



Genomic and proteomic studies on the effects of the insect growth regulator diflubenzuron in the model beetle species *Tribolium castaneum*

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ABSTRACT

Several benzoylphenyl urea-derived insecticides such as diflubenzuron (DFB, Dimilin®) are in wide use to control various insect pests. Although this class of compounds is known to disrupt molting and to affect chitin content, their precise mode of action is still not understood. To gain a broader insight into the mechanism underlying the insecticidal effects of benzoylphenyl urea compounds, we conducted a comprehensive study with the model beetle species and stored product pest *Tribolium castaneum* (red flour beetle) utilizing genomic and proteomic approaches. DFB was added to a wheat flour-based diet at various concentrations and fed to larvae and adults. We observed abortive molting, hatching defects and reduced chitin amounts in the larval cuticle, the peritrophic matrix and eggs. Electron microscopic examination of the larval cuticle revealed major structural changes and a loss of lamellate structure of the procuticle. We used a genomic tiling array for determining relative expression levels of about 11,000 genes predicted by the GLEAN algorithm. About 6% of all predicted genes were more than 2-fold up- or down-regulated in response to DFB treatment. Genes encoding enzymes involved in chitin metabolism were unexpectedly unaffected, but many genes encoding cuticle proteins were affected. In addition, several genes presumably involved in detoxification pathways were up-regulated. Comparative 2D gel electrophoresis of proteins extracted from the midgut revealed 388 protein spots, of which 7% were significantly affected in their levels by DFB treatment as determined by laser densitometry. Mass spectrometric identification revealed that UDP-*N*-acetylglucosamine pyrophosphorylase and glutathione synthetase were up-regulated. In summary, the red flour beetle turned out to be a good model organism for investigating the global effects of bioactive materials such as insect growth regulators and other insecticides. The results of this study recapitulate all of the different DFB-induced symptoms in a single model insect, which have been previously found in several different insect species, and further illustrate that DFB treatment causes a wide range of effects at the molecular level.

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Abbreviations: CHS, chitin synthase; DFB, diflubenzuron; CFW, calcofluor white; IGR, insect growth regulator; GlcNAc, *N*-acetylglucosamine; PM, peritrophic matrix; SU, sulfonyleurea; SUR, sulfonyleurea receptor.

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1. Introduction

Insect growth regulators (IGRs) are in wide use to control a variety of insect pests by interfering with their growth and/or development (Yu, 2008). The main IGR categories are distinguished by their mode of action. Ecdysone agonists such as tebufenozide or methoxyfenozide, and juvenile hormone mimics such

as fenoxycarb, methoprene or pyriproxyfen interfere with the hormonal control of molting and metamorphosis (Dhadialla et al., 1998). In contrast, benzoylphenyl urea compounds such as DFB [1-(4-chlorophenyl)-3-(2,6-diflubenzoyl)urea] and its derivatives prevent the formation of chitinous structures in many insect orders. They have been shown to disturb cuticle formation causing abortive molting. Ultrastructural analysis revealed abnormal deposition of procuticular layers (Gijswijt et al., 1979; Grosscurt, 1978b; Lim and Lee, 1982; Mulder and Gijswijt, 1973; Verloop and Ferrell, 1977). Moreover, DFB also prevents normal formation of the peritrophic matrix (PM), which protects the midgut epithelium from various harms (Becker, 1978; Clarke et al., 1977; Soltani, 1984). The underlying mechanism(s) of DFB's insecticidal activity was the subject of several investigations utilizing several different insect species. An early suggestion was that DFB efficiently inhibited chitin synthesis, because the incorporation of radiolabeled precursors into the growing chitin chain was impaired (Clarke and Jewess, 1990; Hajjar and Casida, 1978; Mayer et al., 1980; Post and Vincent, 1973). Chitin is a polymer of $\beta(1,4)$ -linked *N*-acetylglucosamine, which is synthesized from UDP-*N*-acetylglucosamine precursors by chitin synthase (CHS), an integral membrane protein that belongs to the family of β -glycosyltransferases (Merzendorfer, 2006). As the catalytic domain of CHS is predicted to face the cytoplasm, the nascent chitin chain has to be translocated across the plasma membrane before it can be deposited into the cuticle or PM. In contrast to polyoxins or nikkomycins, which as competitive inhibitors directly inhibit CHS in vitro by binding in the active site of this enzyme, DFB inhibits chitin synthesis only in intact cellular systems and tissues, but not in solubilized membrane fractions (Cohen and Casida, 1980; Kitahara et al., 1983; Mayer et al., 1981; Zimoch et al., 2005). It also does not block any of the metabolic reactions that are necessary for the production of UDP-*N*-acetylglucosamine, nor does it affect chitin synthesis in fungal systems (Cohen, 1987; Verloop and Ferrell, 1977). Based on these and other findings, it was suggested that DFB does not act directly on the catalytic step of chitin synthesis (Cohen, 2001). Over time, many suggestions for DFB's mode of action have been made including direct or indirect effects on the activities of chitinases, phenoloxidasases, glycolytic enzymes, and microsomal oxidases, as well as on processes controlled by molting hormones (DeLoach et al., 1981; Ishaaya and Ascher, 1977; Ishaaya and Cohen, 1974; Mitlin et al., 1977; Soltani et al., 1984). DFB is structurally similar to sulfonylureas (SUs), which are used in the treatment of type II diabetes in humans. SUs are known to bind to membrane-bound sulfonylurea receptors (SURs), which act as regulatory subunits of a voltage-gated inward rectifying potassium channel (K_{ir} channel) (Ashcroft, 1988). Indeed, a gene encoding an SUR ortholog was identified in the *Drosophila melanogaster* genome, and expression of this gene in *Xenopus* oocytes leads to potassium channel activity, which is sensitive to glibenclamide, an SU derivative (Nasonkin et al., 1999). A more recent study suggested that a SUR may be the target for DFB in insects (Abo-Elghar et al., 2004). This conclusion was deduced from competitive binding assays with radiolabeled glibenclamide, an SU compound and structural analog of DFB, which was displaced from its binding site by the addition of unlabeled DFB. As in the case of vertebrates, the insect SUR may act as a regulatory subunit of ATP-sensitive K^+ channels (Akasaka et al., 2006). Modulation of the activity of SUR/ K_{ir} could affect the membrane potential in a way that the activity of voltage-gated Ca^{2+} channels is affected disturbing Ca^{2+} homeostasis. As a result, Ca^{2+} -dependent vesicle fusion, which is necessary for the secretion of proteins involved in cuticle and PM formation, might be blocked. In support of this hypothesis, Ca^{2+} uptake experiments performed with a vesicle preparation from the integument of *Blattella germanica*

demonstrated that Ca^{2+} uptake by isolated cuticular vesicles is inhibited in the presence of glibenclamide or DFB (Abo-Elghar et al., 2004). However, the significance of this finding remains unclear.

As the target molecule of DFB and other benzoylphenyl urea derivatives is still not identified, we aimed to establish a genetic model to examine the insecticidal effects of benzoylphenyl urea compounds. The model beetle and stored product pest *Tribolium castaneum* (red flour beetle) is a well-established insect model amenable to genetic and genomic approaches (Brown et al., 2003; Richards et al., 2008). Therefore, we examined DFB effects in *T. castaneum* by monitoring its toxicity in the course of development, measuring chitin amounts in whole larvae and isolated midguts, and by investigating ultrastructural changes in the cuticle. In addition, we conducted a comprehensive study utilizing genomic tiling arrays to detect changes in transcript levels and computerized 2D gel electrophoresis to document changes in the proteome of larval midguts.

2. Materials and methods

2.1. Insects and DFB treatment

T. castaneum strain GA-1 (Haliscak and Beeman, 1983) and the *pu11* enhancer trap line (Lorenzen et al., 2003) were used in this study. Insects were reared in whole wheat flour containing 5% yeast at 30 °C. For various experiments the flour was supplemented with DFB as follows: per 50 g of flour in a beaker, 50 ml of acetone containing the desired amount of DFB (a kind gift from Dr. Kun Yan Zhu, Kansas State University) was added and the suspension was vigorously mixed for 30 min at room temperature. Then the acetone was completely evaporated under a fume hood over four days. Control diet was prepared exactly as described above but using acetone without the inclusion of DFB. Rearing of insects was performed in standard insect glass vials closed with cork stoppers with a maximum of 50 animals per 10 g of diet. For most evaluations *T. castaneum* larvae with a body weight of 1.50 ± 0.05 mg were fed with the control diet or the diet containing 100 ppm DFB, and feeding was continued until a mortality of 30% was achieved, which usually occurred after 2–3 d. Only live larvae with no obvious signs of intoxication were analyzed for transcript measurements and proteomic analyses.

2.2. Array design and probe synthesis

Global gene expression analysis was performed with Roche NimbleGen HD2 whole tiling microarray. The arrays consist of 1050 by 4200 Perfect Matching (PM)-only oligonucleotide probes. The sizes of the probes ranged from 50-mer to 64-mer. The probes were aligned to the latest *T. castaneum* genome (Tcas3). 30,983 control probes (Random_GC) representing GC-richness of non-coding regions were randomly placed on each of the chips. These random probes were used to measure special bias and to derive background noise by maximum-likelihood estimation.

Total RNA from 3 (replicate 1) or 10 larvae (replicates 2 and 3) was prepared using a combined protocol of Trizol extraction (Invitrogen) and RNAeasy column purification (Qiagen). cDNA synthesis was performed with the Superscript cDNA synthesis kit (Invitrogen) using about 10 μ g of total RNA and random hexamers (Shippy et al., 2008). The resulting cDNA was used as the template for Cy3/Cy5 labeling and competitive hybridization (Squazzo et al., 2006). Specifically, control and DFB treated larval cDNA were differentially amplified/labeled with Cy3- and Cy5-coupled non-amers, respectively, for the first two replicates. The third replicate was dye-swapped. Fifteen μ g of each of the Cy3/Cy5-labeled cDNA

samples were co-hybridized to a single microarray and the array was processed according to the manufacturer's protocol (http://www.nimblegen.com/products/lit/expression_userguide_v5p0.pdf). The arrays were scanned using an Axon GenePix 4000B microarray scanner from Molecular Devices. The fluorescence intensities of the probes were quantitated and represented in a GFF file.

2.3. Obtaining relative expression values

Model-based analysis was applied (Li and Wong, 2001). However, the Mismatch (MM) signal was replaced by the global background (MM_G), so that the actual intensity level for each probe was obtained using the values of $PM-MM_G$, with PM being the perfect match signal. When the $PM-MM_G$ difference is less than zero, the negative value was replaced with 1. Finally, the actual intensity levels were log-transformed to the base 2. MM_G was derived under the statistical inference on the random probes. The intensity values of the random probes for each array were scattered against the x -axis and y -axis (Fig. S1). The plots showed that all intensity values except for a few outliers fall around approximately the same mean with approximately the same standard deviation along the x -axis and y -axis. The result indicated that spatial bias would not significantly affect the analysis and that the global background for MM_G would be appropriate for each array being analyzed. It also allowed us to assume that the intensity values of the random probes are normally distributed on the probability density function given the mean (μ) and standard deviation (σ):

$$f(x; \mu, \sigma^2) = \frac{1}{\sqrt{2\pi\sigma^2}} e^{-\frac{(x-\mu)^2}{2\sigma^2}}, \quad x \in R$$

By using the UNIVARIATE procedure in SAS, fitted normal curves were described on the histograms of the intensity values (x) for each array and the quantiles for each of the curves were estimated (Fig. S2 and Table S1). Initially the 99 percentile with a 1% false discovery rate (FDR) and the 95 percentile with a 5% FDR were selected to obtain the best cut-off of the global background. Each of the selected cut-offs was applied to $\log_2(PM-MM_G)$. The results were visualized through GBrowse (Fig. S3). The 95 percentile was adequate for the first and third replicates, which have higher standard deviations, while the 99 percentile was adequate for the second replicate, which has a two-fold lower standard deviation.

The PM-MM intensity scores for each GLEAN gene annotation covering at least four tiles were averaged after removing the minimum and maximum values. The average values for each gene model between the control (no DFB treatment) and DFB treated samples were compared to each other. The ratio was expressed as the average value obtained for the control group divided by that for the DFB treated group (relative expression value, rE-value).

2.4. Protein extraction from midguts and 2D gel electrophoresis

Ten midguts were surgically removed from control and DFB treated larvae. The pooled midguts were frozen in liquid nitrogen and to each sample 100 μ l of osmotic lysis buffer (OLB, 10 mM Tris-HCl, pH 7.4, 0.3% SDS, supplemented with nuclease and protease inhibitor solutions purchased from Kendrick Labs, Madison, WI) was added. The probes were homogenized in micro-cups using appropriate pestles (Eppendorf). After two freeze/thaw cycles, one volume of SDS boiling buffer (5% SDS, 10% glycerol, 60 mM Tris-HCl, pH 6.8) was added and the samples were incubated for 5 min in a boiling water bath. After insoluble material was removed by centrifugation for 5 min at 12,000 \times g and 4 °C, the

protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce). Two-dimensional gel electrophoresis was performed with 150 μ g of midgut proteins using the carrier ampholine method of isoelectric focusing by Kendrick Labs, Inc. (Madison, WI) (O'Farrell, 1975). Isoelectric focusing was carried out in glass tubes of inner diameter 2.0 mm, using 2% pH 3.5–10 (GE Healthcare, Piscataway, NJ) for 20,000 V-h. 100 ng of an IEF internal standard, tropomyosin (MW 33 kDa and pI 5.2), was added to each sample prior to loading. The enclosed pH gradient plot for these ampholines was determined using a surface pH electrode. After equilibrium in SDS sample buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 0.0625 M Tris-HCl, pH 6.8), each tube gel was sealed to the top of a stacking gel that overlays a 10% acrylamide slab gel (1.0 mm thick). SDS slab gel electrophoresis was carried out for about 5 h at 25 mA/gel. The following proteins (Sigma Chemical Co, St. Louis, MO) were added as molecular weight markers: myosin (220 kDa), phosphorylase A (94 kDa), catalase (60 kDa), actin (43 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa). The gels were stained with silver for computerized comparison and with Coomassie blue for mass spectrometric protein identification. Duplicate gels were obtained from each sample and were scanned with a laser densitometer (Model PDSI, Molecular Dynamics Inc., Sunnyvale, CA). The images were analyzed using Progenesis PG240 software with TT900 (version 2006, Nonlinear Dynamics). Spot % is equal to spot integrated density above background (volume) expressed as a percentage of total density above background of all spots measured. Difference is defined as the fold-change of spot percentages.

2.5. MALDI-MS analysis

Gel spots were transferred to clean tubes and completely hydrated by adding deionized water. After washing the gel spots twice for 20 min with 100 μ l 50 mM Tris-HCl (pH 8.5)/30% (v/v) acetonitrile and for 1–2 min with 100% acetonitrile, they were dried in a Speed-Vac concentrator. Gels were digested overnight at 32 °C after adding 15 μ l 25 mM Tris-HCl (pH 8.5) supplemented with 0.06 μ g trypsin (sequencing grade, Roche). Peptides were extracted with 2 \times 50 μ l 50% acetonitrile/2% TFA, and the combined extracts were dried and resuspended in matrix solution (4-hydroxy- α -cyanocinnamic acid in 50% acetonitrile/0.1% TFA with two internal standards, angiotensin and ACTH 7–37 peptide). The dried gel samples were dissolved in matrix solution and 0.5 μ l aliquots were spotted onto the sample plate, completely dried and washed twice with water. MALDI mass spectrometry was performed using an Applied Biosystems Voyager DE Pro mass spectrometer in linear mode. Peptide masses were entered into search programs to identify proteins in the NCBI or GenPept databases. Programs used were ProFound (<http://prowl.rockefeller.edu/prowl/cgi/profound.exe>) and MS-Fit (<http://prospector.ucsf.exe>). Cysteine residues were modified by acrylamide. Error tolerance was set to 0.5 Da for average masses.

2.6. Other methods

Measurements of chitin content were performed as described previously (Arakane et al., 2005). In brief, whole larvae or isolated midguts were precisely weighed, homogenized with zirconium beads and hydrolyzed by incubating the samples at 80 °C for 90 min in 6% KOH. After washing, the samples were digested with chitinase from *Trichoderma viride* (5 mg/ml in PBS, Sigma-Aldrich), and the released GlcNAc was measured by a modified Morgan-Elson assay (Reissig et al., 1955). The chitin content of whole larvae or of isolated midguts from pools of ten control or ten DFB treated larvae were measured separately. The chitin measurements were

repeated three times, and the values are given as means \pm SD. Cryoprotection, tissue embedding and cryosectioning were performed as described previously (Zimoch and Merzendorfer, 2002). To visualize cuticular chitin in *T. castaneum*, transverse sections of untreated and DFB treated larvae were stained for 30 min in 0.001% (w/v) Calcofluor white (CFW, Fluostain, Sigma–Aldrich, Munich) dissolved in PBS buffer (pH 7.6). Excess CFW was removed by washing with PBS. To detect chitin in the PM, isolated midguts were stained with a fluorescein-conjugated chitin-binding domain probe (FITC-CBD, New England BioLabs) as reported previously (Arakane et al., 2005). Fluorescence was recorded using light of appropriate excitation and emission wavelengths. Ultra-sectioning and transmission electron microscopy was carried out as described elsewhere (Chaudhari et al., 2011). In brief, decapitated larvae were fixed for 1–16 h at room temperature in a 0.1 M sodium cacodylate buffer (pH 7.4) containing 2% (w/v) paraformaldehyde and 2% glutaraldehyde (v/v). Post-fixation was performed with 1–2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature with constant rotation for 2 h. After washing, the samples were stained with 2% uranyl acetate (w/v; in H₂O) for 1 h. After dehydration in an ascending series of ethanol (50–100% ethanol, 10% steps, 3 \times 5 min each) the samples were infiltrated with EMBED 812/Araldite resin at room temperature and embedded in flat mold capsule. Samples were thin sectioned and imaged on a CM-100 TEM (FEI Co.). Polymerization of the resin was carried out in a drying oven at 65 °C for 24–48 h. RT-PCR for evaluating relative mRNA-amounts in control and DFB treated larvae was performed as described previously (Broehan et al., 2010).

3. Results

3.1. DFB perturbs molting and egg hatching in *T. castaneum*

To monitor the insecticidal effects of DFB in *T. castaneum* (GA-1 strain; Haliscak and Beeman, 1983), we fed larvae and adults with a wheat flour-based diet containing DFB at various concentrations. Larvae were significantly impaired by DFB when compared with control animals (Fig. 1). Younger larvae (1.5 mg at the start of treatment) were generally highly susceptible to the insecticide and died during the larval-to-larval molts (Fig. 2A and B). Only a few of them (about 10%) molted to the pupal stage but these eventually died during the pupal-to-adult molt (Fig. 2D). In contrast, older larvae (2.5 mg at the start of treatment) died primarily during the larval-to-pupal molt (Fig. 2C). In Fig. S4, the typical distribution of larval, pupal, adult and dead insects is shown for various DFB concentrations at different exposure times. Adult beetles that were

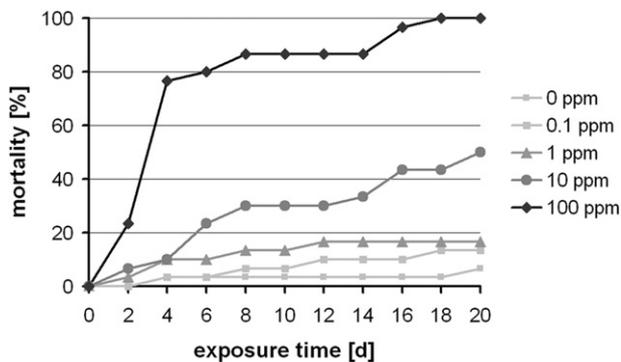


Fig. 1. Dose response of mortality of *T. castaneum* fed a diet containing DFB. DFB was added to a wheat flour-based diet and fed to larvae at the indicated concentrations. Mortality was monitored at indicated exposure times.

fed with a DFB-containing diet three days after emergence were not affected by this IGR and did not show increased mortality compared to control beetles during a three month period of observation (Fig. 2E). Oviposition and subsequent embryonic development appeared to be normal after DFB treatment of adult females (Fig. 2F). However, egg hatch was completely inhibited by DFB treatment and the unhatched embryos became twisted and enlarged (Fig. 2G). The larval, pupal, adult and embryonic phenotypes generally resembled those described for animals injected with dsRNA for the *TcCHS1* (or *TcCHS-A*) and *TcCHT10* genes encoding chitin synthase A and chitinase 10 of *T. castaneum*, respectively (Arakane et al., 2005; Zhu et al., 2008).

3.2. DFB treatment reduces chitin levels in the cuticle, PM and eggs, and alters procuticle structure

To analyze the effects of DFB on chitin levels in the larval cuticle, we performed longitudinal cryosectioning of whole body and stained for chitin with Calcofluor white (CWF). As shown in Fig. 3A, CWF fluorescence is significantly reduced after DFB treatment, indicating decreased chitin levels in the cuticle (Meissner et al., 2010). Similar results were obtained when we stained midguts isolated from control and DFB treated larvae with a fluorescein-conjugated chitin-binding domain probe which is a more specific probe for detecting chitin present in the PM (Arakane et al., 2005). Midguts from DFB treated larvae showed a lower fluorescence than control larvae (Fig. 3B). To quantify the reduction in chitin levels, we determined the chitin content of untreated and DFB treated larvae using a modified Morgan-Elson assay (Arakane et al., 2005; Reissig et al., 1955). In comparison to control insects, DFB treated larvae showed a significant reduction in whole body chitin content (Table 1). The mean chitin content per insect was equivalent to \sim 70 nmol GlcNAc per larva and hence only about one half of the value for the untreated larvae, which was \sim 150 nmol GlcNAc per larva. Similarly, DFB treatment led to a reduction in chitin content of the PM from larval midguts by about 40%, and of eggs obtained from female beetles fed a DFB-containing diet by about 60% (Table 1). To examine possible ultrastructural changes within the cuticle in response to DFB treatment, we performed transmission electron microscopy (TEM). For this purpose, defined abdominal segments (A6) of control larvae and larvae fed with a diet containing either 10 or 100 ppm DFB were fixed, stained with uranyl acetate and subjected to ultrathin sectioning. The dorsal cuticles were examined by TEM analysis. As depicted in Fig. 4A and D, control larvae had a characteristic laminar cuticle with numerous layers. While the old procuticle of larvae treated with 10 ppm DFB exhibited a clear reduction in the numbers of layers (Fig. 4B and E), the new cuticle of larvae treated with either 10 or 100 ppm DFB appeared completely amorphous (Fig. 4C and F). Hence, DFB produces dramatic changes in the lamellar organization of the cuticle.

3.3. DFB does not significantly accelerate or retard larval development

An important prerequisite for genome-wide analysis of DFB effects is the precise monitoring of larval development. Differences in the developmental rate between control and DFB treated animals could cause significant shifts in global gene expression patterns. As the precise larval stages are difficult to assign in *T. castaneum*, we used the *pu11* enhancer trap line to monitor development by means of a stage-specific marker. The strain *pu11* is a transgenic line, which shows onset of EGFP expression in the wing and elytral imaginal discs in the last larval instar as well as constitutive expression in the eye due to the presence of the 3xP3 artificial Pax6

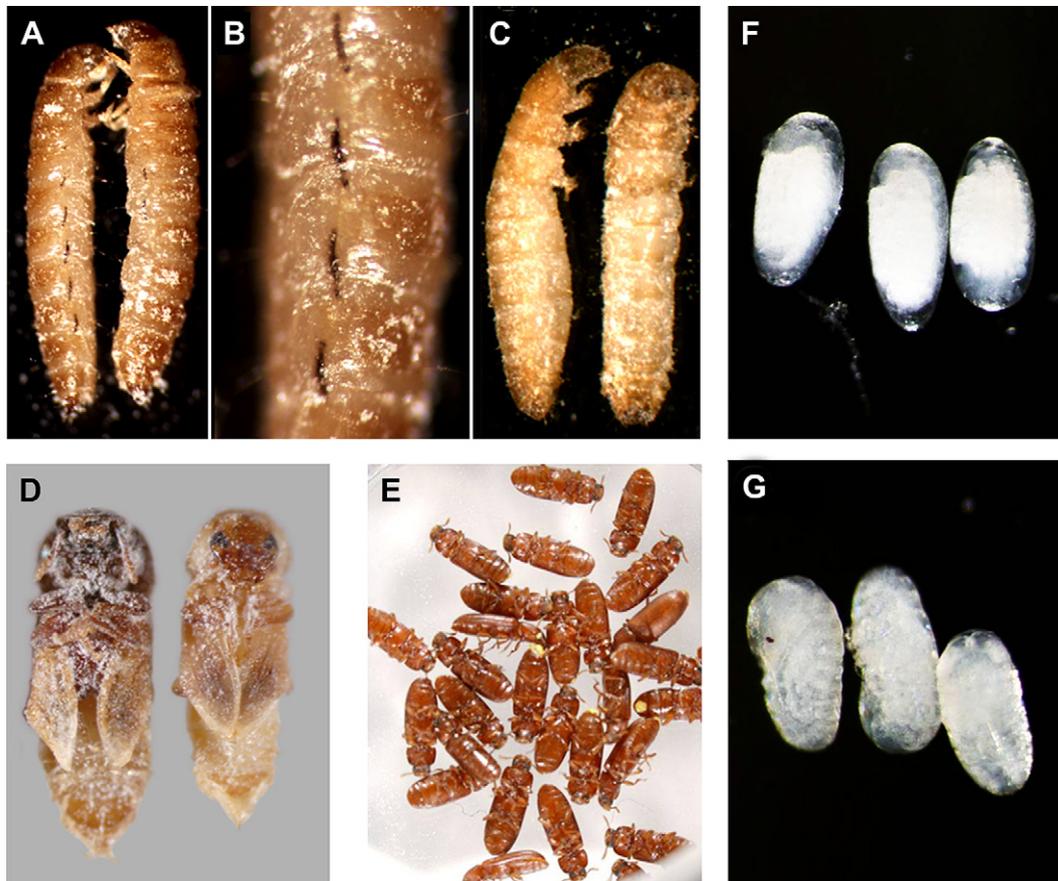


Fig. 2. Effects of DFB on molting, egg-laying and hatching of *T. castaneum*. Larvae and adults were fed with a flour-brewer's yeast diet containing 100 ppm DFB. Whereas particularly younger larvae were significantly impaired by DFB, adults did not exhibit any increase in mortality when compared with control insects. Death was observed during the larval-to-larval molts (A, B), the larval-to-pupal molt (C), and the pupal-to-adult molt (D). Note that the larval spiracles are melanized extensively (B). Adults were not affected by DFB (E). Oviposition and subsequent embryonic development appeared to be normal after DFB treatment of adult females (G). However, egg hatch was completely inhibited by DFB treatment and the development was arrested (F).

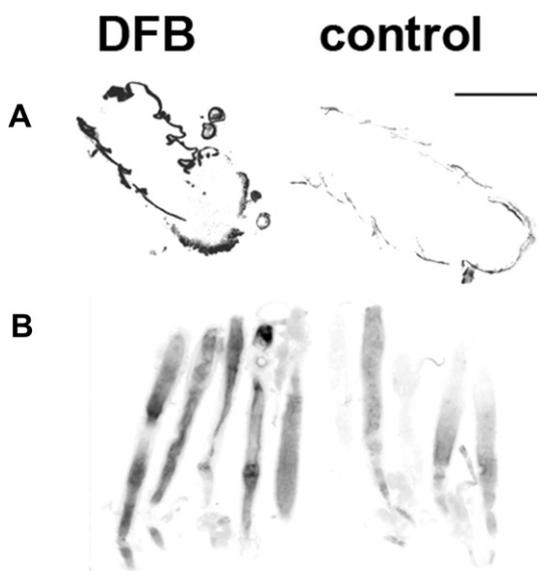


Fig. 3. Labeling of chitin in larval cuticles and midgut peritrophic matrices from *T. castaneum*. Larvae (1.5 mg) were fed a control diet or a diet containing 100 ppm DFB. (A) Cryosections of larvae were stained with Calcofluor White. (B) Midguts were isolated and stained with a FITC-conjugated chitin-binding domain. Gray scales were inverted for better visualization. Bar size = 500 μ m.

element (Lorenzen et al., 2003; Tomoyasu and Denell, 2004). When we fed *pu11* larvae (1.5 mg at the start of treatment) with a flour-based diet containing 100 ppm DFB, these larvae showed the same response to the insecticide as those of the GA-1 strain. Onset of GFP expression in the wing and elytral imaginal discs occurred at about the same time in the last larval instar as in animals fed with the control diet, indicating that larval development is not significantly altered during the three days of observation (Fig. S5, left panels). Those larvae that had molted to the pupal stage showed expression of GFP in the wings and elytra, indicating that development was not arrested by DFB treatment during the pupal stage (Fig. S5, right panels).

3.4. DFB effects on global gene expression

To conduct an unbiased study of changes in expression of all genes that are potentially affected by DFB treatment, we utilized a genome-wide tiling array for hybridization with differentially labeled cDNA derived from total RNA of control and DFB treated (100 ppm) larvae. \log_2 values of fluorescence intensities from three biological replicates were averaged over all tiles (50–64 nucleotides) available for the respective gene. Intensity values were corrected for background fluorescence determined by means of Random-GC oligonucleotides distributed throughout the chips (see Materials and Methods). We predict that these averaged and background-corrected \log_2 values provide a confident measure for the respective gene expression level, as it is based on values from

Table 1
Chitin amounts in whole larvae, midguts and eggs of *T. castaneum*.

Exp #	nmol GlcNAc per larva		nmol GlcNAc per gut		nmol GlcNAc per 100 eggs	
	Control	DFB	Control	DFB	Control	DFB
1	159.68 ± 13.27	76.91 ± 8.16	4.87 ± 3.54	2.15 ± 0.05	11.03 ± 6.31	7.62 ± 0.19
2	171.92 ± 15.36	79.13 ± 8.84	4.10 ± 0.07	2.99 ± 0.06	54.41 ± 6.50	19.87 ± 1.98
3	126.45 ± 13.61	61.65 ± 17.45	3.78 ± 0.10	2.57 ± 0.03	60.36 ± 1.39	24.30 ± 1.41
Mean	152.68 ± 14.08	75.56 ± 11.58	4.25 ± 1.24	2.57 ± 0.05	41.93 ± 4.73	17.26 ± 1.19
%	100.00	49.49	100.00	60.47	100.00	41.16

numerous, individual hybridizations covering the entire gene sequence predicted by GLEAN. To evaluate the effects of DFB on the expression level (as measured by steady-state transcript levels), we calculated relative expression values (rE-values). We categorized rE-values values as follows: <0.5, substantially up-regulated; 0.5–0.9, marginally up-regulated; 0.9–1.1, not affected; 1.1–2.0 marginally down-regulated; >2.0 substantially down-regulated. The frequency distribution indicates that significantly more genes are down-regulated than up-regulated (Fig. S6). The expression of most genes (10,491 of 11,213) predicted by the GLEAN algorithm as measured by steady-state transcript levels were not affected at all or only marginally altered by DFB treatment as indicated by rE-values that ranged between 0.5 and 2.0. In total, 722 genes were substantially up- or down-regulated. Hence, about 6% of all of the genes predicted by GLEAN showed significant changes in expression levels in response to the DFB treatment. As illustrated in Fig. S7, among the 400 genes that were most strongly up- or down-regulated, there were many involved in protein, lipid, nucleotide and sugar metabolism (7, 10, 4%; total, up-regulated and down-regulated, respectively), replication, transcription and translation (8, 11, 4%), development (9, 9, 8%), signal transduction (6, 6, 5%), cuticle formation (5, 7, 3%), or genes encoding channels,

transporters and receptors (5, 4, 5%). In contrast, genes involved in detoxification (2, 3, 0%), energy metabolism (2, 1, 2%), formation of the cytoskeleton and nucleoskeleton (1, 1, 0%), exo-/endocytosis and vesicle transport (2, 2, 1%), and stress and immune responses (1, 1, 2%) constituted only a smaller fraction of all genes that were altered in their expression levels. The group of the 200 genes that were most up-regulated contained also 1% transposon-like genes. The tiling array data can be found in the Excel file provided in the supplementary data ([Spreadsheet.xls](#)).

Our measurements of the chitin levels after a DFB challenge indicated that the chitin content is significantly reduced in *T. castaneum* larvae. Therefore, we focused on the expression levels particularly of those genes encoding enzymes involved in the chitin biosynthetic pathway including hexokinase (*TcHEX*), trehalase (*TcTRE1/2*), GlcNAc-6-phosphate deacetylase (*TcNAGA*), GlcNAc kinase (*TcNAGK*), glucosamine-6-phosphate deaminase (*TcGNPDA*), glutamine: fructose-6-P aminotransferase (*TcGFAT*), glucosamine-6-P acetyltransferase (*TcGNPAT*), phosphoacetylglucosamine mutase (*TcPAGM*), UDP-GlcNAc pyrophosphorylase (*TcUAP1*) and chitin synthase (*TcCHS1/2*). We considered also chitin modifying and degrading enzymes such as chitin deacetylases (*TcCDA1/2*), *N*-acetylglucosaminidases (*TcNAG1/2/3* and *TcFDL*), and

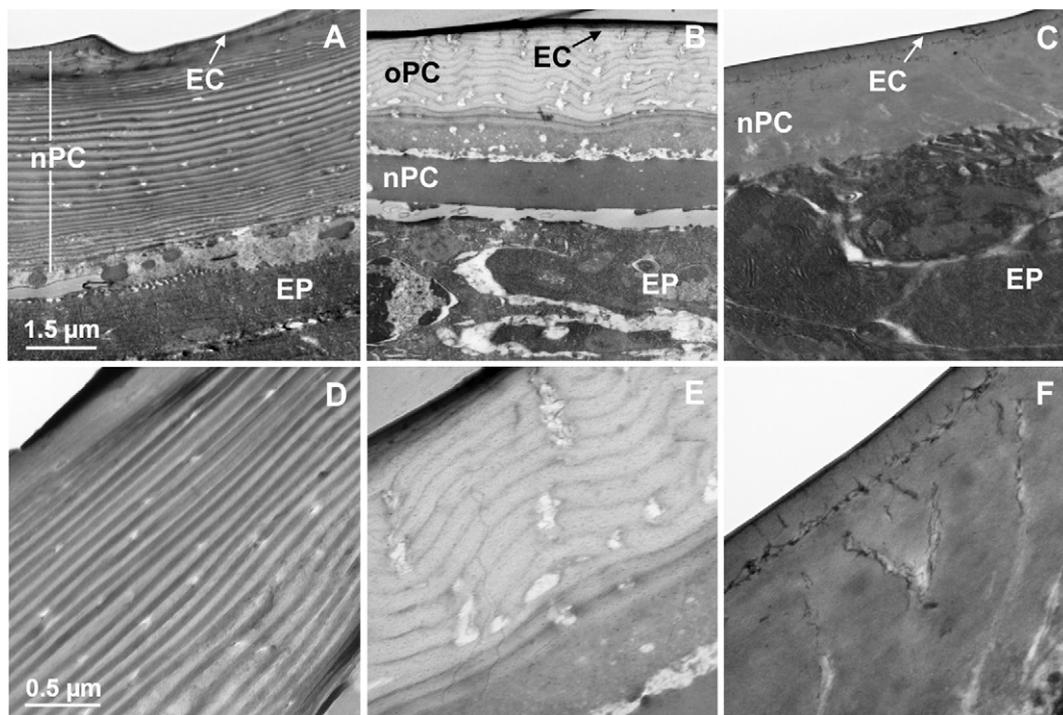


Fig. 4. Effects of DFB on the ultrastructure of the epicuticle and procuticle from penultimate instar larvae of *T. castaneum*. Defined abdominal segments (A6) of control larvae (A & D) and larvae fed with a diet containing either 10 ppm (B & E) or 100 ppm DFB (C & F) were fixed, stained with uranyl acetate and subjected to ultrathin sectioning. The new procuticle (nPC) of larvae treated with either 10 or 100 ppm DFB appears completely amorphous. The number of layers in the old procuticle (oPC) of larvae treated with 10 ppm DFB is reduced when compared to control larvae. EC, epicuticle; EP, epidermis; Magnifications: 25,000× (A–C) and 7,900× (D–F).

several chitinases (*TcCHT4/5/7/10*). As shown in Table 2, none of these genes is significantly up- or down-regulated, a finding which is in line with the results from RT-PCR performed for several of these genes (Fig. S8). Hence, the analysis of global changes in gene expression after a DFB challenge indicates that the transcription of genes involved in chitin metabolism, modification and degradation is not affected significantly by DFB. Most notably, among the genes that were significantly affected by the DFB treatment were numerous genes encoding cuticle structural proteins of the RR1 and RR2 subfamilies (*TcCPRs*), as were genes that are known to be involved in detoxification of insecticides such as those encoding cytochrome P450 4c3 (*TcCYP4c3*), glutathione S-transferase (*TcGST*), sulfotransferase (*TcSULT*), glucosyl/glucuronosyl-transferase (*TcGLCT*) and subfamily C ABC transporter C5 K (*TcABC-C5 K*) (Fig. S8 and Table 2).

3.5. DFB effects on the larval midgut proteome

In silico comparison of proteins extracted from midgut of control and DFB treated larvae and resolved by 2D gel electrophoresis revealed changes in the midgut proteome due to DFB treatment. Among 388 proteins that were analyzed in duplicate gels (see Fig. 5), only 26 proteins (~7%) were significantly changed as determined by laser densitometry, comprising 21 up-regulated and 5 down-regulated proteins (Table 3 and Fig. S9). Of these, ten proteins whose levels changed more than 2.5-fold were identified by MALDI-TOF analysis of tryptic peptides (Table S3). For nine of these proteins high quality mass spectra were obtained. Two of the protein spots are probably due to sample contamination from human keratin. Among the remaining seven midgut proteins from *T. castaneum* were UDP-*N*-acetylglucosamine pyrophosphorylase 1 (UAP1), glutathione synthetase (GS), two forms of the NADH-ubiquinone oxidoreductase 75 kDa subunit (complex I), paramyosin (PMYO), actin-2 (ACT2) and the 12 kDa hemolymph protein b (TcHP12b). All of these proteins appeared to be up-regulated between 3- and 6-fold. As the corresponding mRNA levels for these proteins were not affected significantly (see Table 2), the observed changes may be due to post-transcriptional regulation. However, transcriptional regulation in the midgut cannot be completely excluded, because mRNA levels were measured using whole body preparations, which blurred any possible changes in the midgut transcriptome.

4. Discussion

DFB was developed as a commercial insecticide in the early 1970s by Philips-Duphar B.V. (Netherlands) and many other benzoylphenyl urea derivatives have been successfully established as even more potent insecticides, several of which are still in wide use in agriculture and forestry. Different effects of DFB and other benzoylphenyl urea compounds have been examined in single insect species from various orders including Diptera, Lepidoptera, Coleoptera, Acari, and Hemiptera (Grosscurt, 1978a). Because the important stored product pest *T. castaneum* has been developed as a genomic model for studies of development and pest biology (Brown et al., 2009), we aimed to use this beetle model for thoroughly investigating the effects of DFB and related insecticides in a single insect species.

4.1. DFB effects on growth and development of *T. castaneum*

By feeding *T. castaneum* larvae a diet with DFB for three days, we were able to confirm the toxic effects described previously for this species (Gazit et al., 1989). The LC₅₀ values for DFB were reported previously to be approximately 13 ppm for fourth instar larvae until

pupation and 11 ppm until emergence. Our data are generally in good agreement with those data as DFB exhibited LC₅₀ values of 22.6 ppm ($R^2 = 0.81$) for 1.5 mg larvae until pupation and 12.8 ppm ($R^2 = 0.85$) until emergence (data not shown). We also found that the toxicity of DFB was significantly higher in younger than in older larvae, a finding that is in line with a previous report (Ishaaya and Ascher, 1977). Albeit oviposition is not affected, DFB treatment has been reported to have ovicidal effects in *T. castaneum* as hatchability of the eggs laid by DFB treated females was significantly decreased at relatively low concentrations (Carter, 1975). We also observed in our experiments that the number of eggs that were laid by female beetles fed a diet containing 100 ppm DFB was nearly the same as for females fed a control diet without DFB. Most of the embryos developed normally inside the egg shell but they failed to hatch, indicative of a weakened larval cuticle that lacked the mechanical strength to break through the egg shell. This phenotype is similar to the one we have reported for eggs derived from females subjected to RNAi for the *CHS-A* gene (Arakane et al., 2008). Our direct chitin measurements on the developing embryos indeed confirmed that DFB substantially inhibited the increase in chitin that occurs during larval cuticle deposition during embryonic development.

DFB has been reported to interfere with normal endocuticle deposition during larval development (Mulder and Gijswijk, 1973). More recently, Gangishetti et al. (2009) have shown in *D. melanogaster* that DFB treatment of adult females results in embryos that have lower chitin staining and lack the chitin laminae in the developing larval cuticle as shown by transmission electron microscopy. In our studies with *T. castaneum* penultimate instar larvae, we found that compared to control larvae, the procuticle of larvae treated with 10 ppm DFB showed a reduction in the number of laminae that were also less compact than in the control. After treatment with 100 ppm DFB, the newly synthesized larval cuticle was completely devoid of laminae and appeared to be amorphous.

The latter findings were similar to observations made in *Manduca sexta* and *Lucilia cuprina* (Binnington, 1985; Hassan and Charnley, 1987). Furthermore, in line with studies reporting a decrease in cuticular chitin amounts in response to DFB treatment (Hajjar and Casida, 1978; Post et al., 1974; van Eck, 1979), we determined that the decrease of the total chitin content was about 50% in *T. castaneum* larvae consistent with the observed reduction in CFW fluorescence in the cryosections of larval cuticles. The TEM data provide further evidence for the loss of chitin-containing laminae in the larval cuticle in a manner that is dependent on the dose of DFB. These results suggest that the steady-state level of chitin in the integument is significantly inhibited by DFB. According to our measurements of total and midgut chitin amounts, cuticular chitin may account for more than 90% of total chitin content, while PM-associated chitin represents less than 10%. Although generally low, we found that the chitin content in the PMs of isolated midguts from DFB treated *T. castaneum* larvae, which were freed of fore- and hindguts, was reduced by about 40%. This observation in turn is in line with the reduction in fluorescence intensity of midguts stained with FITC-CBD in larvae fed a DFB-containing diet and with previous reports from several insect species in which DFB appeared to block chitin synthesis during the production of the PM (Clarke et al., 1977; Cohen and Casida, 1980; Soltani, 1984).

To examine potential changes in the developmental rate, which could contribute to a reduction in chitin content at a particular stage in development, we monitored the onset of the developmental marker 3xP3-EGFP in the transgenic *T. castaneum* line *pu11* during the relevant observation time of three days. Until pupation we could not detect significant changes in the onset of the developmental marker, and those beetles that progressed to the pupal stage continued to express 3xP3-EGFP. The only study that

Table 2

Relative expression levels of various genes in response to DFB treatment as revealed by genomic tiling array chip hybridization.

Genes	Function	NCBI ref. seq.	Glean	rE-value	Transcript levels
Chitin biosynthesis					
<i>TcHEX</i>	Hexokinase	XM_965552.1	00318	1.064	±
<i>TcTRE1</i>	Trehalase	XM_968826.2	06698	0.999	±
<i>TcTRE2</i>	Trehalase	XM_967517.2	04791	1.062	±
<i>TcNAGA</i>	GlcNAc-6-phosphate deacetylase	XM_968918.2	07801	0.849	+
<i>TcNAGK</i>	GlcNAc kinase	XM_967098.2	12681	0.947	±
<i>TcGNPDA</i>	glucosamine-6-phosphate deaminase	XM_970016.2	02924	1.087	±
<i>TcGFAT</i>	Glutamine: fructose-6-P aminotransferase	XM_961795.1	02585	0.976	±
<i>TcPAGM</i>	Phosphoacetylglucosamine mutase	XM_968253.2	11920	0.932	±
<i>TcGNPAT</i>	Glucosamine-6-P acetyltransferase	XM_966988.1	09619	1.081	±
<i>TcUAP1*</i>	UDP-GlcNAc pyrophosphorylase	XM_963976.2	01751	1.005	±
<i>TcUAP2*</i>	UDP-GlcNAc pyrophosphorylase	XM_968988.2	05593	0.962	±
<i>TcCHS1*</i>	Chitin synthase	NM_001039402.1	14634	0.999	±
<i>TcCHS2*</i>	Chitin synthase	NM_001039403.1	N.A.	N.A.	N.A.
Chitin modifying/degrading					
<i>TcCHT4*</i>	Chitinase	NM_001080098.1	09180	1.049	±
<i>TcCHT5</i>	Chitinase	NM_001039435.1	01770	1.054	±
<i>TcCHT7</i>	Chitinase	NM_001042570.1	15481	0.875	+
<i>TcCHT10</i>	Chitinase	NM_001042602.1	12734	0.941	±
<i>TcNAG1</i>	N-acetylglucosaminidase	NM_001098848.1	09808	0.964	±
<i>TcNAG2</i>	N-acetylglucosaminidase	NM_001098828.1	11540	1.109	–
<i>TcNAG3</i>	N-acetylglucosaminidase	NM_001098827.1	01116	1.310	–
<i>TcFDL</i>	N-acetylglucosaminidase	NM_001098826.1	09779	0.971	±
<i>TcCDA1*</i>	Chitin deacetylase	NM_001102476.1	14100	0.986	±
<i>TcCDA2*</i>	Chitin deacetylase	NM_001102577.1	14101	0.984	±
Cuticle proteins (nomenclature according to Dittmer et al., 2011)					
<i>TcCPR12</i>	RR2 type cuticle protein	XM_001815995.1	00853	3.083	– – –
<i>TcCPR15</i>	RR2 type cuticle protein	XM_965862.1	03109	2.738	– – –
<i>TcCPR17</i>	RR2 type cuticle protein	NM_001167848.1	03363	0.643	++
<i>TcCPR20</i>	RR2 type cuticle protein	XM_970249.1	02908	0.564	++
<i>TcCPR25</i>	RR1 type cuticle protein	XM_963573.1	03834	1.950	– –
<i>TcCPR26*</i>	RR1 type cuticle protein	XM_963646.2	03835	0.465	+++
<i>TcCPR51</i>	RR2 type cuticle protein	XM_001809938.1	13814	3.048	– – –
<i>TcCPR54</i>	RR2 type cuticle protein	XM_963430.1	13992	0.574	++
<i>TcCPR55</i>	RR2 type cuticle protein	XM_963581.2	13810	2.425	– –
<i>TcCPR59</i>	RR1 type cuticle protein	NC_007420.2	13306	3.733	– – –
<i>TcCPR67</i>	RR1 type cuticle protein	XM_968570.1	13139	0.690	++
<i>TcCPR79</i>	RR1 type cuticle protein	XM_968926.1	13129	0.669	++
<i>TcCPR84</i>	RR1 type cuticle protein	XM_964170.1	14770	0.653	++
<i>TcCPR88</i>	RR2 type cuticle protein	XM_965123.1	15901	0.540	++
<i>TcCPR89*</i>	RR1 type cuticle protein	XM_961240.2	09873	0.997	±
<i>TcCPR96*</i>	RR2 type cuticle protein	XM_001816297.1	08767	3769	– – –
<i>TcCPR100</i>	RR2 type cuticle protein	XM_965802.1	11148	3474	– – –
<i>TcCPR103</i>	RR2 type cuticle protein, Pro-resilin	XM_001806976.1	03362	1.454	–
<i>TcCPR104</i>	RR1 type cuticle protein	XM_001811735.1	01120	0.873	+
Phenoloxidases					
<i>TcLAC1</i>	Laccase, diphenol oxidase	NM_001039425.1	00821	1.210	–
<i>TcLAC2*</i>	Laccase, diphenol oxidase	NM_001039398.1	10489	1.002	±
<i>TcTYR1</i>	Tyrosinase, monophenol oxidase	NM_001039404.1	N.A.	N.A.	N.A.
<i>TcTYR2</i>	Tyrosinase, monophenol oxidase	NM_001039433.1	15848	1.011	±
Detoxification					
<i>TcSULT*</i>	Sulfotransferase	XM_961924.1	12015	0.465	+++
<i>TcGLCT</i>	Glucosyl/glucuronosyl-transferase	XM_967666.2	10152	0.596	++
<i>TcCHE</i>	Carboxy/cholinesterase	XM_962191.2	02194	4.626	– – –
<i>TcGST</i>	Glutathione S-transferase	XM_969955.2	00522	0.673	++
<i>TcGS</i>	Glutathione synthetase	XM_962977.2	09391	0.982	±
<i>TcCYP4c3</i>	Cytochrome P450 monooxygenase	XM_968307.2	15293	0.599	++
Others					
<i>TcRPS6*</i>	Ribosomal protein S6	NM_001172390.1	10830	1.054	±
<i>TcACT2</i>	Actin-2	XM_970977.2	06296	0.991	±
<i>TcPMP2C*</i>	Peritrophic matrix protein	NM_001168449.1	09580	0.970	±
<i>TcSTE24*</i>	Zinc-dependent metalloprotease	XM_963563.1	00463	1.176	–
<i>TcABC-C9A (TcSur1)*</i>	ABC transporter subfamily C	XM_964261.2	12252	0.971	±
<i>TcABC-C5K</i>	ABC transporter subfamily C	XM_966647.2	14381	0.635	++
<i>TcHP12b</i>	Odorant binding protein	XM_962784.2	N.A.	N.A.	N.A.
<i>TcY-e</i>	Yellow protein	NM_001168307.1	06227	0.608	++
<i>TcY-k</i>	Yellow protein	XM_971253.1	06225	4.715	– – –
<i>TcY-2</i>	Yellow protein	NM_001168312.1	03539	5.162	– – –

Genes whose expression levels were also analyzed by RT-PCR (see Fig. S8) are marked with an asterisk. Strong, moderate and weak up or down regulation of transcript levels are indicated with (+++), (++) , (+) or (– – –), (– –), (–), respectively, and genes whose expression was unaltered are indicated with (±). Genes that were not predicted by the GLEAN algorithm were not assigned (N.A.).

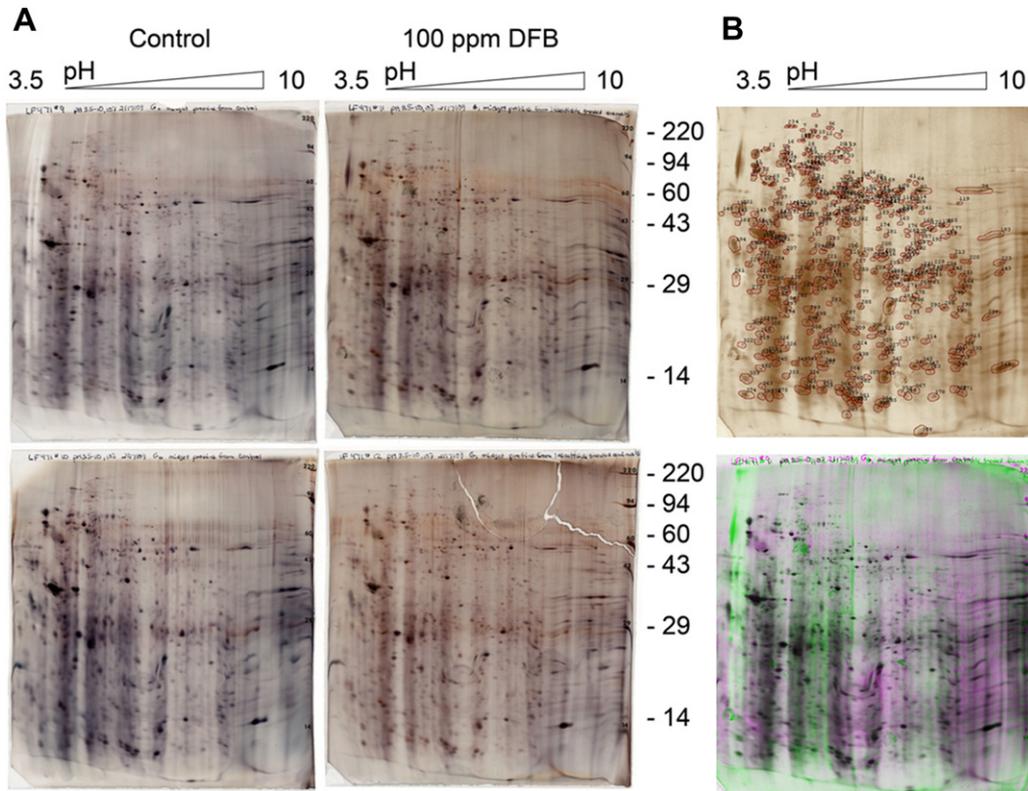


Fig. 5. *In silico* comparison of larval midgut proteins from *T. castaneum* resolved by two-dimensional gel electrophoresis. (A) Proteins were SDS extracted from midguts of larvae that were fed a control diet or a diet containing 100 ppm DFB. The proteins were separated in duplicate gels by 2D electrophoresis. The gels were scanned with a laser densitometer, aligned, and compared to each other. (B) Reference gel showing all spot numbering (top). Overlay image showing midgut proteins from DFB treated animals in green overlaying midgut proteins from control in magenta (bottom). Image appears black where spots overlay each other. A total of 388 protein spots were identified, but only the levels of 26 proteins were changed more than 2-fold.

Table 3
Molecular masses, isoelectric points and MALDI-TOF analysis of midgut proteins from *T. castaneum* whose amount significantly changed in response to DFB treatment.

Spot #	pI	MW	DFB	Control	DFB/Control	DFB/Control	MALDI-MS Identified protein spots
			Spot %	Spot %	Difference	T-test (p)	
8	5.7	112,068	0.077	0.021	3.6	0.009	XP_970719.1, paramyosin
12	5.9	92,018	0.012	0.006	1.9	0.029	N.D.
17	6.2	83,232	0.023	0.006	3.7	0.025	XP_973797.1, NADH-ubiquinone oxidoreductase 75 kDa subunit
19	6.3	82,382	0.054	0.017	3.2	0.015	XP_973797.1, NADH-ubiquinone oxidoreductase 75 kDa subunit, modified
30	6.2	75,584	0.099	0.042	2.3	0.024	N.D.
35	6.1	71,193	0.041	0.004	9.5	0.020	Keratin contamination
39	5.7	69,280	0.036	0.018	2.0	0.045	N.D.
56	6.8	59,520	0.095	0.018	5.3	0.182	NP_001164533.1, UDP-GlcNAc pyrophosphorylase I
82	6.8	51,143	0.055	0.010	5.7	0.002	XP_968070.1, glutathione synthetase
87	6.0	50,477	0.156	0.088	1.8	0.022	N.D.
90	6.2	50,286	0.101	0.056	1.8	0.037	N.D.
119	8.1	46,875	0.037	0.016	2.4	0.039	N.D.
153	7.0	43,867	0.059	0.029	2.0	0.046	N.D.
161	4.6	39,311	0.054	0.029	1.9	0.026	N.D.
168	7.6	38,283	0.172	0.096	1.8	0.027	N.D.
242	7.9	27,975	0.475	0.276	1.7	0.022	N.D.
262	5.8	26,911	1.269	0.645	2.0	0.031	N.D.
267	5.9	26,573	0.222	0.116	1.9	0.020	N.D.
297	6.3	22,166	0.523	0.207	2.5	0.044	N.D.
307	5.9	20,324	0.251	0.087	2.9	0.037	NP_001165843.1, actin-2
385	7.1	11,933	0.167	0.055	3.1	0.196	XP_967877.1, 12 kDa hemolymph protein b
89	6.1	50,286	0.008	0.020	-2.6	0.025	Poor spectrum, no ID
294	5.3	22,495	0.283	0.563	-2.0	0.019	N.D.
311	7.1	19,855	0.202	0.341	-1.7	0.027	N.D.
361	5.8	13,290	0.029	0.216	-7.5	0.264	Keratin contamination
384	7.1	12,035	0.713	1.325	-1.9	0.036	N.D.

previously analyzed the effects of DFB on developmental rate in *T. castaneum* reported a small but significant effect for very young larvae (0–3 h old) that were continuously exposed to DFB until emergence. In this case the developmental period to emergence was reduced by only about 15% (Ishaaya and Ascher, 1977).

4.2. DFB-effects on global gene expression

Although many scientists have attempted to elucidate DFB's mode of action, the question of how it interferes with insect molting and egg hatching is still a matter of speculation (Gangishetti et al., 2009; Muthukrishnan et al., 2012). Therefore, we decided to adopt an unbiased genome-wide approach profiling changes in gene expression levels in response to DFB treatment of larvae. Since conventional microarrays usually only use a few probes to interrogate a gene, and do not provide data on unknown transcriptionally active regions, we probed Roche NimbleGen HD2 whole genome DNA tiling array chips covering two million features of the *T. castaneum* genome. We developed a statistically evaluated procedure for background subtraction based on "Random-GC oligonucleotides" that were randomly placed on each chip, and an evaluation method to obtain gene expression levels based on averaged intensity scores obtained from numerous features interrogating all exons of each GLEAN-model. Hence, we for the most part obtained expression levels from a high number of individual hybridization events per gene. For instance, GLEAN_09580 (NM_001168449.1) encoding PM protein 2C (PMP2C; Jasrapuria et al., 2010) comprises three predicted exons represented by 48 individual tiles (see Fig. S3). After background subtraction, the mean intensity values for these tiles averaged for three biological replicates were 13.09 ± 1.24 for the control and 13.47 ± 0.75 for the DFB treated animals. The corresponding rE-value of 0.97 indicates no significant changes in the expression levels of this gene in response to DFB treatment. Our study revealed that only comparatively few *T. castaneum* genes exhibit significant changes in steady-state transcript levels in response to DFB treatment. They included genes involved in protein, lipid, nucleotide and sugar metabolism, replication, transcription and translation, development, signal transduction, cuticle formation and degradation, and genes encoding channels, transporters and receptors. Of particular interest are those functional categories that include more genes that are up- rather than down-regulated, as is the case for genes involved in the metabolism and modification of proteins, lipids, nucleotides and sugars, as well as replication, transcription and translation. While up-regulation of the latter genes may reflect a general stress response to DFB, other genes that are up-regulated may be involved in specific metabolic pathways involved in detoxification of this compound or in cuticle repair processes that compensate for malformations of the cuticle.

4.3. Induction of genes potentially involved in the elimination of xenobiotics

Among the genes that were up-regulated in response to DFB treatment were several that are known to be associated with insecticide detoxification by phase I and II reactions. Phase I reactions are commonly mediated by carboxy-esterases and cytochrome P450 enzymes (CYPs), while phase II reactions involve sulfotransferases, glucosyl/glucuronosyl-transferases and glutathione S-transferase (Ahmad and Forgash, 1976; Ffrench-Constant et al., 2004; Ishaaya, 1993). However, it should be pointed out that the up-regulation of genes known to be involved in detoxification may reflect a general response to xenobiotic stress, without necessarily playing a specific role in the detoxification of DFB.

Experiments with *T. castaneum* using esterase inhibitors suggested that the major metabolic pathway in the larva for DFB is by hydrolytic cleavage of the amide bond at the benzoyl carbon to yield 4-chlorophenylurea and 2,6-difluorobenzoic acid, which may be further converted into 4-chloroaniline, CO₂, and 2,6-difluorobenzamide (Gazit et al., 1989). The DFB hydrolase sensitive to the esterase inhibitors has not yet been identified, and there is no obvious hydrolase-encoding gene in *T. castaneum*, which is up-regulated in response to DFB treatment.

The tiling array data indicate furthermore that the transcript levels of genes encoding sulfotransferase, glucosyl/glucuronosyl-transferase, glutathione S-transferase and one particular cytochrome P450 isoform (TcCyp4C3) are up-regulated, suggesting that aromatic metabolites formed after cleavage may require hydroxylation and conjugation to small hydrophilic molecules in order to get eliminated. This is, however, in contrast to the house fly *Musca domestica* and the boll weevil *Anthonomus grandis*, in which direct oxidation appears to be the major route for DFB detoxification, because the major metabolites in these two species are hydroxylated forms of DFB (Chang, 1978; Chang and Stokes, 1979).

In addition to the detoxifying enzymes, ATP binding cassette (ABC) transporters of subfamilies B, C and G have been implicated in the insect defense response against xenobiotics including insecticides (Labbe et al., 2011). Experiments performed with *Aedes caspius* revealed synergistic effects between DFB and the ABC transporter inhibitor, verapamil, increasing DFB toxicity significantly when both compounds are co-administered (Porretta et al., 2008). The latter finding suggests that certain ABC transporters could act as efflux pumps for DFB or its metabolites. Indeed, the tiling array data revealed several genes encoding ABC transporters whose expression was altered in DFB treated *T. castaneum* larvae. However, among the 200 most up-regulated genes was only one encoding an ABC multidrug transporter of subfamily C, *TcABC-C5 K*. Hence, *TcABC-C5 K* might have a function during the course of DFB elimination. In contrast, the expression of *TcABC-G9B (TcWhite)* as well as *TcABC-C7A* was found to be down-regulated. However, many of the ABC transporter-encoding genes such as *TcABC-G8A* were unaltered in their expression levels. Particularly, we could not detect changes in expression levels of *TcABC-C9A* and *TcABC-C4A*, which encode two homologs of the *D. melanogaster* sulfonylurea receptor, DmSUR, a ABC transporter of the C subfamily, which has been suggested to act as a receptor for DFB based on the ability of DFB to compete with glibenclamide binding (Abo-Elghar et al., 2004). By studying Ca²⁺ uptake into vesicles prepared from abdominal integuments of *B. germanica* nymphs and the effects of DFB in this process, it has been proposed that DFB binding to SUR inhibits a GTP- or ATP-dependent Ca²⁺ and/or K⁺ transport system (Nakagawa and Matsumura, 1994). The mere absence of transcriptional responsiveness of SUR homologs from *T. castaneum* does not exclude the possibility that they are involved in DFB toxicity.

4.4. Genes involved in cuticulogenesis and PM formation

It is well-established that the addition of DFB neither inhibits chitin synthesis in cell-free membrane preparations that are actively making chitin nor does it inhibit the activity of the partially purified chitin synthase preparations (Cohen, 2001; Merzendorfer, 2006). These results are inconsistent with the well-known effect of DFB and other structurally related "chitin inhibitors" to bring about drastic reductions in chitin levels in cuticles and PMs of insects exposed to these compounds. We therefore examined genes involved in chitin metabolism with the expectation that there will be alterations in their expression at the transcriptional level. Down-regulation of these genes could directly affect the enzyme levels available for chitin synthesis and turnover and up-regulation could

reflect transcriptional mechanisms to compensate for the loss in chitin production. Increased *AqCHS1* mRNA levels associated with decreased chitin synthesis have been reported, for example, in *Anopheles quadrimaculatus* exposed to DFB (Zhang and Zhu, 2006). In contrast to our expectation, however, the expression levels of genes encoding trehalases, various enzymes of the Leloir pathway including UDP-GlcNAc pyrophosphorylases, chitin synthases, chitin deacetylases and chitinolytic enzymes were not significantly altered in response to DFB. Our findings are in line with previous observations obtained for embryos of two different *Drosophila* strains, where the expression of neither *mmy* that encodes UDP-GlcNAc pyrophosphorylase nor *kkv* that encodes chitin synthase 1 (CHS1) was changed in response to treatment with either 100 ppm DFB or lufenuron as measured by qRT-PCR (Gangishetti et al., 2009). Similarly, another study performed utilizing several different insect species also reported no significant alterations in the expression levels of *CHS* genes due to the treatment with benzoylphenyl urea compounds (Ashfaq et al., 2007).

Although the expression of genes involved in chitin metabolism was unaffected, many other genes encoding cuticle proteins (CPs) were significantly up- or down-regulated in response to DFB treatment, a finding that to our knowledge has not been described so far. Therefore, it is likely that in addition to the loss of chitin microfibrils, altered CP composition may contribute to the major structural changes in the procuticle induced by benzoylphenyl urea compounds, as observed in this and many other studies (Binnington, 1985; Gangishetti et al., 2009; Ker, 1977). Altered mechanical properties of the cuticle may be a major problem in successfully shedding the old cuticle and hence one of the contributing causes of abortive molting.

4.5. Changes in the midgut proteome

Another remarkable outcome of the tiling array analysis is that the expression levels of genes encoding PM proteins (PMPs) are widely unchanged, while many CP-encoding genes were altered significantly. Although structurally different from CPs, PMPs are believed to play similar roles, as they bind and cross-link chitin microfibrils via chitin-binding (peritrophin A) domains and finally help to organize the PM (Hegedus et al., 2009; Jasarapuria et al., 2010; Wang et al., 2004). The possibility remains that PMP levels could be changed at a post-transcriptional level. To examine DFB-induced changes in the midgut proteome, we performed 2D gel electrophoresis, densitometric quantification of protein spots and mass spectrometric identification of proteins that significantly changed in their amounts. Similar to the tiling array data, we found that about 7% of the detected midgut proteins changed significantly in their levels. Again, no PMP was among the proteins, whose steady-state levels were altered by more than 2.5-fold. However, the extraction conditions that we have used may have been too mild to extract tightly bound PMPs. Interestingly, UDP-GlcNAc pyrophosphorylase I (TcUAP1) was increased more than 5-fold in DFB treated larvae. There are two genes encoding UDP-GlcNAc pyrophosphorylases in *T. castaneum*, but only TcUAP1 has been shown to be required for chitin synthesis in the cuticle and PM (Arakane et al., 2011). As the *TcUAP1* gene expression level is unaltered following DFB treatment, increased protein levels in the midgut appear rather to be due to increased translation or to a decrease in the turnover of this protein. We currently do not know whether TcUAP1 protein levels are also increased in the cuticle. An increase in TcUAP1 may reflect a compensatory mechanism to elevate cytosolic levels of UDP-GlcNAc for chitin synthesis. However, at least in the cuticle it would also be necessary to accommodate the greater need for this substrate arising from increased protein glycosylation, as many genes encoding CPs are

up-regulated at this stage. At least some CPs from different insect species including *T. castaneum* are likely to be glycoproteins (Lemoine et al., 1990; Missios et al., 2000; Silvert et al., 1984; Stiles, 1991). Another protein that was significantly increased in amount was glutathione synthetase, whose gene was also found to be slightly up-regulated in the tiling array. The increase in glutathione synthetase levels may provide additional reduced form of glutathione in midgut cells, which is utilized for the elimination of radical oxygen species and for glutathionylation of DFB metabolites (Forcella et al., 2007). It is possible that the up-regulation of several other proteins in the midgut may be in response to metabolic stress induced by the insecticide. As a consequence of increased levels of the mitochondrial NADH-ubiquinone oxidoreductase additional oxidative stress and mitochondrial dysfunction may arise, which can trigger apoptosis (Jezek and Hlavata, 2005). Likewise, paramyosin and actin-2 have been frequently observed to be affected in response to metabolic stress resulting in the formation of stress fibers to remodel both tissues and extracellular matrix (Pellegrin and Mellor, 2007). Finally, we found that a 12 kDa hemolymph protein to be up-regulated in the midgut in response to DFB. This protein belongs to a family of proteins known to function as carriers that bind hydrophobic compounds such as odorants and pheromones, and transport them through aqueous solutions (Vogt and Riddiford, 1981). Some of these have direct roles in sensual perception (Pelosi and Maida, 1995), but for many of them a clear function has not been ascribed (Graham et al., 2003). The finding of increased amounts for the 12 kDa hemolymph protein in the midgut of DFB treated *T. castaneum* larvae suggests that it may be involved in transport and inactivation of DFB, which is a highly hydrophobic compound as long it is not conjugated with a hydrophilic agent.

5. Conclusions

Our study on DFB-mediated changes revealed expected effects on chitin content in cuticle and midgut, cuticle structure and egg hatching in a single insect model species, which have been previously found in several different insect species. However, we were unable to document significant changes in expression of several genes of chitin metabolism, indicating that net changes in chitin content following DFB treatment must be due to events that are downstream of transcription of these genes. The negligible changes in developmental rate after DFB treatment in surviving animals and the availability of genomic resources along with the amenability to systemic RNAi make the red flour beetle a good model organism for investigating the mode of action of DFB and other benzoylphenyl urea-based IGRs as well as other kinds of insecticides.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ibmb.2011.12.008.

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