separately. The effect of treatment was analyzed for each; if replicate
1006 was non-significant, all replicates were combined.

1007 **Suppl. Table 5.** Effect of pesticide treatment on germinated spores from oral diet disks.

1008 **Suppl. Table 6.** Survival after septic Bt infection.

1009

1010 **Supplemental Figure Legends**

1011 **Suppl. Figure 1.** Experimental design schematic. Larvae from six distinct populations were
1012 exposed to control, organophosphate (OP), or pyrethroid (Pyr) diets for at least eight generations.
1013 Adults from Gen. 8 pesticide-selected and control populations were allowed to reproduce on
1014 control diets, and twelve-day old F2 larvae from each population were exposed to control and
1015 pesticide diets with and without *Btt*.

1016 **Suppl. Figure 2.** *In* vitro growth rate (r) of *Btt* under control and pesticide conditions. **A.** *In vitro*
1017 growth rate (r) of *Btt* under control and OP conditions. Data were not normally distributed, so
1018 non-parametric tests were used. There was a significant difference between replicates, so the
1019 effect of treatment was analyzed for each replicate separately (Suppl. Table 4). K-W test
1020 revealed significant positive effect of OP on *Btt* growth rate for three of the four replicates (reps
1021 2-4; Suppl. Table 4). **B.** *In vitro* growth rate (r) of *Btt* under control and Pyr conditions. Data
1022 were not normally distributed, so non-parametric tests were used. There was no significant
1023 difference between replicates or with the effect of treatment (Suppl. Table 4).

1024 **Suppl. Fig. 3.** Germination of spores from diet disks in the absence or presence of either
1025 pesticide (Bt, OP + *Btt* or Pyr + *Btt*: n=10, control: n=6). There was no significant difference
1026 between Bt control and Bt pesticide diet disks (Suppl. Table 5).

1027 **Suppl. Figure 4.** Top significantly enriched GO terms for differentially expressed gene sets. **A.**
1028 Biological process GO terms, **B.** Molecular function GO terms. Differentially expressed gene
1029 sets are denoted on the x-axis and refer to the DESeq2 model (no P: = no pesticide model, OP: =
1030 OP model, Pyr: = Pyr model) and the factor corresponding to the differentially expressed gene
1031 set (BtTx = main effect of *Btt* treatment; OP Reg, Pyr Reg = main effect of OP and Pyr selection
1032 regimes, respectively; OP PTx, Pyr PTx = main effect of OP and Pyr exposure, respectively;

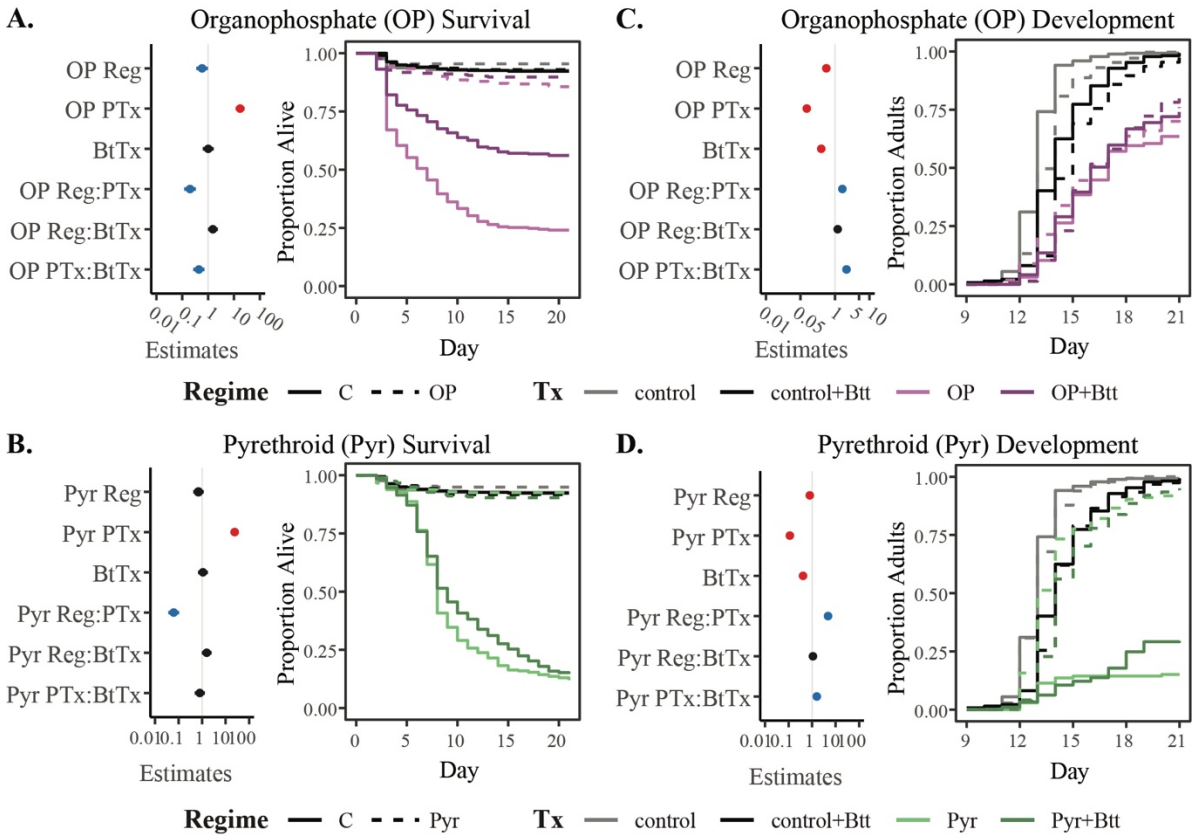
1033 OPReg:PTx, PyrReg:PTx = interaction between OP and Pyr regime and exposure, respectively;
1034 PyrReg:BtTx = interaction between Pyr regime and *Btt* treatment; OPTx:BtTx = interaction
1035 between OP exposure and *Btt* treatment).
1036 **Suppl. Fig. 5.** Canonical detoxification genes significantly differentially expressed (padj < 0.05)
1037 with **A.** OP exposure (OP PTx), OP selection regime (OP Reg), *Btt* exposure (BtTx), and the
1038 interaction between OP regime and exposure (OP Reg:PTx) and **B.** Pyr exposure (Pyr PTx), Pyr
1039 selection regime (Pyr Reg), *Btt* exposure (BtTx), and the interaction between Pyr regime and
1040 exposure (Pyr Reg:PTx).
1041 **Suppl. Fig. 6.** Cuticle-associated genes significantly differentially expressed (padj < 0.05) with
1042 **A.** OP exposure (OP PTx), OP selection regime (OP Reg), *Btt* exposure (BtTx), the interaction
1043 between OP regime and exposure (OP Reg:PTx), and the interaction between OP and *Btt*
1044 exposure (OP PTx:BtTx), and **B.** Pyr exposure (Pyr PTx), Pyr selection regime (Pyr Reg), *Btt*
1045 exposure (BtTx), the interaction between Pyr regime and exposure (Pyr Reg:PTx), the interaction
1046 between Pyr regime and *Btt* exposure (Pyr Reg:BtTx), and the interaction between Pyr and *Btt*
1047 exposure (Pyr PTx:BtTx).
1048 **Suppl. Fig. 7.** WGCNA gene modules and significant relationships with experimental factors.
1049 The total number of transcripts (n) and number of differentially expressed (DE) transcripts in
1050 each module are listed, and significant relationships with experimental factors are indicated with
1051 black stars. Top values within each cell indicate the correlation strengths (corresponding to the
1052 heat map colors) and bottom values in parentheses indicate p-value significance for the
1053 relationship of each module with the different experimental factors. Significant ($p < 0.05$)
1054 positive correlation values and red cells indicate that the transcripts within the module are

1055 commonly upregulated; negative significant correlation values and green cells indicate that the

1056 transcripts within the module are commonly downregulated.

1057 **Figures**

1058



1059

1060 **Figure 1.**

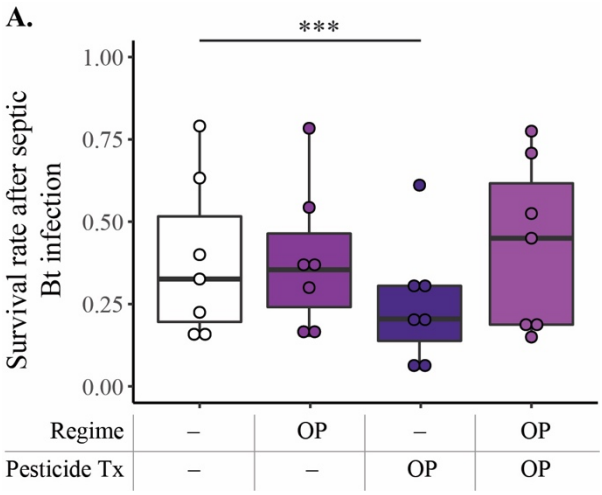

A.
***
1.00
0.75
0.50
0.25
0.00
Survival rate after septic Bt infection
Regime
–
OP
–
OP
Pesticide Tx
–
–
OP
OP

Figure 2.

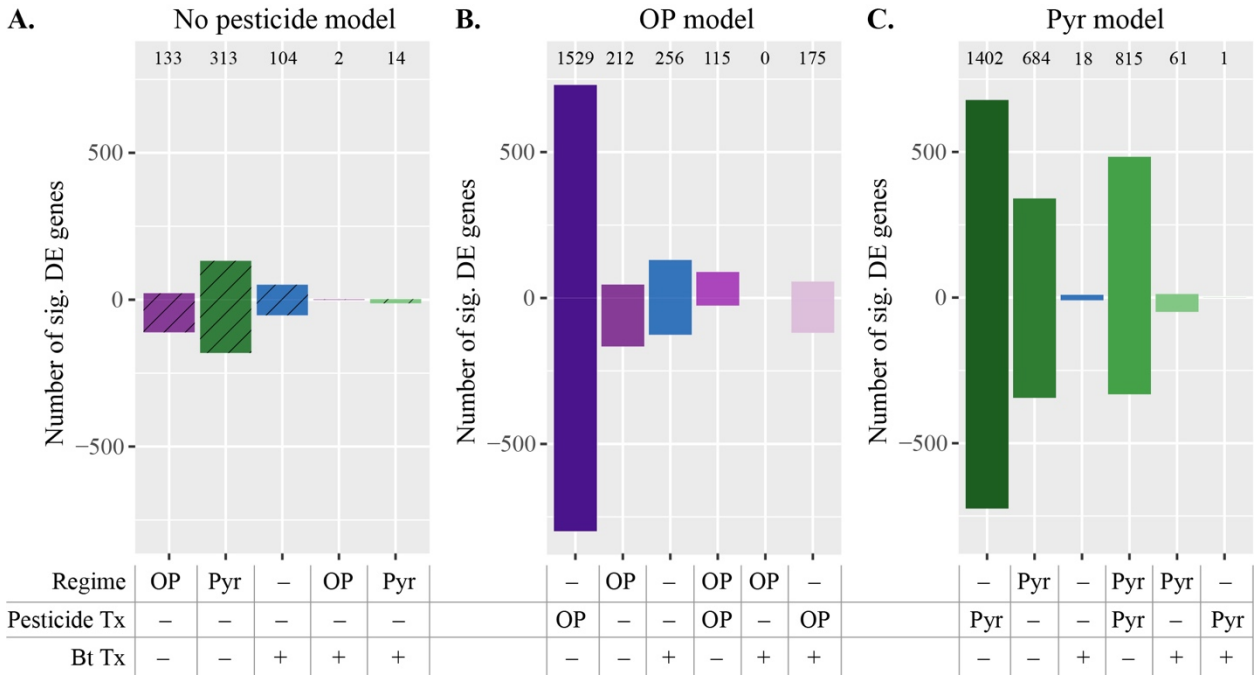

A.
No pesticide model
133
313
104
2
14
500
0
−500
Number of sig. DE genes
Regime
OP
Pyr
–
OP
Pyr
Pesticide Tx
–
–
–
–
–
Bt Tx
–
–
+
+
+
B.
OP model
1529
212
256
115
0
175
500
0
−500
Number of sig. DE genes
–
OP
–
OP
OP
–
OP
–
–
OP
–
OP
–
–
+
–
+
+
C.
Pyr model
1402
684
18
815
61
1
500
0
−500
Number of sig. DE genes
–
Pyr
–
Pyr
Pyr
–
Pyr
–
–
Pyr
–
Pyr
–
–
+
–
+
+

Figure 3.

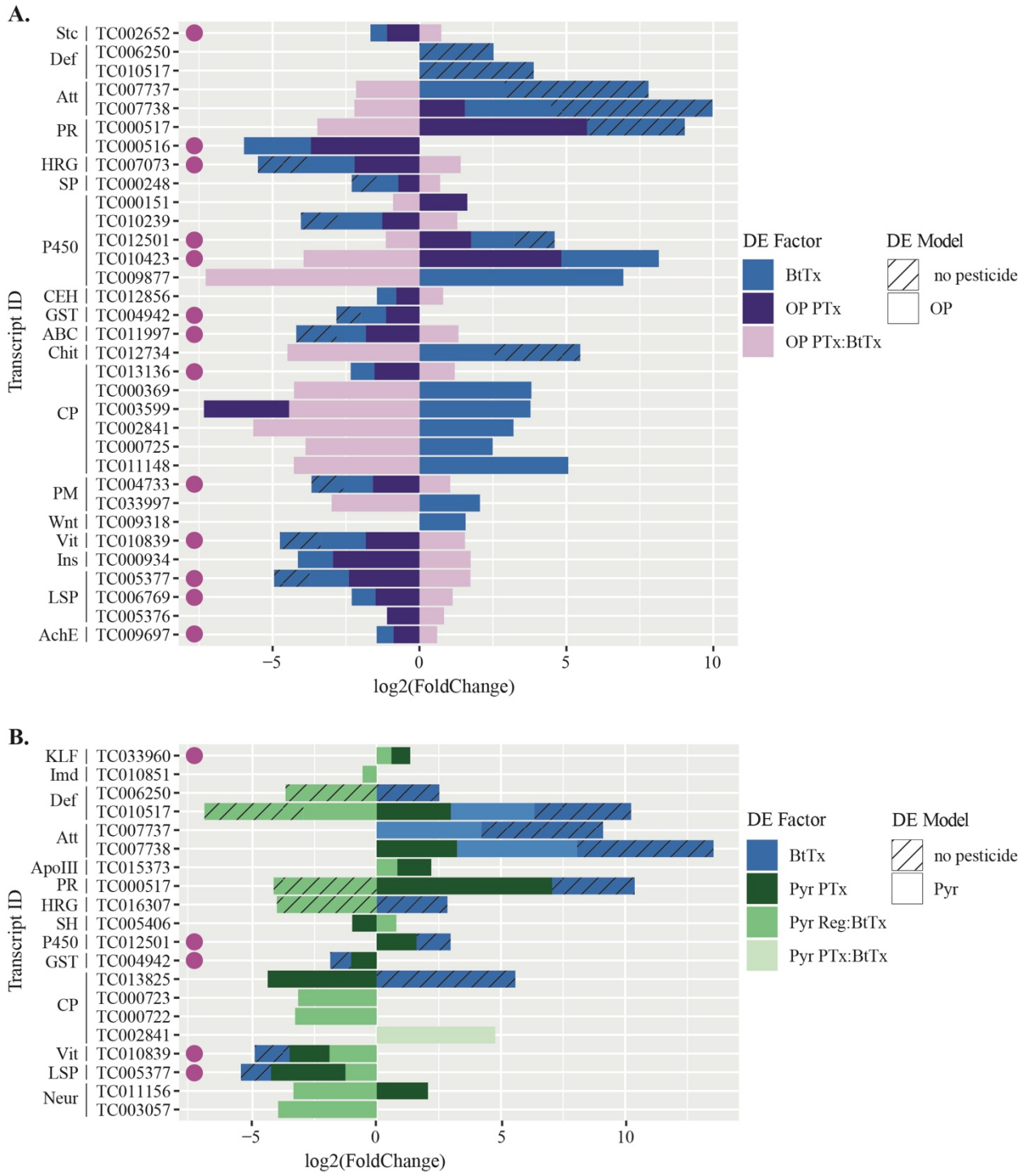

A.
Stc | TC002652
Def | TC006250
TC010517
Att | TC007737
TC007738
PR | TC000517
TC000516
HRG | TC007073
SP | TC000248
P450 | TC000151
TC010239
TC012501
TC010423
TC009877
CEH | TC012856
GST | TC004942
ABC | TC011997
Chit | TC012734
CP | TC013136
TC000369
TC003599
TC002841
TC000725
TC011148
PM | TC004733
TC033997
Wnt | TC009318
Vit | TC010839
Ins | TC000934
LSP | TC005377
TC006769
TC005376
AchE | TC009697
Transcript ID
−5
0
5
10
log2(FoldChange)
DE Factor
BtTx
OP PTx
OP PTx:BtTx
DE Model
no pesticide
OP
B.
KLF | TC033960
Imd | TC010851
Def | TC006250
TC010517
Att | TC007737
TC007738
ApoIII | TC015373
PR | TC000517
HRG | TC016307
SH | TC005406
P450 | TC012501
GST | TC004942
CP | TC013825
TC000723
TC000722
TC002841
Vit | TC010839
LSP | TC005377
Neur | TC011156
TC003057
Transcript ID
−5
0
5
10
log2(FoldChange)
DE Factor
BtTx
Pyr PTx
Pyr Reg:BtTx
Pyr PTx:BtTx
DE Model
no pesticide
Pyr

Figure 4.

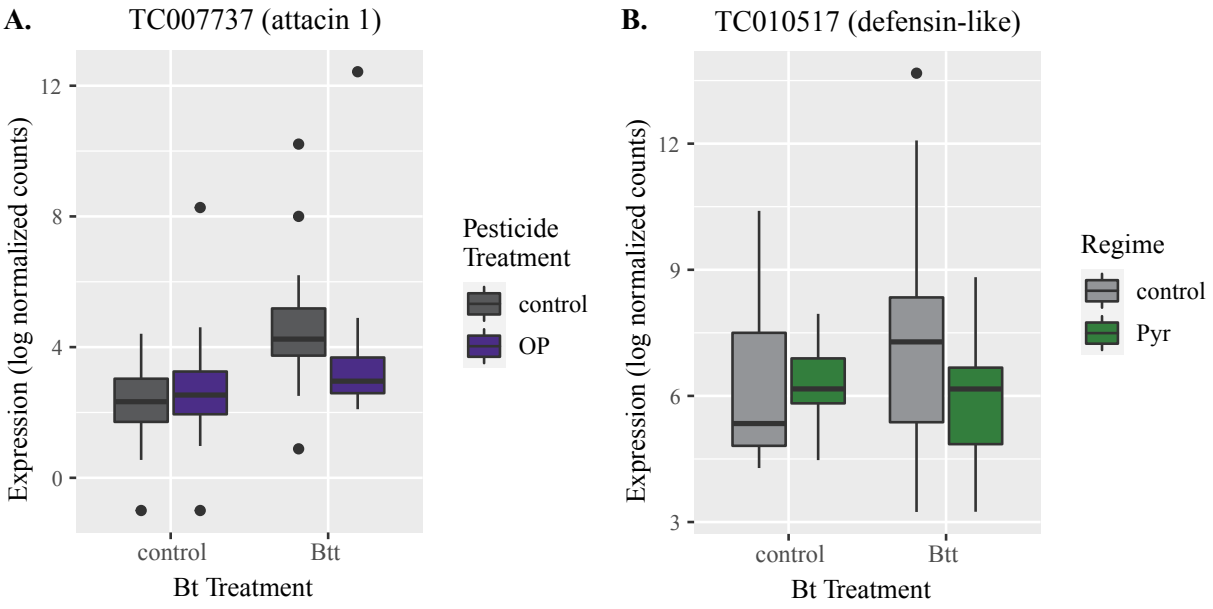
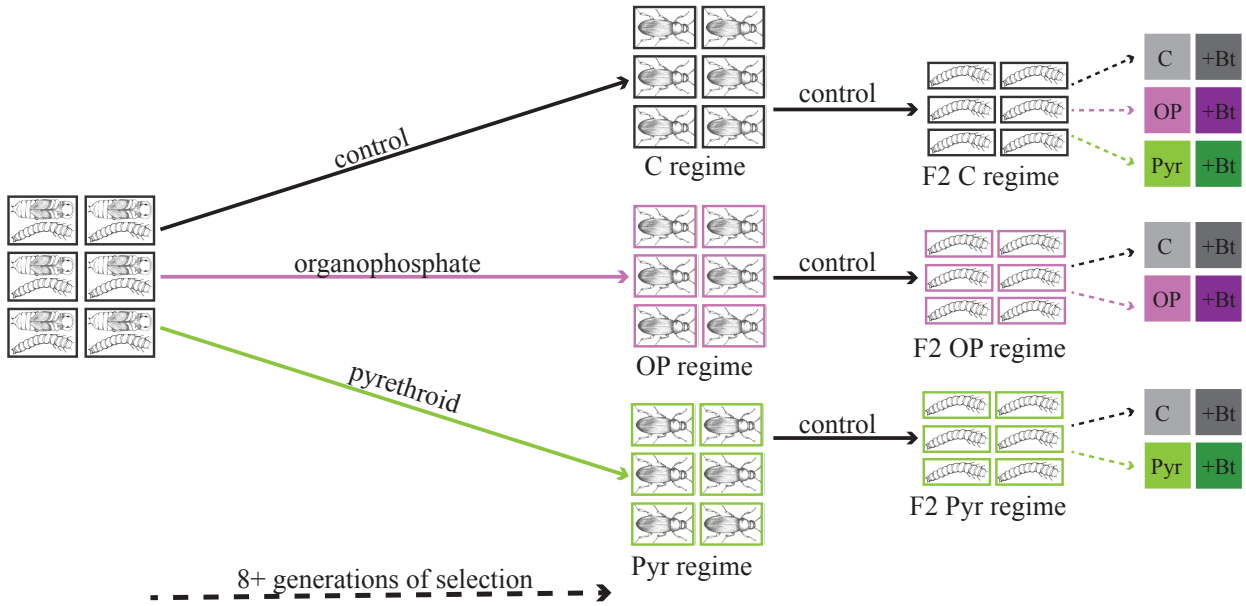
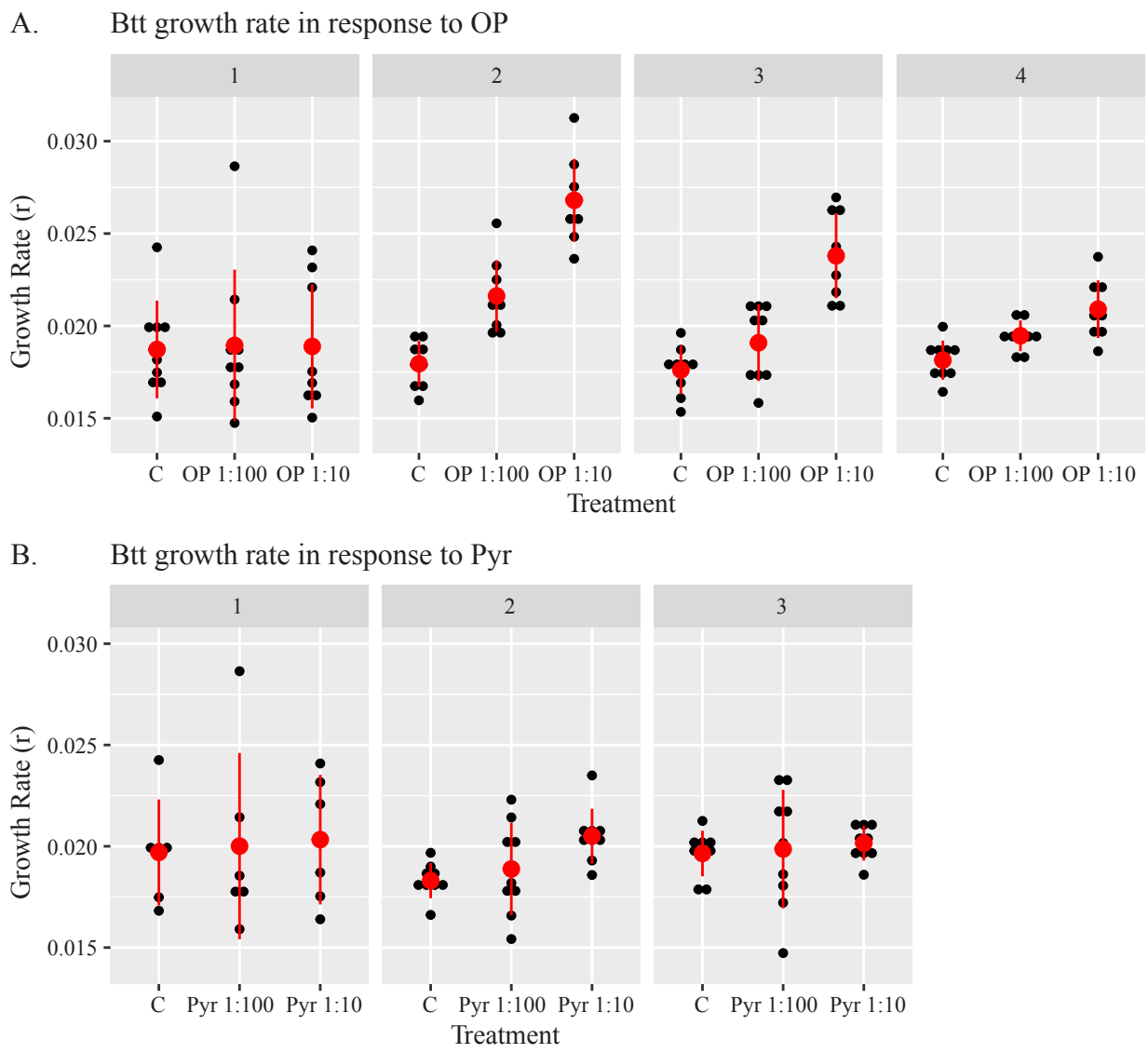

A.
TC007737 (attacin 1)
Expression (log normalized counts)
12
8
4
0
control
Btt
Bt Treatment
Pesticide Treatment
control
OP
B.
TC010517 (defensin-like)
Expression (log normalized counts)
12
9
6
3
control
Btt
Bt Treatment
Regime
control
Pyr

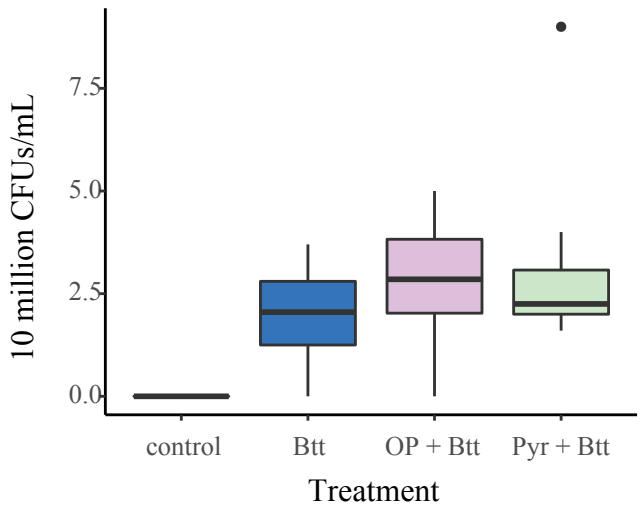
Figure 5.



Suppl. Figure 1.

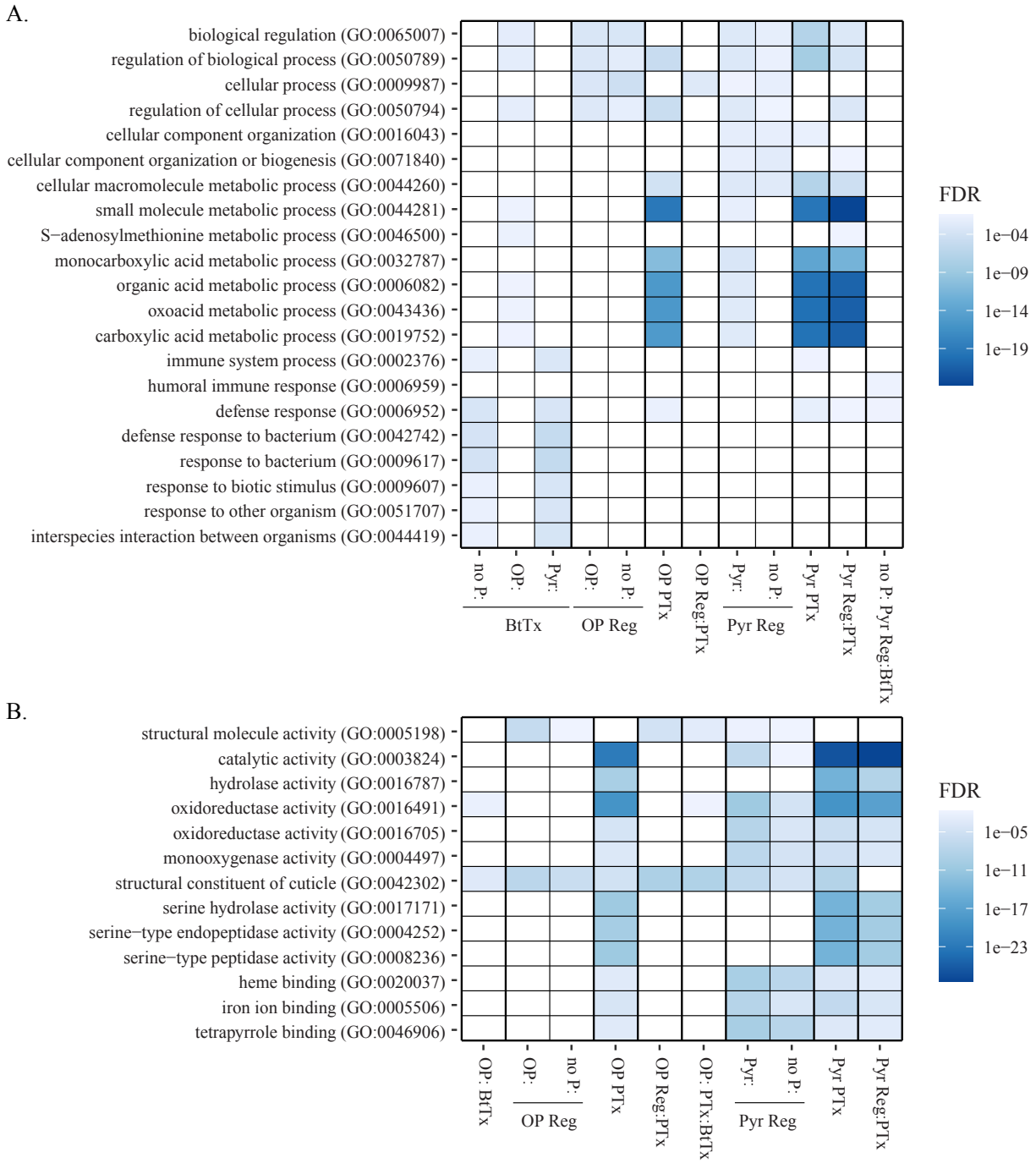


Suppl. Figure 2.

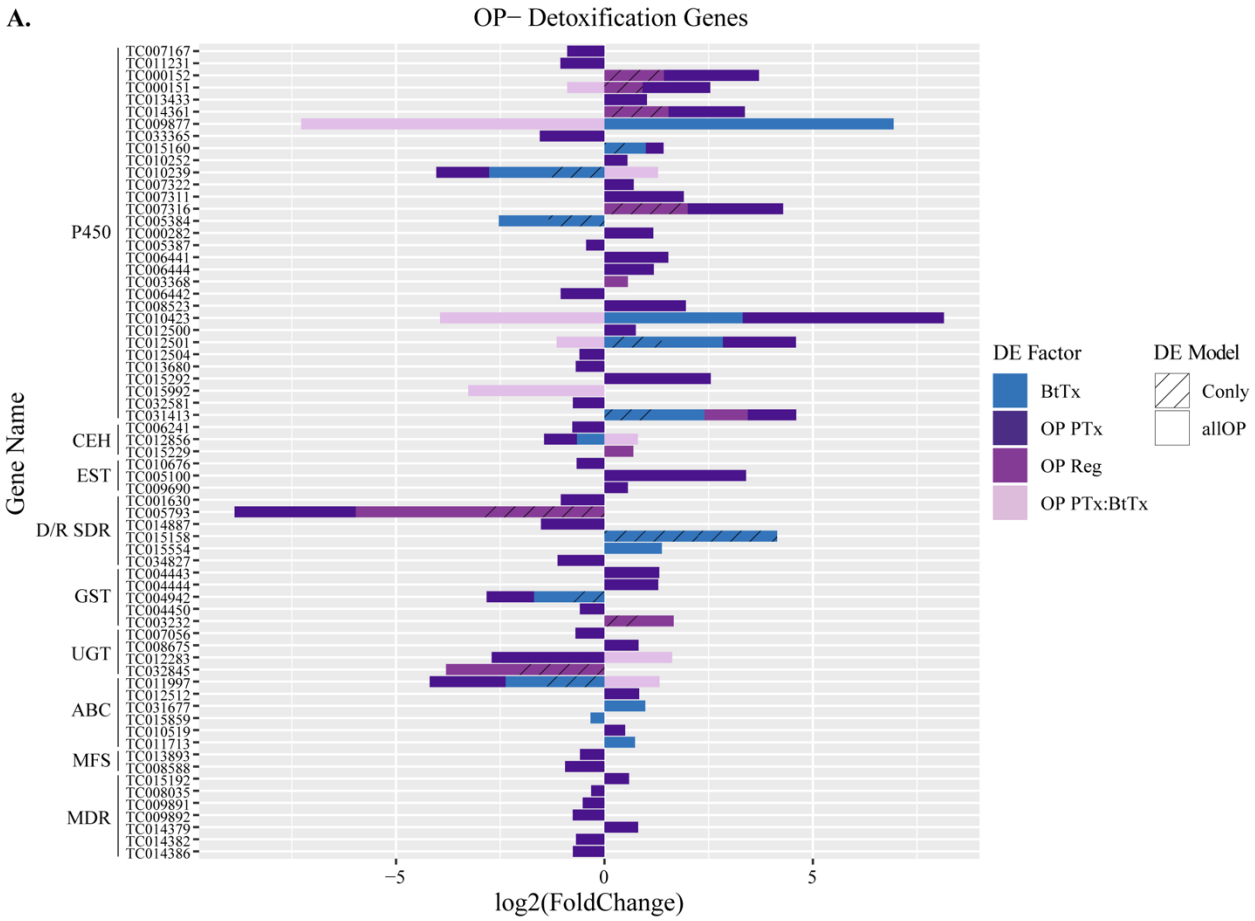


1073

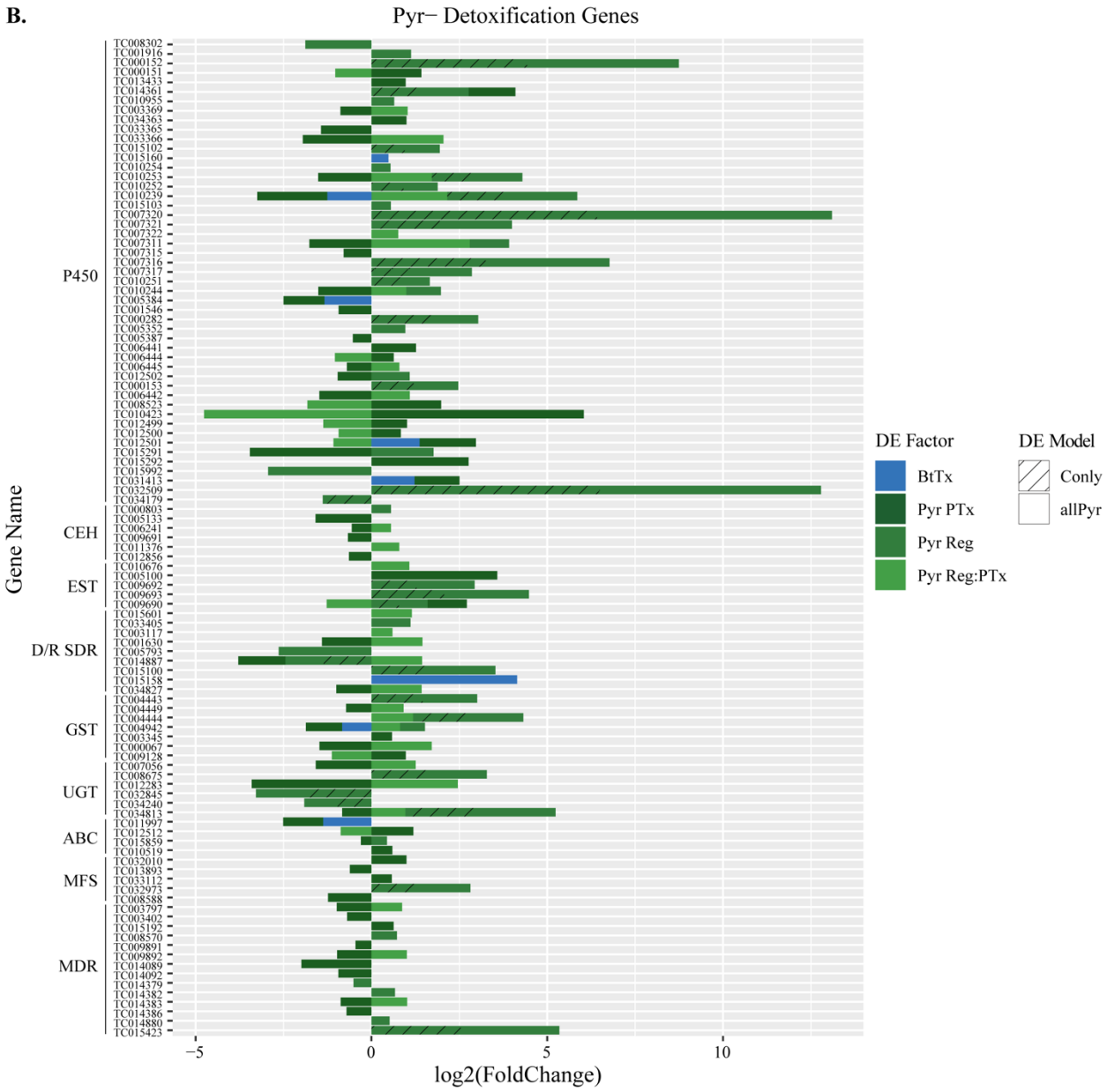
1074 **Suppl. Fig. 3.**



Suppl. Figure 4.

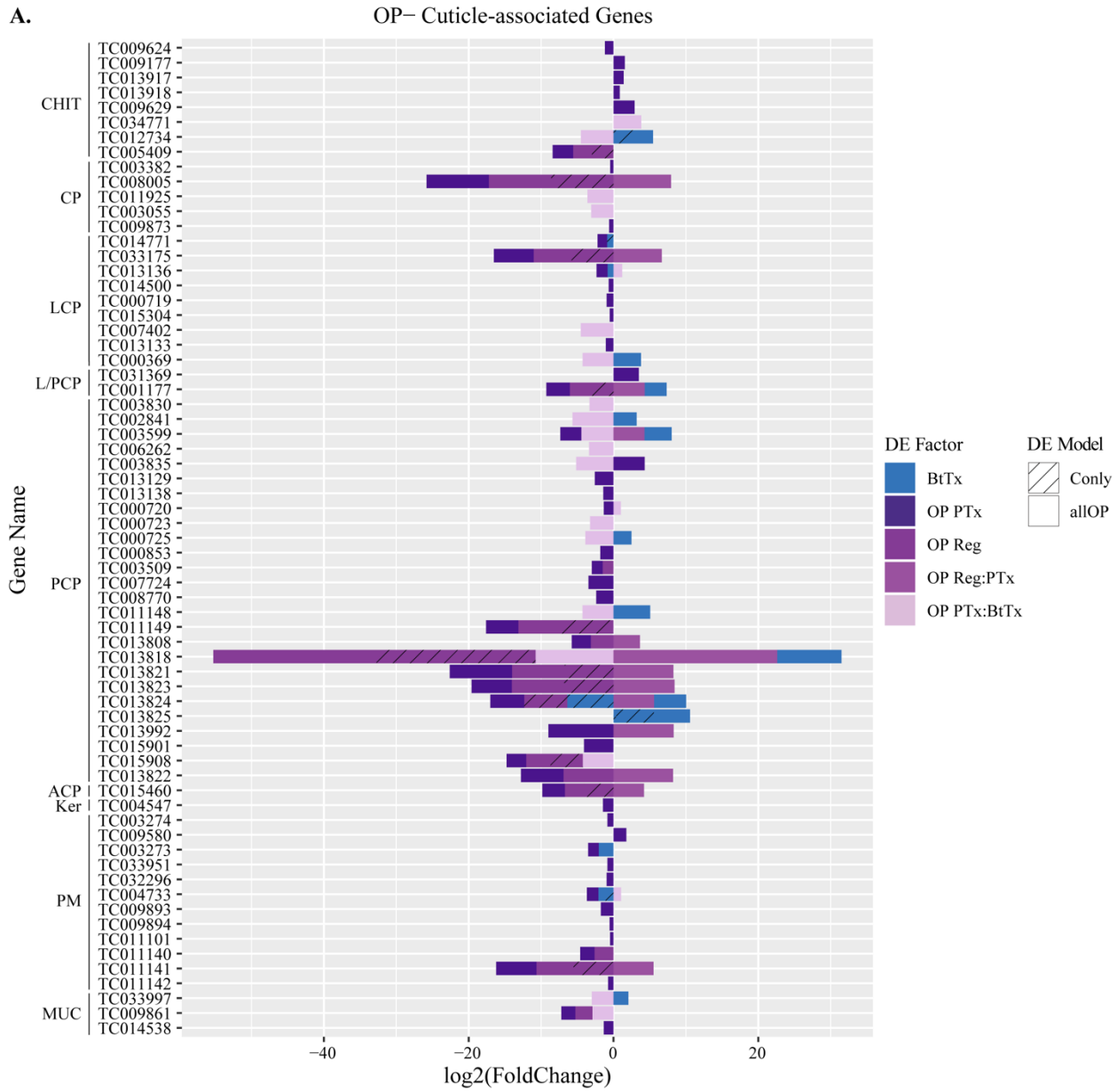

A.
OP− Detoxification Genes
Gene Name
P450
CEH
EST
D/R SDR
GST
UGT
ABC
MFS
MDR
TC007167
TC011231
TC000152
TC000151
TC013433
TC014361
TC009877
TC033365
TC015160
TC010252
TC010239
TC007322
TC007311
TC007316
TC005384
TC000282
TC005387
TC006441
TC006444
TC003368
TC006442
TC008523
TC010423
TC012500
TC012501
TC012504
TC013680
TC015292
TC015992
TC032581
TC031413
TC006241
TC012856
TC015229
TC010676
TC005100
TC009690
TC001630
TC005793
TC014887
TC015158
TC015554
TC034827
TC004443
TC004444
TC004942
TC004450
TC003232
TC007056
TC008675
TC012283
TC032845
TC011997
TC012512
TC031677
TC015859
TC010519
TC011713
TC013893
TC008588
TC015192
TC008035
TC009891
TC009892
TC014379
TC014382
TC014386
−5
0
5
log2(FoldChange)
DE Factor
BtTx
OP PTx
OP Reg
OP PTx:BtTx
DE Model
Conly
allOP

1077

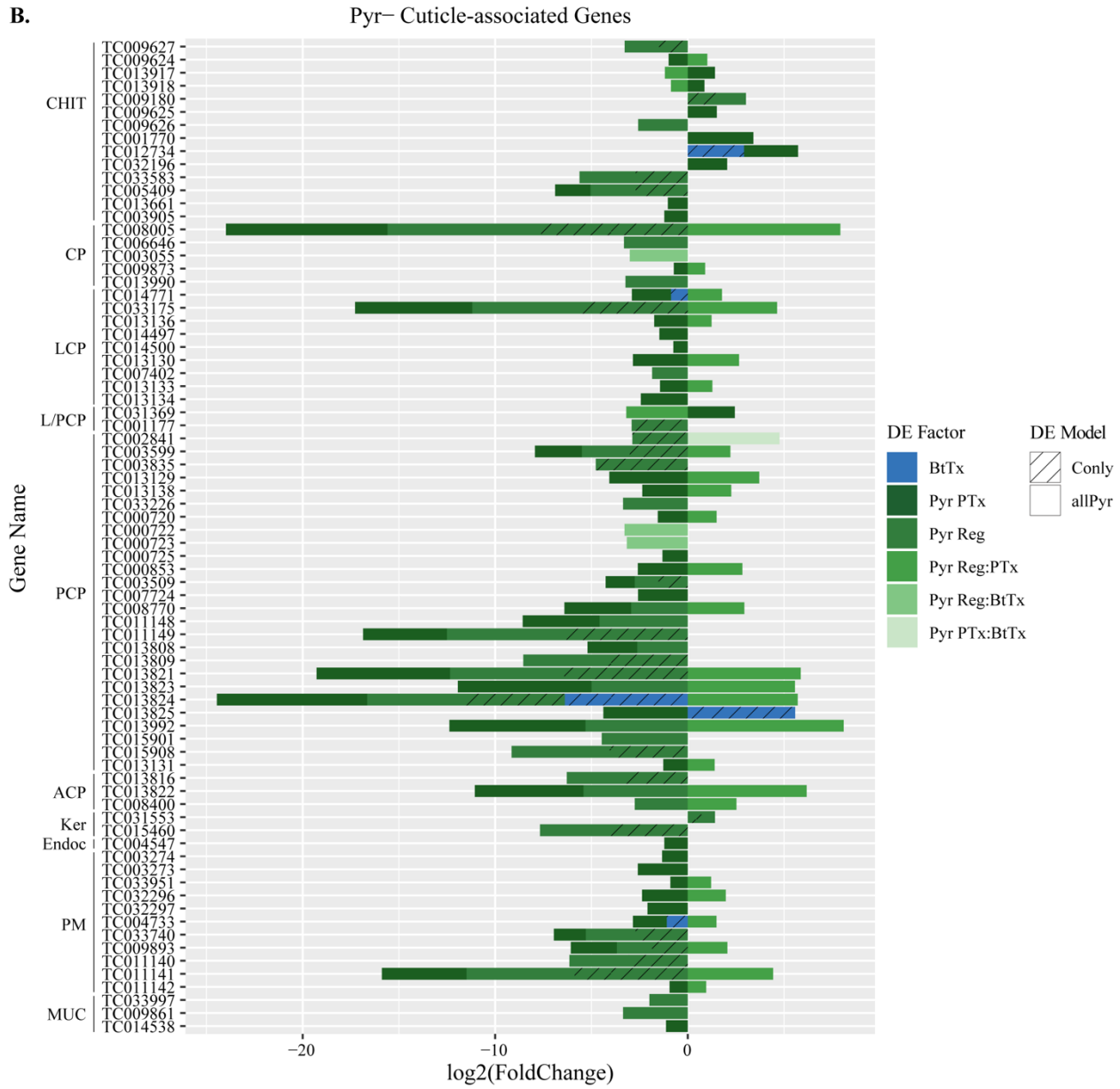


1078

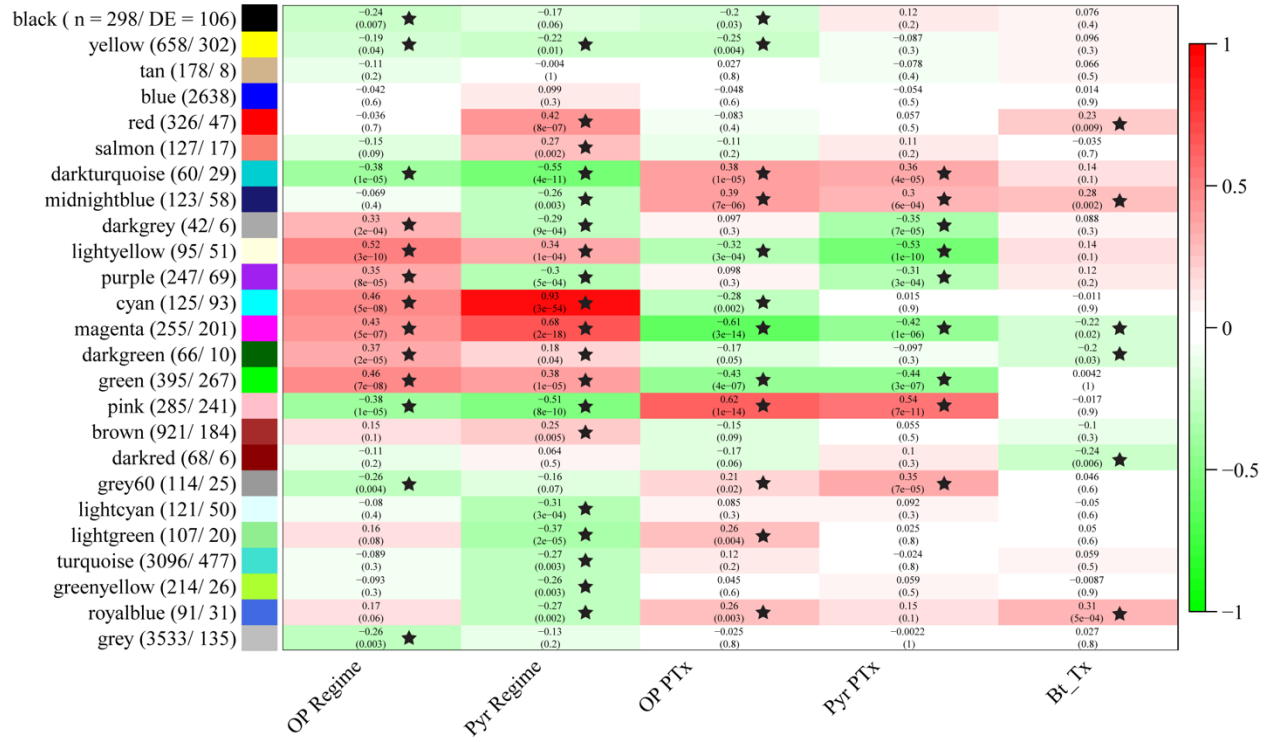
1079 **Suppl. Fig. 5.**


A.
OP− Cuticle-associated Genes
Gene Name
CHIT
CP
LCP
L/PCP
PCP
ACP
Ker
PM
MUC
TC009624
TC009177
TC013917
TC013918
TC009629
TC034771
TC012734
TC005409
TC003382
TC008005
TC011925
TC003055
TC009873
TC014771
TC033175
TC013136
TC014500
TC000719
TC015304
TC007402
TC013133
TC000369
TC031369
TC001177
TC003830
TC002841
TC003599
TC006262
TC003835
TC013129
TC013138
TC000720
TC000723
TC000725
TC000853
TC003509
TC007724
TC008770
TC011148
TC011149
TC013808
TC013818
TC013821
TC013823
TC013824
TC013825
TC013992
TC015901
TC015908
TC013822
TC015460
TC004547
TC003274
TC009580
TC003273
TC033951
TC032296
TC004733
TC009893
TC009894
TC011101
TC011140
TC011141
TC011142
TC033997
TC009861
TC014538
−40
−20
0
20
log2(FoldChange)
DE Factor
BtTx
OP PTx
OP Reg
OP Reg:PTx
OP PTx:BtTx
DE Model
Conly
allOP

1080


B.
Pyr− Cuticle-associated Genes
Gene Name
CHIT
CP
LCP
L/PCP
PCP
ACP
Ker
Endoc
PM
MUC
TC009627
TC009624
TC013917
TC013918
TC009180
TC009625
TC009626
TC001770
TC012734
TC032196
TC033583
TC005409
TC013661
TC003905
TC008005
TC006646
TC003055
TC009873
TC013990
TC014771
TC033175
TC013136
TC014497
TC014500
TC013130
TC007402
TC013133
TC013134
TC031369
TC001177
TC002841
TC003599
TC003835
TC013129
TC013138
TC033226
TC000720
TC000722
TC000723
TC000725
TC000853
TC003509
TC007724
TC008770
TC011148
TC011149
TC013808
TC013809
TC013821
TC013823
TC013824
TC013825
TC013992
TC015901
TC015908
TC013131
TC013816
TC013822
TC008400
TC031553
TC015460
TC004547
TC003274
TC003273
TC033951
TC032296
TC032297
TC004733
TC033740
TC009893
TC011140
TC011141
TC011142
TC033997
TC009861
TC014538
−20
−10
0
log2(FoldChange)
DE Factor
BtTx
Pyr PTx
Pyr Reg
Pyr Reg:PTx
Pyr Reg:BtTx
Pyr PTx:BtTx
DE Model
Conly
allPyr

Suppl. Fig. 6.


black (n = 298/ DE = 106)
yellow (658/ 302)
tan (178/ 8)
blue (2638)
red (326/ 47)
salmon (127/ 17)
darkturquoise (60/ 29)
midnightblue (123/ 58)
darkgrey (42/ 6)
lightyellow (95/ 51)
purple (247/ 69)
cyan (125/ 93)
magenta (255/ 201)
darkgreen (66/ 10)
green (395/ 267)
pink (285/ 241)
brown (921/ 184)
darkred (68/ 6)
grey60 (114/ 25)
lightcyan (121/ 50)
lightgreen (107/ 20)
turquoise (3096/ 477)
greenyellow (214/ 26)
royalblue (91/ 31)
grey (3533/ 135)
OP Regime
Pyr Regime
OP PTx
Pyr PTx
Bt_Tx
1
0.5
0
−0.5
−1

Suppl. Fig. 7.