Bioremediation of industrial pollutants by insects expressing a fungal laccase

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Abstract

Inadequate management of household and industrial wastes pose major challenges to human and environmental health. Advances in synthetic biology may help address these challenges by engineering biological systems to perform new functions such as biomanufacturing of high-value compounds from low-value waste streams and bioremediation of industrial pollutants. The current emphasis on microbial systems for biomanufacturing, which often require highly pre-processed inputs and sophisticated infrastructure, is not feasible for many waste streams. Concerns about transgene biocontainment have limited the release of engineered microbes or plants for bioremediation. Engineering animals may provide opportunities for utilizing various waste streams that are not suitable for microbial biomanufacturing while effective transgene biocontainment options should enable *in situ* bioremediation. Here, we engineer the model insect *Drosophila melanogaster* to express a functional laccase from the fungus *Trametes trogii.* Laccase expressing flies reduced concentrations of the endocrine disruptor bisphenol A by more than 50% when present in their growth media. A lyophilized powder made from engineered adult flies retained substantial enzymatic activity, degrading more than 90% of bisphenol A and the textile dye indigo carmine in aqueous solutions. Our results demonstrate that transgenic animals may be used to bioremediate environmental contaminants *in vivo* and serve as novel production platforms for industrial enzymes. These results support further development of insects, and possibly other animals, as bioproduction platforms and their potential use in bioremediation.

34 **Main Text**

35 Developing economically viable approaches for sustainable waste management and
36 mitigation of environmental pollution are important for ensuring a high quality of life[1]. At
37 present, over half of all municipal solid waste is either landfilled[2], where it is estimated to
38 contribute to 11% of global methane emissions[3,4], or openly dumped, where it contaminates
39 drinking water and disrupts natural ecosystems[5]. Pollution from industrial wastes includes a
40 variety of persistent compounds which have been linked to neurological defects, cancer, and
41 species extinction[6–10].

42 Regulatory changes including prohibiting discharge of industrial pollutants and reducing the
43 landfilling of food waste are helpful, however, the cost of compliance can be substantial[11].
44 Generating value from a variety of waste-streams and developing inexpensive methods to
45 treat pollutants *in situ* will reduce the costs of achieving sustainability targets and help
46 support a circular economy[12].

47 We propose developing solutions by expanding the enzymatic repertoire of select animals to
48 consume waste, degrade xenobiotic chemicals, and produce high-value compounds (Fig. 1).
49 Microbial and fungal enzymes can enable animals to breakdown pollutants *in situ*. These may
50 include herbivores that degrade pollutants accumulating in plants, fish that secrete enzymes
51 to degrade aquatic pollutants, or invertebrates engineered to interrupt the biomagnification
52 of xenobiotics in food webs. Effective transgene biocontainment safeguards are currently a
53 challenge for plants/microbes[13,14], but are possible in animals via surgical sterilization or
54 engineered genetic incompatibilities[15]. Developing insects as a biomanufacturing platforms
55 for industrial enzymes, lipids, or other molecules is also an unexplored avenue for waste
56 valorisation.

57 Insects have advantages over bacteria and fungi for processing many waste-streams. Their
58 complex digestive systems, including mastication, allow them to consume a variety of low-
59 value wastes, including municipal organic waste[16] and livestock manure[17]. Crude waste can
60 be fed directly to insects without pre-processing, refinement, and sterilization necessary for
61 microbial fermentation[18,19]. Some insect species, such as Black soldier flies (*Hermetia*
62 *illucens*), which are already used to manage waste have excellent tolerance to bacteria and
63 fungi[20] and are easily separated from the organic waste for downstream processing[21]. Insect
64 biotechnology is readily scalable for mass production, as the infrastructure can be relatively

65 unsophisticated, and the installation costs and land use requirements are low[22].
66 Furthermore, utilising organic waste means crops that could otherwise be used for food do
67 not need to be diverted as feedstocks for fermentation, and will not threaten food security.

68 There is a long history of using lepidopteran insect larvae to make recombinant proteins
69 starting with *Bombyx mori* expressing human α-interferon[23]. However, these utilize transient
70 baculoviral systems to infect the larvae. This approach is not practical for large-scale or long-
71 term, multi-generational use. Here we explore the potential of stably engineered insects as
72 platforms for the production of an industrially relevant fungal laccase and bioremediation of
73 polluted sites. Laccases are multicopper oxidases that are present in many taxa. Fungal
74 laccases were selected due to their demonstrated utility for bioremediation of a wide variety
75 of industrial pollutants such as bisphenols[24], industrial dyes[25], pharmaceuticals[26], phenolics[27],
76 polycyclic aromatic hydrocarbons[28], perfluoroalkyl and polyfluoroalkyl substances (PFAS)[29,30],
77 plastics[31], pesticides[32], and mycotoxin contaminants such as aflatoxins[33]. Fungal laccases also
78 have diverse industrial applications in the textile, paper and pulp, food, pharmaceutical,
79 chemical synthesis, and forestry industries[34–36]. They have been applied to treat distillery[37],
80 paper and pulp[38], and olive mill[39] industry wastewater. Ligninolytic enzymes, such as fungal
81 laccases, also show potential application in the delignification of feedstocks for biofuel
82 production[40]. However, fungal laccases have not yet reached appreciable commercial scale
83 production. This is because the native hosts for laccases often produce low yields and
84 although this has been improved using heterologous hosts, the production levels are still too
85 low for many commercial applications[41].

86 We engineered the common fruit fly (*Drosophila melanogaster*) to express a laccase from the
87 fungus *Trametes trogii.* Transgenic flies degraded bisphenol A (BPA), a widespread endocrine
88 disruptor[42], when present in their diet. We also demonstrate that aqueous solutions of BPA
89 and indigo carmine (IC), a textile dye and aquatic pollutant[43], can be treated with a lyophilized
90 powder from adult transgenic flies to degrade both compounds. Our results describe the first
91 instance of engineering an animal to perform an ecosystem service by heterologous
92 expression of a fungal enzyme and demonstrate the potential for using transgenic strains of
93 insects for enzyme bioproduction.

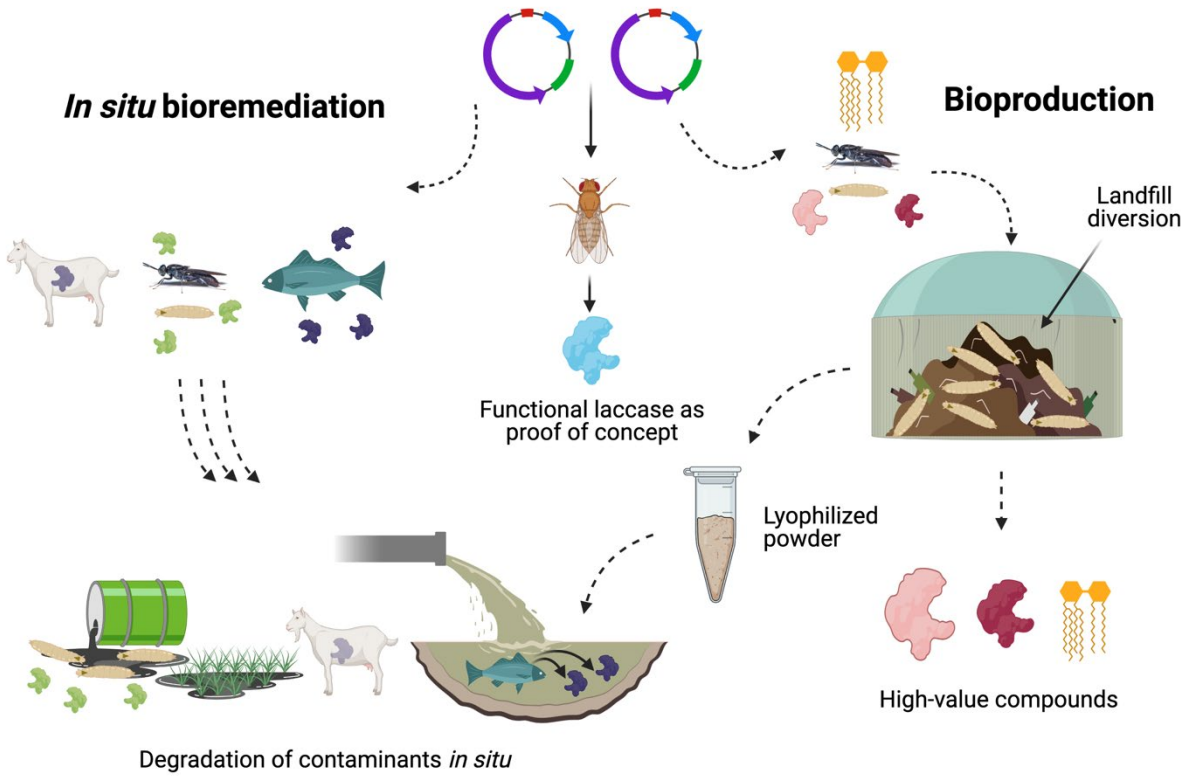

Fig. 1: Applications of transgenic animals for bioremediation and bioproduction.

Results

Screening for fungal laccase activity in transgenic *D. melanogaster*

D. melanogaster and other insects express endogenous laccases involved in immune defense[44]. However, it was unknown if an insect could heterologously express a functional fungal laccase or if its expression would be toxic. We were also uncertain which tissues would provide the optimal expression of a functional laccase for either *in vitro* or *in vivo* activity. We selected a short tubulin promoter, previously used to express a toxic gene[45], with the goal of achieving moderate expression across many tissues. The native fungal signal peptides were replaced with the *D. melanogaster* larval cuticle protein 9[46] signal peptide to facilitate extracellular secretion.

We generated homozygous transgenic strains expressing laccases from two fungal species, *T. trogii* and *Polyporus brumalis*, known to have a broad substrate range and high redox potential (Table 1). Fly lysates from both were assayed for oxidation of the chromogenic laccase substrate 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The

111 engineered *D. melanogaster* strain Dm/Tt.Lcc1, which expresses a laccase from *T. trogii,*
112 oxidized ABTS (Fig. 2a). There was no activity observed in the wild-type or from the
113 engineered Dm/Pb.Lac1 strain expressing a laccase from *P. brumalis* (Fig. 2a). Moving
114 forward, we focused on characterizing Dm/Tt.Lcc1.

115 We next determined if Dm/Tt.Lcc1 flies are capable of degrading bisphenol A (BPA), an
116 endocrine disruptor and emerging environmental contaminant found in many consumer
117 products[47]. BPA has been shown to be a substrate of *T. trogii* laccase when expressed from
118 its native host[48]. We incubated lysates from flies with 25 µg/mL BPA for 21 hours and
119 measured BPA concentrations by liquid-chromatography mass spectrometry (LC-MS). Lysates
120 from Dm/Tt.Lcc1 reduced BPA concentrations by 95% compared to the no-fly controls and
121 the wild-type fly lysate (Fig. 2b-c).

122 **Table 1. The fungal laccases engineered into *D. melanogaster***

Gene name	Native host	Genbank accession	Redox potential (V vs. NHE, pH 5)	Laccase substrates
Lcc1	*Trametes trogii*	AJ294820.1	*E*◦ 0.79 V [49]	Aminophenols, polyphenols, industrial dyes[50–52].
Lac1	*Polyporus brumalis*	ABN13591.1	*E*◦ 0.80 V [53]	Industrial dyes, aromatic hydrocarbons [53].

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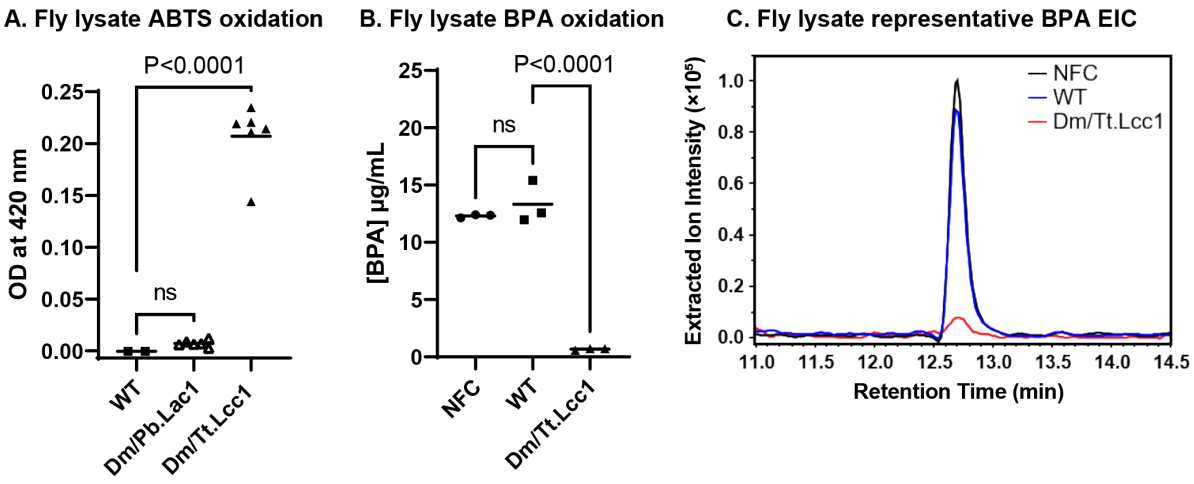

Fig. 2: *In vitro* laccase substrate oxidation in fly lysates. (A) Fly lysate oxidation of 1 mg/mL ABTS for 2 hours. *n* = 2 biologically independent replicates for WT, *n* = 6 for all others. **(B)** Fly lysate degradation of 25 µg/mL BPA for 21 h. *n* = 3 biologically independent replicates. **(C)** Overlaid representative extracted ion chromatograms (EIC) (m/z 227.15) displaying the BPA peak for samples shown in **B**. Dm/Tt.Lcc1 = *D. melanogaster* engineered with *T. trogii* laccase, Dm/Pb.Lac1 = *D. melanogaster* engineered with *P. brumalis* laccase, WT = wild-type, NFC = No-Fly control. The statistical analysis was conducted using one-way ANOVA using Dunnett's method compared to WT, where ns = not significant.

In vivo laccase activity

We evaluated the potential of laccase expressing insects to oxidize substrates present in their environment by secreted enzymes. Flies were reared on a minimal media to avoid binding of substrates to insoluble components present in standard media. The minimal media included 1 mM $CuSO_4$ to supply the laccase co-factor. First, we reared 3 adult males and 3 adult females on minimal diet including 1 mg/mL ABTS for 2 days, after which they were removed. Larvae of the mated flies were grown for an additional 5 days. Dm/Tt.Lcc1 flies oxidized ABTS as indicated by blue colorization of the fly media, however, no oxidation was observed with wild-type flies (Fig. 3a). We controlled for the possibility that the ABTS oxidation was due to laccase that may have been leaking from dead larvae by incubating 6 male flies in ABTS media. After 11 days, only the Dm/Tt.Lcc1 males had oxidized ABTS (Fig. 3b). This result indicates that the oxidation was due to enzyme secreted into the media.

Next, we reared 5 male and 5 female flies in a minimal diet containing 25 µg/mL BPA. Adults were removed after three days. On day 10, BPA was extracted from their diet and quantified by Ultra-High-Performance Liquid Chromatography coupled with fluorescence detection

152 (UHPLC-FLD). Both Dm/Tt.Lcc1 and wild-type flies had reduced BPA concentrations by 36%
153 and 45%, respectively, compared to no-fly controls (Fig. 3c). There was no significant
154 difference between Dm/Tt.Lcc1 and wild-type indicating that the reduction in BPA
155 concentration was not due to the activity of the laccase transgene. The reduction in BPA
156 concentration for wild-type flies relative to the no-fly control may be in part due to BPA
157 absorbed by the adult flies which were removed after three days. Another possibility is that
158 BPA detoxification pathways have been characterized in mammals[54] and it is possible *D.*
159 *melanogaster* also contains endogenous pathways capable of detoxifying BPA.

160 Some laccase substrates, such as ABTS, can function as redox mediators to facilitate the
161 oxidation of additional compounds[55]. When *in vivo* BPA degradation was tested in the
162 presence of ABTS, Dm/Tt.Lcc1 flies effectively reduced BPA concentration by 68% and 56%
163 compared to the no-fly control and wild-type, respectively (Fig. 3d). It is not clear why ABTS
164 was necessary for the *in vivo* assay and not the *in vitro* lysate assays. Adding lysate directly to
165 the media also required ABTS for efficient degredation, suggesting that there is some
166 component of the diet which may be interfering (Fig. S1).

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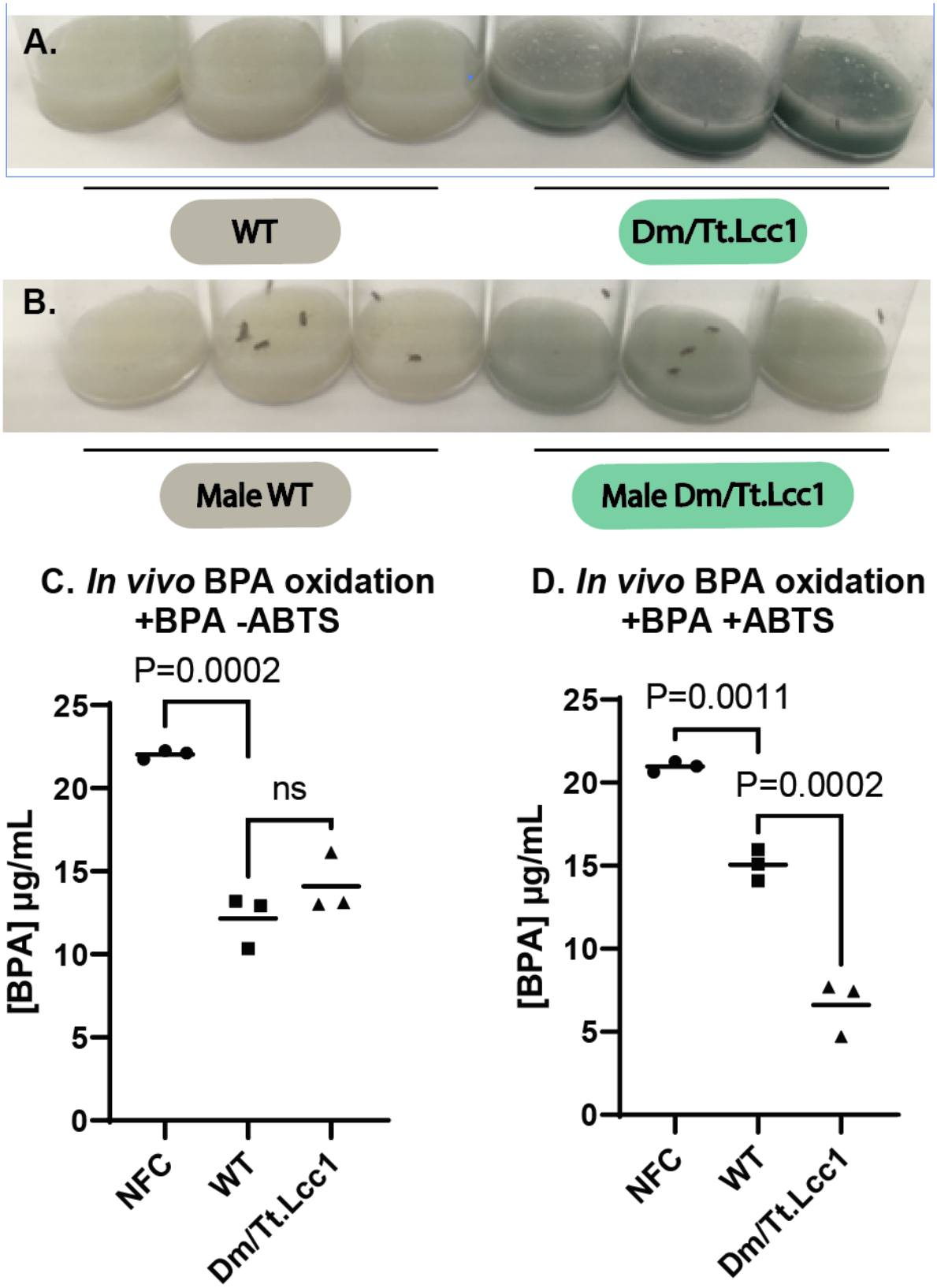

Fig. 3: *In vivo* laccase enzyme activity. (A) *In vivo* oxidation of 1 mg/mL ABTS after 7 days. (**B)** *In vivo* oxidation of 1 mg/mL ABTS after 11 days using only male flies. **(C, D)** *In vivo* degradation of 25 µg/mL BPA without ABTS **(C)** and with 1 mg/mL ABTS **(D).** *n* = 3 biologically

172 independent replicates. Dm/Tt.Lcc1 = *D. melanogaster* expressing *T. trogii* laccase, WT = wild-
173 type, NFC = No-Fly control. The statistical analysis was conducted using one-way ANOVA using
174 Dunnett's method compared to WT, where ns = not significant.

175 **Lyophilized insect powder laccase activity**

176 We next examined the potential of insects as platforms to produce a lyophilized laccase which
177 can be easily stored and transported before use. A crude lyophilized powder from Dm/Tt.Lcc1
178 was compared to a commercially available purified laccase from *Trametes versicolor* using
179 ABTS as a substrate. The lyophilized Dm/Tt.Lcc1 displayed a total laccase activity of 1.4 U/g ±
180 0.1 SEM dry weight, while the commercial purified laccase displayed a specific activity of 75.3
181 U/g ± 6 SEM dry weight.

182 We next tested if Dm/Tt.Lcc1 lyophilized whole-fly powder had activity against indigo
183 carmine, a textile industry dye and pollutant[43]. Incubating 5 mg/mL of Dm/Tt.Lcc1 lyophilized
184 powder in water containing 100 mg/L indigo carmine resulted in 90% decolorization after 48
185 h; however, WT fly lyophilized powder also decolorized 55% of the dye (Fig. 4a). We observed
186 that insoluble material in the powder appeared to adsorb the dye. However, incubating dye
187 using supernatant from centrifuged lyophilized Dm/Tt.Lcc1 in water did not appear to
188 decolorize indigo carmine compared to controls (Fig. 4b). When the Cu^{2+} co-factor was
189 included, supernatant from centrifuged lyophilized powder in water decolorized nearly 100%
190 of indigo carmine in Dm/Tt.Lcc1 and 25% from WT flies after 90 hours (Fig. 4c-d).

191 We also evaluated Dm/Tt.Lcc1 lyophilized powder activity against BPA. Incubating 12.5 µg/mL
192 BPA with soluble enzyme from the lyophilized powder in the presence of Cu^{2+} co-factor
193 resulted in a 96% reduction of BPA from Dm/Tt.Lcc1 fly powder after 90 hours. Incubation
194 with WT fly powder resulted in a 3% reduction of BPA (Fig. 4e-f).

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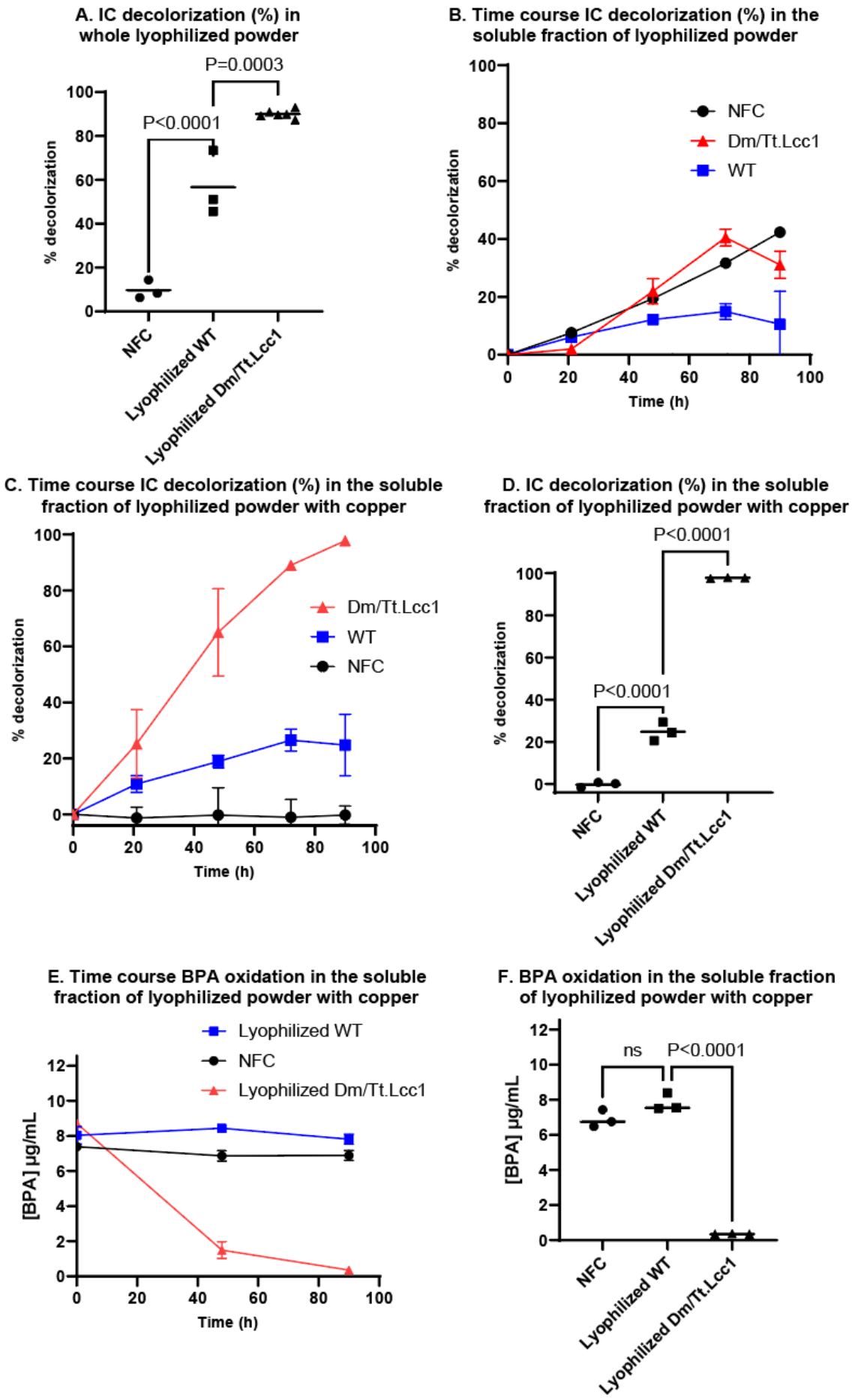
A. IC decolorization (%) in whole lyophilized powder
P=0.0003
P<0.0001
% decolorization
NFC
Lyophilized WT
Lyophilized Dm/Tt.Lcc1
B. Time course IC decolorization (%) in the soluble fraction of lyophilized powder
NFC
Dm/Tt.Lcc1
WT
% decolorization
Time (h)
C. Time course IC decolorization (%) in the soluble fraction of lyophilized powder with copper
Dm/Tt.Lcc1
WT
NFC
% decolorization
Time (h)
D. IC decolorization (%) in the soluble fraction of lyophilized powder with copper
P<0.0001
P<0.0001
% decolorization
NFC
Lyophilized WT
Lyophilized Dm/Tt.Lcc1
E. Time course BPA oxidation in the soluble fraction of lyophilized powder with copper
Lyophilized WT
NFC
Lyophilized Dm/Tt.Lcc1
[BPA] µg/mL
Time (h)
F. BPA oxidation in the soluble fraction of lyophilized powder with copper
ns
P<0.0001
[BPA] µg/mL
NFC
Lyophilized WT
Lyophilized Dm/Tt.Lcc1

198 **Fig. 4: *In vitro* activity of lyophilized Dm/Tt.Lcc1 whole-fly powder on laccase substrates. (A)**
199 Decolorization (%) of 100mg/L indigo carmine at 610 nm in whole lyophilized powder
200 resuspended in water after 48 hours. *n* = 3 biologically independent replicates. **(B-D)** Time
201 course decolorization (%) of 100 mg/mL indigo carmine in the soluble fraction of lyophilized
202 powder in water **(B)** or supplemented with 10 mM $CuSO_4$ **(C)**. Statistical analysis of **C** was
203 performed at end point **(D)**. *n* = 3 biologically independent replicates. **(E,F)** Time course
204 degradation of 12.5 µg/mL BPA in the soluble fraction of lyophilized samples in water
205 supplemented with 10 mM $CuSO_4$ **(E)**. Statistical analysis of **E** was performed at the end point
206 **(F).** *n* = 3 biologically independent replicates. Dm/Tt.Lcc1 = *D. melanogaster* engineered with
207 *T. trogii* laccase, WT = wild-type, NFC = No-Fly control. The statistical analysis was conducted
208 using one-way ANOVA using Dunnett's method compared to WT, where ns = not significant.

209 **Discussion**

210 Engineering animals to heterologously express fungal/microbial enzymes may improve
211 sustainable waste management, bioremediation, and facilitate the use of low-value waste
212 streams as inputs for the production of high-value products such as industrial enzymes. Here,
213 we engineer *D. melanogaster* to express a functional laccase from the fungus *T. trogii*. Lysates
214 of this engineered fly (Dm/Tt.Lcc1) oxidized the endocrine disruptor BPA by 95% *in vitro*
215 compared to the WT flies. Engineered flies also degraded BPA by 56% *in vivo* when added to
216 their diet. Further, a powder prepared from lyophilized engineered flies also degraded BPA
217 by 96% and the textile dye indigo carmine by almost 100% *in vitro*.

218 Mediator compounds are often used to improve the substrate range and activity of laccases[56].
219 Although Dm/Tt.Lcc1 lyophilized powder did not require the addition of ABTS as a mediator
220 for degrading BPA, *in vivo* BPA degradation did. White rot fungi such as *Phanerochaete*
221 *chrysosporium* and *Pycnoporus cinnabarinus* synthesize and secrete endogenous redox
222 mediators[57,58] and it may be possible to engineer animals to secrete redox mediators to also
223 enhance their bioremediation activities. Alternatively, colloidal lignin or derivatives of lignin
224 also show potential to act as redox mediators[59,60], and including them may be feasible to assist
225 with *in vivo* activity.

226 Using transgenic insects as platforms to convert waste to high-value products, such as
227 industrial enzymes, will require high production titres. Dm/Tt.Lcc1 lyophilized powder made
228 from whole flies had 1.4 U/g of laccase activity without any attempt at optimization compared

229 to 75.3 U/g of activity from a commercially available purified *T. versicolor* laccase. This is a
230 very promising start and developing tools to genetically manipulate more relevant hosts and
231 strain engineering is needed for insects to reach their potential as bioproduction platforms.

232 In summary, our findings demonstrate for the first time that insects can be stably engineered
233 to express a functional fungal laccase, which demonstrated *in vivo and in vitro* laccase activity.
234 Transgenic flies were capable of oxidizing laccase substrates in their environment and they
235 can be used as a lyophilized powder to oxidize substrates in aqueous solutions. These results
236 provide strong support for further research in using transgenic animals as platforms for
237 bioremediation, waste stream management, and recombinant protein production.

238 **Methods**

239 **Construct design and assembly**

240 NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs) was used to assemble all
241 constructs. pMBO2744[15] was used as a backbone to generate the enzyme expression
242 plasmids. A gBLOCK (IDT®) was synthesized that encodes a short alpha-tubulin promoter, a
243 downstream SV40 polyadenylation sequence, and a NotI restriction site in between. This
244 gBLOCK was installed into NotI linearized pMB02744 by HiFi assembly to produce pMC-1-1-1
245 (Supplementary Table 4). This plasmid was then linearized with NotI, and gBLOCKs that
246 encode for *T. trogii* laccase or *P. brumalis* laccase enzyme were installed with HiFi assembly
247 to generate pMC-1-2-3 and pMC-1-2-6, respectively (Supplementary Table 4). The DNA
248 sequences were codon optimized for expression in *D. melanogaster* using the IDT® codon
249 optimization tool. The native fungal signal peptides were replaced with the larval cuticle
250 protein 9 signal peptide. The Kozak sequence, AATCTTACAAA, was upstream of the start
251 codon. See Supplementary Table 2 for plasmid descriptions and Supplementary Table 3 for
252 primers used in this study.

253

254 **Insect rearing and generation of transgenic fly strains**

255 Canton S wild-type flies were from the Bloomington Stock Centre (64349, RRID: BDSC 64349).
256 Enzyme expression plasmids were purified using ZymoPURE™ II Plasmid Midiprep Kit (Zymo
257 Research #D4200) and sent to BestGene Inc (Chino Hills, Ca) for ϕC31 mediated integration.
258 All strains were maintained on a cornmeal diet based on the Bloomington standard Nutri-Fly
259 formulation (catalog number 66-113; Genesee Scientific). In preparation for the *in vitro* or *in*

260 *vivo* laccase enzyme activity assay, the diet for both the transgenic and wild-type strains was
261 supplemented with $CuSO_4 \bullet 5H_2O$ (1 mM, Merck, CAS-no: 7758-99-9). Flies were reared in a
262 controlled environment room at 25.0˚C, 75% humidity, and a 12 hour light/dark cycle with a
263 30 minute transition period. See Supplementary Table 1 for all of the fly strains used in this
264 study. Biosafety approval was granted by the Institutional Biosafety Committee of Macquarie
265 University (#5049).

266 ***In vitro* enzyme activity assay**

267 To obtain a fly lysate, ten frozen adult flies were homogenized with a motorized pestle in the
268 stated buffer supplemented with 10 mM CuSO4•5H2O and acid-washed glass beads. The
269 samples were then agitated along a tube rack 10 times. Each tube was incubated for 15
270 minutes at 25 °C and centrifuged twice to remove insoluble debris.

271 For the ABTS oxidation assay, fly lysates were prepared in citrate-phosphate buffer (pH 5) and
272 added to 1 mM ABTS. The tubes were incubated at 25 ˚C for 2.5 hours. The reaction was
273 quenched by adding 5% trichloroacetic acid (TCA, Sigma Aldrich, catalogue #T0699, CAS-
274 no:76-03-9). A UV-Vis spectrophotometer (Jasco V-760 UV-Vis spectrophotometer) was used
275 to measure the absorbance at 420 nm. The measurements were blanked to an equivalent
276 sample that did not contain ABTS.

277 For the BPA degradation assay, fly lysates or controls were prepared in sodium acetate buffer
278 (pH 5) and added to 25 µg/mL BPA. The assay mixtures were incubated at room temperature
279 for 21 hours. The reaction was quenched by adding 5% (v/v) trichloroacetic acid (Sigma
280 Aldrich, catalog #T0699, CAS-no:76-03-9). BPA concentration was analyzed by LC-MS as
281 described below.

282 ***In vivo* laccase activity assays**
283

284 Minimal media:

285 The minimal media components were for 100 mL: 1.58 g yeast, 0.66 g agar, 5 g sugar, 4 mL 1
286 M propionic acid (pH 4.4), 12.4 mg $CuSO_4 \bullet 5H_2O$ (1 mM final concentration). A buffer of 20
287 mM citrate-phosphate (pH 5) was added for the initial ABTS assays, 25 mM sodium acetate
288 buffer (pH 5) was used for all BPA experiments.

289 ABTS only *in vivo* experiments:

290 To minimal media 100 mg ABTS (1 mg/mL final concentration) was added. Three female and
291 three male adult flies were incubated on the media for 48 hours, after which they were
292 removed. For the all-male ABTS assay, the adult flies remained in the vial for 11 days. The
293 appearance of the dark green radical cation was monitored and recorded with photographs.

294 For the ABTS + BPA experiments:

295 To minimal media, BPA was added (25 µg/mL final concentration). ABTS (1 mg/mL final
296 concentration) was added for the +BPA, + ABTS condition.

297 For each condition, the liquid media was pipetted into a 14 mL polypropylene round-bottom
298 culture tube. 5 male and 5 female adult flies were incubated on the media for 3 days and
299 then removed. For the lysate condition, 50 µL of lysate from ~100 flies was added to the
300 tube. After a total of 10 days the enzymatic reaction was stopped with TCA (5% v/v final
301 concentration). The media was melted in a water bath, after which methanol (making ~1:1
302 methanol: aqueous TCA for a two-fold dilution of BPA) was added, followed by vortexing.
303 The tubes were then incubated at 300 rpm and 37 ˚C for 30 minutes. After incubation, the
304 tubes were centrifuged twice with the final supernatant reserved for BPA quantification by
305 UHPLC-FLD as described below.
306

307 **Lyophilized Dm/Tt.Lcc1 enzyme activity assays**

308 Frozen Dm/Tt.Lcc1 or wild-type flies were lyophilized (Alpha 1-4 LDplus, Christ) at -45 °C at
309 0.1 mBar. The freeze-dried flies were homogenized in a porcelain mortar and pestle and
310 stored at 4 °C.

311 For the activity assays, lyophilized Dm/Tt.Lcc1 and commercial *T. versicolor* laccase (Sigma-
312 Aldrich, Catalogue 38429) were resuspended in 25 mM sodium acetate buffer (pH 5)
313 supplemented with 10 mM $CuSO_4 \bullet 5H_2O$. Lyophilized Dm/Tt.Lcc1 did not dissolve, and the
314 soluble fraction was extracted at 25 °C for 15 minutes at 800 rpm and centrifuged twice to
315 remove the insoluble debris. Enzyme serial dilutions were added in duplicate to 1 mM ABTS
316 and ABTS oxidation was monitored on a PHERAStar FS plate reader (BMG Labtech) at 420 nm
317 at 25 °C. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the
318 oxidation of 1 μmol ABTS min^{-1} using the molar extinction coefficient 36 mM^{-1} cm^{-1} [61].

319 For the indigo carmine decolorization assay, 5 mg/mL of lyophilized powder or controls in
320 Millipore water were added to 100 mg/L indigo carmine (Sigma-Aldrich catalog 131164) and
321 incubated at RT. At 0 and 48 hours, indigo carmine decolorization was monitored on a
322 PHERAStar FS plate reader (BMG Labtech) at 610 nm.

323 As indigo carmine appeared to adsorb to the lyophilized powder, the soluble fraction was
324 tested for enzymatic activity. 5 mg/mL lyophilized powder or controls in Millipore water
325 supplemented with and without 10 mM $CuSO_4 \bullet 5H_2O$ were extracted at 25 °C for 15 minutes
326 at 800 rpm. The samples were centrifuged twice to remove the insoluble debris. The
327 supernatant of the lyophilized powder and controls were added to 100 mg/L indigo carmine
328 and incubated at room temperature. Indigo carmine decolorization was monitored on a
329 PHERAStar FS plate reader (BMG Labtech) at 610 nm at 0, 21, 48, 72, and 90 hours.

330 For the BPA degradation assay, the soluble fraction of the lyophilized powder was extracted
331 as described in the indigo carmine assay. The supernatant of the lyophilized powder and
332 controls were added to 12.5 µg/mL BPA and incubated at room temperature. At 0, 48, and 96
333 hours, 100 µL aliquots were removed and the reaction was stopped using a 3 KDa nominal
334 molecular weight limit (NMWL) centrifuge filter (Merck Amicon Ultra 0.5mL Centrifugal
335 Filters, UFC500396). BPA concentration was analyzed by UHPLC-FLD as described below.

336 **BPA quantification**

337 BPA in samples was either quantified by UHPLC-FLD analysis or by LC-MS. UHPLC-FLD analysis
338 was performed using an Agilent RRHD ZORBAX Eclipse Plus C18 column (3.0 x 150 mm 1.8 µm,
339 p/n 959759-302) equipped with a UHPLC Eclipse Plus C18 Guard Column, (2.1 mm 1.8 µm,
340 p/n 821725-901). The isocratic mobile phase consisted of acetonitrile (ACN):10 mM KH_2PO_4
341 (50:50 v/v) at a flow rate of 0.5 mL/min. A gradient wash step was introduced after each run
342 with eluent B 100% ACN and eluent A 10mM KH_2PO_4, starting with 45% B1 from 0 - 1 minute,
343 70% B from 1 – 6 minutes, 5% B from 6 – 11 minutes, and finally 50% B from 11 - 16 minutes
344 at 0.3 mL/min. BPA was detected by setting FLD excitation wavelength at 229 nm, and
345 emission wavelength at 316 nm and a gain setting of 12. Linear regression of 7-point
346 standards from 12.5 ng/mL to 12,500 ng/mL BPA was performed to calibrate BPA
347 concentration. All samples and standards were either filtered using syringe filters (Filtropur S
348 0.2 µm, Sarstedt, 83.1826.001) or centrifuge filters (Merck Amicon Ultra 0.5mL Centrifugal
349 Filters, UFC500396) prior to injection into the UHPLC.

350 LC-MS validation and quantification of BPA was performed externally at the Mass
351 Spectrometry Facility at the University of Sydney. LC separation was performed using a
352 Waters Sunfire C18 column (5 μm, 2.1 mm ID x 15 mm) coupled to a Bruker amaZon SL mass
353 spectrometer. Gradient elution was performed with a mobile phase consisting of (A)
354 methanol and (B) water with 0.1% acetic acid at a flow rate of 0.3 mL/min. The gradient
355 consisted of 20% A from 0 - 9.3 minutes, 76% A from 9.3 - 17.3 minutes, 92% A from 17.3 –
356 26 minutes, and finally 20% A from 26 - 35 minutes. MS analysis was performed using
357 Atmospheric Pressure Chemical Ionisation (ACPI) under positive and negative ionization mode
358 over a range of 200-1400 m/z. The operation parameters were: Nebulizer 27.3 psi, Dry Gas
359 4.0 L/min, Dry temperature 180 °C, APCI Vaporisation temperature: 400 °C, APCI Corona
360 needle current: 6000 nA, Mass mode: enhanced resolution, SPS (Smart parameter setting)
361 Target mass 150 m/z, compound stability 100%. Linear regression of 6-point BPA standards
362 from 20 ng/mL to 10 µg/mL BPA was performed to calibrate BPA concentration. An internal
363 deuterated BPA-D_{16} standard was spiked into the samples prior to injection to calibrate
364 ionization in the sample matrix compared to the standard curve matrix prepared in methanol.
365 All samples were centrifuge filtered (Merck Amicon Ultra 0.5mL Centrifugal Filters,
366 UFC500396) prior to injection into the LC-MS.

367 **Statistical analysis**

368 Figures were generated in Prism 9 by GraphPad. For statistical analysis, a one-way ANOVA
369 using Dunnet's method was conducted. The significance level for the analysis was 0.05, and
370 all error bars represent the standard error of the mean. The exact *P* values, statistical tests
371 used, and sample numbers (*n*) can be found in the figure legend.

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377 Contributions:** MC, KT, and MM conceived of this study. MC, KT, KP, AS, and MM designed
378 experiments and analyzed data. MC, KT, KP, and SK performed the experiments. All authors
379 contributed to writing the manuscript. **Competing Interests:** The authors have filed a patent

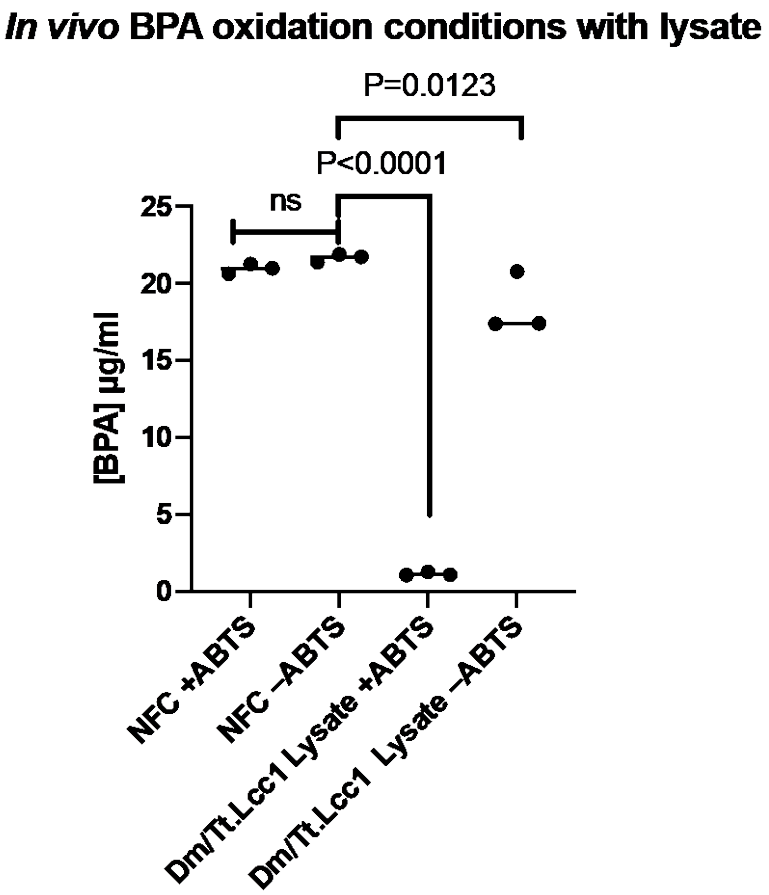
380 application for aspects of this work. **Data and materials availability:** All data is available
381 upon request.

382 **Supplementary Materials:**

383 Supplementary Figure 1
384 Supplementary Tables 1-4
385 Supplementary Data File
386

387 **Supplementary information**

388



389

390 **Supplementary figure 1:** *In vivo* BPA degradation conditions comparing Dm/Tt.Lcc1 lysate to
391 NFC. Fly lysate was added to media that contained 25 µg/mL BPA with and without 1 mg/mL
392 ABTS. Dm/Tt.Lcc1 lysate was unable to degrade BPA without ABTS. *n* = 3 biologically
393 independent replicates. The statistical analysis was a one-way ANOVA with Dunnett's
394 method. ns= not significant, Dm/Tt.Lcc1 = *D. melanogaster* engineered with *T. trogii* laccase,
395 NFC = no fly control.

396

397

Supplementary Table 1: *D. melanogaster* strains used in this study

Strain	Description	Reference	BDSC #
Wild Type	Canton S	[62]	64349
2nd Chr. attP3	y^1 $w^{1118;}$ PBac{y[+]-attP-3B}VK00037	[63]	9752
2nd Chr. Bal, Tm2/Tm6b	w^{1118}; Gla/CyO; Tm2/Tm6b	This study	
2nd Chr. Bal, Tm2/Tm6b	w^{1118}; Bl/CyO; Tm2/Tm6b	This study	
Dm/Tt.Lcc1	y^1 $w^{1118;}$ PBac{Tt.Lcc1}attP-3B	This study	
Dm/Pb.Lac1	y^1 $w^{1118;}$ PBac{Pb.Lac1}attP-3B	This study	

398

Supplementary Table 2: Plasmids used in this study

Plasmid	Integrated in Fly Strain	Description	Reference/ GenBank Accession #
pMB02744	NA	Contains a mini-white gene for *D. melanogaster* selection, an *AttB* site for genomic integration, and an ampicillin resistance cassette for cloning in *E. coli*.	[15]
pMC-1-1-1	NA	Empty vector, with short alpha-tubulin promoter and SV40 regulatory sequences	This study
pMC-1-2-3	Dm/Tt.Lcc1	Short alpha-tubulin promoter driving expression of *Lcc1* from *Trametes trogii*	This study
pMC-1-2-6	Dm/Pb.Lac1	Short alpha-tubulin promoter driving expression of *Lac1* from *Polyporus brumalis*	This study

399

Supplementary table 3: Primers used in this study

Name	**Sequence (5' → 3')**	**Notes**
1stconstruct_F_2.0	GCTTGGATTTCACTGGAACTAG	pMC-1-1-1 sequencing
1stconstruct_R	TCGAGACCGTGACCTACATC	pMC-1-1-1 sequencing
2ndconstruct_F_2.0	cgtttgtcaagcctcatagc	pMC-1-2-3/pMC-1-2-6 sequencing
2ndconstruct_R_2.0	ttcattttatgtttcaggttcaggg	pMC-1-2-3/pMC-1-2-6 sequencing
P.bru_F	TGACTTTCAGGTGCCCGATC	pMC-1-2-6 sequencing
P.bru_R	GCCGGGAATGTGAGTTCGATG	pMC-1-2-6 sequencing

T.tro_F	ATGGCCCTGCATTCGTCAAC	pMC-1-2-3 sequencing
T.tro_R	CGTTGCTGGCAAGCTGATTTC	pMC-1-2-3 sequencing

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Supplementary table 4: gblocks used to construct laccase expression plasmids:	
Name and description	**Sequence (5' → 3')**
1st construct Gblock that includes short alpha-tubulin and SV40 regulatory sequences for expression of the enzymes in *Drosophila melanogaster*. Contains a NotI site between the promoter and terminator for assembly of enzyme gene gblocks. Generates pMC-1-1-1 from pMBO2744	AGCGTAAGCTTCGTACGTAGCGACCGTCTCAAAGTACTGCCTTTCTGCGTTGGAAAAC ATCGCCTTTTTCGTCCAAAAGGAGTCCCCAGGTTCGATCCGCATGGCGTTGTGCGTGC GTGCCTTTCTTTTCAAATGATTACGGCTATTAACTTGGGGGCGTTAAGTTGGAAACAC GTAAATTGCAGACTGCGATTAGAGTGACCATGAGTAGGAGTTCAAAATCTCCTGACA TCATTTTCTTAAAACCTGCTTTGTTTTTTACATTTCTATTTAATATAACTCCTATTTGAAT AAAAAAACAAAACAAGTTTAGATGTTAAGATATTAACTACATCCTTTGCTCCAAAGGG AGAGGGGAAGTTATGGAGTTAATTAATTTGCTGTTGGAAATCAATATGGAGTCAGAA ATATAATGATTTACTAAACCTTATTGAATCGGTAACGATGCGAATTTATATTAAAATA GCTTTTATGAAACATTCAACAAAAATATTATTAATGTTGGCCCACTTTAGCAACCGGTT AGGTCTACCGGTTGGGCAAGCAAAGATTCACGCCCTGGTTCGAGTCCCAACTAGTCC TGCAAAATACCGCAGCAAGTTTTAGAGAGACCAAGTGCCATTACCTCTCCCACTTCAG TTATCGGTTATGCGGCGTTTAAGTCGACAGCTTGCCGTCTCTAGCTCCGGTGCCTATA TAAAGCAGCCCGCTTTCCACATTTCATATTCGTTTTACGTTTGTCAAGCCTCATAGCCG GCAGTTCGAACGTATACGCTCTCTGAGTCAGACCTCGAAATCGTAGCTCTACACAATT CTGTGAATTTTCCTTGTCGCGTGTGAAACACTTCCAAGGCGGCCGCCCGACTCTAGAT CATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACC TCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCA GCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTT TTTCACTGCATTCTAGTTGTGGTTTGCCCAAACTCATCAATGTATCTTAGCGGCTCGAG GGTACCTCTAG
P. Bru Lac *Polyporus brumalis* laccase gene, codon optimised for *D. melanogaster* with overlaps to pMC-1-1-1 and larval cuticle 9 signal peptide sequence. Generates pMC-1-2-6	gtgtgaaacacttccaagGCaatcttacaaaATGAAGTTTGTTATTGTTCTGGCTTGCCTGTTGG CAGTTGTCTTTGCCGCTATTGGCCCTGTTACCGACTTGACGGTTACGGATGCCAACAT CAGTCCCGACGGATTTACGCGCGCCGGTATAGTGGTCAACAACGTGTTCCCCGCTCC CCTGATTACGGGACAAAAAGGCGACAGGTTTCAGCTGAATCTGGTCAACCAAATGAG TAATCATACGATGTTGAAGACAACTTCGATACACTGGCACGGTTTTTTTCAAAAGGGA ACTAATTGGGCGGATGGACCCGCATTCGTGAATCAGTGTCCTATCGCTAGCGGTAAT TCGTTTCTGTATGACTTTCAGGTGCCCGATCAAGCTGGAACTTTCTGGTACCACTCCCA CCTGTCCACGCAGTATTGTGACGGTCTCCGCGGACCTTTCGTAGTTTATGACCCAACT GACCCTCACCTGTCGCTGTATGACGTGGATGACGATAGCACAGTTATAACGCTGGCG GATTGGTACCATGTGGCCGCACGCCTGGGACCGCGCTTCCCGTTGGGTGCTGACTCC ACTCTGATCAACGGTTTGGGCAGGTCCACCGCGACTCCGACTGCTGATCTGGCTGTG ATCAGTGTAACCAAAGGTAAACGTTACCGGTTTAGGCTGGTGAGTATTTCCTGCGAT CCGAACCATACCTTCAGCATTGACGGTCACAAGCTCACCGTCATCGAAGCTGATGGC ATTTCCACTCAACCCGTCACTGGCATTGACTCCATTCAAATTTTTGCCGCACAGAGGTA CAGCTTCGTCCTCACCGCCGACCAGGATGTAGACAATTACTGGGTTAGGGCTAACCCT AATTTTGGTACTACTGGATTTGCCGGAGGCATTAACAGCGCGATTCTCCGTTATGATG GTGCACCTGCGGTTGAACCCACTACCTCCCAAACCGGTACGAATCTGCTCGTGGAGA CAGATCTGCATCCTCTGAGCACAATGCCCGTTCCGGGACTGCCGACACAAGGTGGTG CCGATTTTAATCTCAATCTCGCCTTTAACTTTAACGGTTCGGACTTCTTTATTAACGGT GCTTCGTTCGTTCCACCGACCGTCCCTGTCCTCCTCCAGATAATCAGCGGTGCGAACA GCGCCCAAGATTTGCTCCCAAGTGGTTCGGTATATGCACTCCCCTCGAATAGCAGCAT CGAACTCACATTCCCGGCCACTGCTGCAGCGCCAGGCGCCCCCCATCCATTCCACTTG CATGGCCACGCTTTTGCCGTAGTACGCAGTGCTGGAAGTACTGTTTATAACTACGACA

	ACCCGGTATTCAGGGATGTAGTTTCCACTGGTACGCCCGCTGCGGGCGACAACGTCA CCATCCGATTCCAAACGGACAATCCTGGACCATGGTTTTTGCATTGTCACATCGACTT CCACCTCGATGCTGGCTTCGCTGTCGTGTTCGCGGAAGACCTCCCGGACGTTGTAAGT GCAAACCCCGTGCCCCAAGCGTGGAGCGATTTGTGCCCCATCTATAACGCTCTCGATC CGTCGGATCAGTAAGCccgactctagatcataatc
<u>T. tro. Lac</u> *Trametes trogii* laccase gene, codon optimised for *D. melanogaster* with overlaps to pMC-1-1-1 and larval cuticle 9 signal peptide sequence. Generates pMC-1-2-3	gtgtgaaacacttccaagGCaatcttacaaaATGAAGTTTGTTATTGTTCTGGCTTGCCTGTTGG CAGTTGTCTTTGCCGCTATTGGTCCAGTTGCAGATCTCACTATCAGCAACGGAGCGGT TAGTCCTGATGGATTCTCCCGCCAAGCCATATTGGTGAACGATGTGTTCCCGTCGCCG TTGATAACGGGAAATAAAGGTGACAGGTTTCAGCTCAACGTAATAGACAACATGACA AATCACACGATGCTCAAATCCACAAGTATCCATTGGCATGGTTTTTTCCAACATGGCA CAAATTGGGCTGATGGCCCTGCATTCGTCAACCAATGTCCCATTAGTACGGGCCACGC TTTTCTGTATGATTTTCAGGTTCCGGATCAGGCGGGTACCTTCTGGTATCACTCGCATT TGAGCACTCAGTACTGCGACGGCCTGCGTGGTCCAATCGTAGTCTATGATCCCCAGG ACCCGCATAAAAGTCTGTATGATGTTGATGATGACAGTACAGTGATAACCCTCGCCG ACTGGTATCATTTGGCAGCTAAGGTCGGCTCGCCGGTACCAACAGCCGACGCAACAC TCATAAATGGCCTGGGTAGGTCCATAGACACCCTGAACGCAGATTTGGCGGTTATTA CCGTTACAAAAGGCAAGAGGTATCGCTTCCGTTTGGTCAGTTTGAGTTGCGACCCAA ATCATGTTTTCTCCATTGATGGTCATTCGTTGACCGTAATTGAGGCGGATTCCGTAAA CTTGAAGCCTCAAACGGTAGATAGTATCCAAATCTTTGCGGCCCAACGGTATAGCTTC GTCCTGAATGCTGATCAAGATGTGGGCAACTACTGGATTCGAGCTCTGCCGAATAGT GGCACACGCAACTTTGACGGAGGTGTTAATTCGGCGATCCTCCGCTATGATGGCGCC GCGCCCGTCGAACCGACAACCAGTCAAACGCCCAGCACGAATCCACTCGTAGAGTCG GCCCTGACAACATTGGAGGGAACAGCAGCGCCGGGTAGCCCTGCTCCGGGAGGTGT AGACCTCGCTCTGAACATGGCGTTTGGCTTTGCTGGCGGCAAGTTTACCATAAATGG AGCAAGCTTTACTCCCCCGACCGTCCCTGTGCTGTTGCAGATACTCTCCGGTGCGCAG TCGGCCCAAGATCTGCTCCCGTCGGGCTCCGTTTATTCCCTGCCCGCCAATGCAGACA TCGAAATCAGCTTGCCAGCAACGGCTGCCGCTCCCGGTTTCCCACACCCGTTTCATCT CCACGGTCATACTTTTGCTGTGGTTCGCAGCGCAGGCTCCAGTACATACAATTATGAG AATCCCGTCTACCGTGACGTCGTTTCGACTGGCTCGCCCGGCGATAATGTCACCATCC GTTTCCGAACGGATAACCCGGGCCCATGGTTTCTGCATTGTCATATAGACTTTCATTT GGAGGCGGGTTTTGCAGTGGTTATGGCCGAGGATATCCCGGAGGTTGCCGCCACTA ACCCCGTACCACAAGCATGGTCGGATCTGTGCCCCACATACGACGCGCTGTCGCCGG ACGATCAGTAAGCccgactctagatcataatc

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References:

1. UN General Assembly. *Transforming our world : the 2030 Agenda for Sustainable Development*. (2015).

2. Silpa Kaza, Yao, L., Bhada-Tata, P. & Woerden, F. Van. *What a Waste 2.0: A Global Snapshot of Solid Waste Management to 2050*. (Urban Dev. World Bank, Washington, DC, 295, 2018).

3. Weitz, M., Coburn, J. B. & Salinas, E. Estimating National Landfill Methane Emissions: An Application of the 2006 Intergovernmental Panel on Climate Change Waste Model in Panama. *J. Air Waste Manage. Assoc.* **58**, 636–640 (2008).

4. Du, M. *et al.* Quantification of methane emissions from municipal solid waste landfills in China during the past decade. *Renewable and Sustainable Energy Reviews* **78**, 272–279 (2017).

5. Przydatek, G. & Kanownik, W. Impact of small municipal solid waste landfill on groundwater quality. *Environ. Monit. Assess.* **191**, 1–14 (2019).

6. Landrigan, P. J. *et al.* The Lancet Commission on pollution and health. *Lancet* **391**, 462–512 (2018).

7. Fuller, R. Hazardous Waste and Toxic Hotspots. in *Textbook of Children's Environmental Health* 254–261 (Oxford University Press, 2014). doi:10.1093/med/9780199929573.003.0027

8. Lanphear, B. P. *et al.* Low-level environmental lead exposure and children's intellectual function: An international pooled analysis. *Environ. Health Perspect.* **113**, 894–899 (2005).

9. Kingsford, R. T. *et al.* Major Conservation Policy Issues for Biodiversity in Oceania. *Conserv. Biol.* **23**, 834–840 (2009).

10. Malaj, E. *et al.* Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 9549–54 (2014).

11. (CIE), C. of I. E. NSW waste regulation. (2014).

12. Stahel, W. R. The circular economy. *Nature* **531**, 435–438 (2016).

13. Clark, M. & Maselko, M. Transgene Biocontainment Strategies for Molecular Farming. *Frontiers in Plant Science* **11**, 210 (2020).

14. Lee, J. W., Chan, C. T. Y., Slomovic, S. & Collins, J. J. Next-generation biocontainment systems for engineered organisms. *Nat. Chem. Biol.* **14**, 530–537 (2018).

15. Maselko, M. *et al.* Engineering multiple species-like genetic incompatibilities in insects. *Nat. Commun.* **11**, 1–7 (2020).

16. Diener, S., Studt Solano, N. M., Roa Gutiérrez, F., Zurbrügg, C. & Tockner, K. Biological treatment of municipal organic waste using black soldier fly larvae. *Waste and Biomass Valorization* **2**, 357–363 (2011).

17. Parodi, A. *et al.* Black soldier fly larvae show a stronger preference for manure than

for a mass-rearing diet. *J. Appl. Entomol.* **144**, 560–565 (2020).

18. Meyer, H.-P., Minas, W. & Schmidhalter, D. Industrial-Scale Fermentation. in *Industrial Biotechnology* 1–53 (Wiley-VCH Verlag GmbH & Co. KGaA, 2016). doi:10.1002/9783527807833.ch1

19. Dahman, Y., Syed, K., Begum, S., Roy, P. & Mohtasebi, B. 14 - Biofuels: Their characteristics and analysis. in *Woodhead Publishing Series in Composites Science and Engineering* (eds. Verma, D., Fortunati, E., Jain, S. & Zhang Biopolymer-Based Materials, and Bioenergy, X. B. T.-B.) 277–325 (Woodhead Publishing, 2019). doi:https://doi.org/10.1016/B978-0-08-102426-3.00014-X

20. Joosten, L. *et al.* Review of insect pathogen risks for the black soldier fly (Hermetia illucens) and guidelines for reliable production. *Entomol. Exp. Appl.* **168**, 432–447 (2020).

21. Dortmans, B., Diener, S., Verstappen, B. & Zurbrugg, C. *Black Soldier Fly Biowaste Processing*. (2017).

22. Kim, C.-H. *et al.* Use of Black Soldier Fly Larvae for Food Waste Treatment and Energy Production in Asian Countries: A Review. *Processes* **9**, (2021).

23. Maeda, S. *et al.* Production of human α-interferon in silkworm using a baculovirus vecto. *Nature* **315**, 592–594 (1985).

24. Zeng, S., Zhao, J. & Xia, L. Simultaneous production of laccase and degradation of bisphenol A with Trametes versicolor cultivated on agricultural wastes. *Bioprocess Biosyst. Eng.* **40**, 1237–1245 (2017).

25. Chivukula, M. & Renganathan, V. Phenolic Azo Dye Oxidation by Laccase from Pyricularia oryzae. *Appl. Environ. Microbiol.* **61**, 4374 LP – 4377 (1995).

26. Alharbi, S. K. *et al.* Degradation of diclofenac, trimethoprim, carbamazepine, and sulfamethoxazole by laccase from Trametes versicolor: Transformation products and toxicity of treated effluent. *Biocatal. Biotransformation* **37**, 399–408 (2019).

27. Collins, P. J., Kotterman, M., Field, J. A. & Dobson, A. Oxidation of Anthracene and Benzo[a]pyrene by Laccases from Trametes versicolor. *Appl. Environ. Microbiol.* **62**, 4563 LP – 4567 (1996).

28. Cañas, A. I. *et al.* Transformation of Polycyclic Aromatic Hydrocarbons by Laccase Is Strongly Enhanced by Phenolic Compounds Present in Soil. *Environ. Sci. Technol.* **41**, 2964–2971 (2007).

29. Luo, Q. *et al.* Laccase-Catalyzed Degradation of Perfluorooctanoic Acid. *Environ. Sci. Technol. Lett.* **2**, 198–203 (2015).

30. Luo, Q., Yan, X., Lu, J. & Huang, Q. Perfluorooctanesulfonate Degrades in a Laccase-Mediator System. *Environ. Sci. Technol.* **52**, 10617–10626 (2018).

31. Sumathi, T., Viswanath, B., Sri Lakshmi, A. & SaiGopal, D. V. R. Production of Laccase by *Cochliobolus* sp. Isolated from Plastic Dumped Soils and Their Ability to Degrade Low Molecular Weight PVC. *Biochem. Res. Int.* **2016**, 9519527 (2016).

32. Jin, X. *et al.* Conditions Optimizing and Application of Laccase-mediator System (LMS) for the Laccase-catalyzed Pesticide Degradation. *Sci. Rep.* **6**, 35787 (2016).

33. Alberts, J. F., Gelderblom, W. C. A., Botha, A. & van Zyl, W. H. Degradation of aflatoxin B1 by fungal laccase enzymes. *Int. J. Food Microbiol.* **135**, 47–52 (2009).

34. Rodríguez Couto, S. & Toca Herrera, J. L. Industrial and biotechnological applications of laccases: A review. *Biotechnol. Adv.* **24**, 500–513 (2006).

35. Widsten, P. & Kandelbauer, A. Laccase applications in the forest products industry: A review. *Enzyme Microb. Technol.* **42**, 293–307 (2008).

36. Witayakran, S. & Ragauskas, A. J. Synthetic Applications of Laccase in Green Chemistry. *Adv. Synth. Catal.* **351**, 1187–1209 (2009).

37. Kharayat, Y. Distillery wastewater: bioremediation approaches. *J. Integr. Environ. Sci.* **9**, 69–91 (2012).

38. Baghel, S., Sahariah, B. P. & Anandkumar, J. Bioremediation of Lignin-Rich Pulp and Paper Industry Effluent BT - Combined Application of Physico-Chemical & Microbiological Processes for Industrial Effluent Treatment Plant. in (eds. Shah, M. & Banerjee, A.) 261–278 (Springer Singapore, 2020). doi:10.1007/978-981-15-0497-6_12

39. Ahmed, P. M., Fernández, P. M., de Figueroa, L. I. C. & Pajot, H. F. Exploitation alternatives of olive mill wastewater: production of value-added compounds useful for industry and agriculture. *Biofuel Res. J.* **6**, 980–994 (2019).

40. Kudanga, T. & Le Roes-Hill, M. Laccase applications in biofuels production: current status and future prospects. *Appl. Microbiol. Biotechnol.* **98**, 6525–6542 (2014).

41. Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A. P. & Narasimha, G. Fungal laccases and their applications in bioremediation. *Enzyme Res.* **2014**, (2014).

42. Rochester, J. R. Bisphenol A and human health: A review of the literature. *Reproductive Toxicology* **42**, 132–155 (2013).

43. Chowdhury, M. F. *et al.* Current treatment technologies and mechanisms for removal of indigo carmine dyes from wastewater: A review. *Journal of Molecular Liquids* **318**, 114061 (2020).

44. Gorman, M. J., Dittmer, N. T., Marshall, J. L. & Kanost, M. R. Characterization of the multicopper oxidase gene family in Anopheles gambiae. *Insect Biochem. Mol. Biol.* **38**, 817–24 (2008).

45. Waters, A. J., Capriotti, P., Gaboriau, D. C. A., Papathanos, P. A. & Windbichler, N. Rationally-engineered reproductive barriers using CRISPR & CRISPRa: an evaluation of the synthetic species concept in Drosophila melanogaster. *Sci. Rep.* **8**, 13125 (2018).

46. Charles, J. P., Chihara, C., Nejad, S. & Riddiford, L. M. Identification of proteins and developmental expression of RNAs encoded by the 65A cuticle protein gene cluster in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **28**, 131–8 (1998).

528 47. Almeida, S., Raposo, A., Almeida-González, M. & Carrascosa, C. Bisphenol A: Food
529 Exposure and Impact on Human Health. *Compr. Rev. Food Sci. Food Saf.* **17**, 1503–
530 1517 (2018).

531 48. Atacag Erkurt, H. Biodegradation and Detoxification of BPA: Involving Laccase and a
532 Mediator. *CLEAN - Soil, Air, Water* **43**, 932–939 (2015).

533 49. Garzillo, A. M. *et al.* Structural and Kinetic Characterization of Native Laccases from
534 Pleurotus ostreatus, Rigidoporus lignosus, and Trametes trogii. *J. Protein Chem.* **20**,
535 191–201 (2001).

536 50. Garzillo, A. M. V *et al.* Laccase from the white-rot fungus Trametes trogii. *Appl.*
537 *Microbiol. Biotechnol.* **49**, 545–551 (1998).

538 51. Atacag Erkurt, H. Biodegradation and Detoxification of BPA: Involving Laccase and a
539 Mediator. *CLEAN – Soil, Air, Water* **43**, 932–939 (2015).

540 52. Colao, M. C., Lupino, S., Garzillo, A. M., Buonocore, V. & Ruzzi, M. Heterologous
541 expression of lcc1 gene from Trametes trogii in Pichia pastoris and characterization of
542 the recombinant enzyme. *Microb. Cell Fact.* **5**, 31 (2006).

543 53. Nakade, K., Nakagawa, Y., Yano, A., Sato, T. & Sakamoto, Y. Characterization of an
544 extracellular laccase, PbLac1, purified from Polyporus brumalis. *Fungal Biol.* **114**, 609–
545 618 (2010).

546 54. Shmarakov, I. O., Borschovetska, V. L. & Blaner, W. S. Hepatic Detoxification of
547 Bisphenol A is Retinoid-Dependent. *Toxicol. Sci.* **157**, 141–155 (2017).

548 55. Bourbonnais, R. & Paice, M. G. Oxidation of non-phenolic substrates: An expanded
549 role for laccase in lignin biodegradation. *FEBS Lett.* **267**, 99–102 (1990).

550 56. Morozova, O. V., Shumakovich, G. P., Shleev, S. V. & Yaropolov, Y. I. Laccase-mediator
551 systems and their applications: A review. *Appl. Biochem. Microbiol.* **43**, 523–535
552 (2007).

553 57. Jensen, K. A., Evans, K. M., Kirk, T. K. & Hammel, K. E. Biosynthetic Pathway for
554 Veratryl Alcohol in the Ligninolytic Fungus Phanerochaete chrysosporium. *Appl.*
555 *Environ. Microbiol.* **60**, 709–714 (1994).

556 58. Eggert, C., Temp, U., Dean, J. F. D. & Eriksson, K.-E. L. A fungal metabolite mediates
557 degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS*
558 *Lett.* **391**, 144–148 (1996).

559 59. Felby, C., Nielsen, B. R., Olesen, P. O. & Skibsted, L. H. Identification and
560 quantification of radical reaction intermediates by electron spin resonance
561 spectrometry of laccase-catalyzed oxidation of wood fibers from beech (Fagus
562 sylvatica). *Appl. Microbiol. Biotechnol.* **48**, 459–464 (1997).

563 60. Camarero, S., Ibarra, D., Martínez, M. J. & Martínez, A. T. Lignin-derived compounds
564 as efficient laccase mediators for decolorization of different types of recalcitrant
565 dyes. *Appl. Environ. Microbiol.* **71**, 1775–1784 (2005).

566 61. Aslam, M. S., Aishy, A., Samra, Z. Q., Gull, I. & Athar, M. A. Identification, Purification

and Characterization of a Novel Extracellular Laccase from Cladosporium Cladosporioides. *Biotechnol. Biotechnol. Equip.* **26**, 3345–3350 (2012).

62. Lindsley, D. L., Grell, E. H. & Bridges, C. B. Genetic Variations of Drosophila melanogaster. *Science (80-.).* **162**, 993–993 (1968).

63. Venken, K. J. T., He, Y., Hoskins, R. A. & Bellen, H. J. P[acman]: A BAC transgenic platform for targeted insertion of large DNA fragments in D. melanogaster. *Science (80-.).* **314**, 1747–1751 (2006).