Phenobarbital Induction of UDP-glucosyltransferase Activity in *Drosophila melanogaster* Meigen[1]

Carolina Rausell, Julia Llorca and M. Dolores Real

Departamento de Genética, Universitat de València, Dr. Moliner 50, 46110-Burjassot, Valencia, Spain

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Abstract The inducibility of UDP-glucosyltransferase activities towards the exogenous substrates 1-naphthol and 2-naphthol and the endogenous metabolite xanthurenic acid was demonstrated in *Drosophila melanogaster* Meigen larvae and adults using phenobarbital as an inducer. In adults, a 3.5-fold increase of glucosyltransferase activity toward xanthurenic acid and a 2.0-fold increase of the activity toward exogenous substrates (1-naphthol and 2-naphthol) was found. In larvae, maximum induction of all three UDP-glucosyltransferase activities (2.5-fold and 1.5-fold increase of the activity toward the exogenous and endogenous substrates, respectively) was achieved when insects, reared on solid medium, were exposed to phenobarbital for 24 h. However, a 24-h exposure to inducer on liquid medium yielded the same level of induction as in solid medium only for xanthurenic acid:UDP-glucosyltransferase activity. Repression of the activities toward exogenous substrates also was noticed. On this medium, comparable induction to that detected on solid medium was found when inducer exposure was reduced to 2.5 h. According to the observed variation resulting from the different conditions of phenobarbital treatment, a differential induction of UDP-glucosyltransferase isoenzymes also was demonstrated. After 2.5 h of inducer exposure, three isoenzymes (every one specific for each of the three analysed substrates) were detected; whereas, after 24 h of inducer treatment, only one isoenzyme, specific for xanthurenic acid, was detected.

Key Words UDP-glucosyltransferase activity; phenobarbital induction; xenobiotics; biotransformation; *drosophila melanogaster*

Uridine disphosphoglucosyltransferase activity plays a major role in the detoxification of many endogenous and xenobiotic substrates in insects (Smith 1968, Real and Ferré 1990, Real et al. 1991, Ahmad and Hopkins 1992, 1993, Hopkins and Kramer 1992). This detoxification is accomplished by transferase-mediated conjugation with glucose resulting in compounds that are efficiently excreted.

Although little information on the role of UDP-glucosyltransferase activity in insects is available, it is well known that this enzyme is involved in the metabolic pathways related to pigmentation and cuticle formation (Morello and Repetto 1979, Väisänen et al. 1983, Real and Ferré 1990, Ahmad and Hopkins 1992, Hopkins and Kramer 1992). Furthermore, we previously demonstrated the occurrence of multiple UDP-glucosyltransferase isoenzymes in three developmental stages of *Drosophila melanogaster* Meigen (Rausell et al. 1997).

UDP-glucosyltransferase activity in *Musca domestica* L. and in *D. sordidula* Kikawa and Peng adults can be induced by phenobarbital treatment (Morello and Repetto 1979, Real and Ferré 1990). Inducibility of other activities involved in detoxification processes, such as cytochrome P-450, glutathione S-transferases and carboxyesterases, also has been demonstrated in insects (Hällstrom and Grafström

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1981, Hällstrom et al. 1983, Hällstrom 1984, Terriere 1984). Enzyme inducers are found in several classes of chemicals, such as drugs, pesticides, solvents, and polycyclic aromatic hydrocarbons. Phenobarbital is one of the most common drugs used in induction studies. Such compounds induce the expression of genes whose products could not be detected otherwise or also can act as inducible enhancers that increase transcription of genes only in the presence of the inducer (Hodgson et al. 1993). This is especially relevant when considering genes coding for enzymes involved in detoxification processes because the increase of the metabolic capacity is considered the major route for resistance development. Detoxification studies in insects have revealed that further versatility in the adaptation of insects to the environment is provided by this induction phenomenon (Terriere 1984), and numerous examples of increase in tolerance due to induction in insects are found in the literature. In *D. melanogaster,* a 50% increase of glutathione S-transferase upon administration of penthamethyl benzene, and up to a 7-fold increase of cytochrome P-450 after treatment with phenobarbital, β-naphthoflavone or polychlorinated biphenyls have been reported (Cochrane and Leblanc 1986, Hällstrom and Grafström 1981). In other insects, such as *Heliothis virescens* F., a 2 to 3-fold increase in tolerance to diazinon induced by tridecanone has been demonstrated (Riskallah et al. 1986a, b). By far, the bulk of scientific reports on insect resistance caused by induction are related to modified or amplified enzymes (glutathione S-transferase, carboxyesterases and cytochrome P-450) which result in increased metabolic detoxification of insecticides (see Georghiou and Saito 1983, Price 1991, Morton 1993).

In this paper, inducibility by phenobarbital of UDP-glucosyltransferase activity towards one endogenous (xanthurenic acid) and two exogenous substrates (1-naphthol and 2-naphthol), in *D. melanogaster* larvae and adults, is demonstrated. The presence of distinct isoenzymes in response to different conditions of phenobarbital treatment also has been determined.

Materials and Methods

Chemicals. Sephadex G-25 and Polybuffer 74 were obtained from Pharmacia LKB Biotechnology Inc (Sweden). Uridine 5′-diphosphoglucose (UDPG), 1-naphthol, 2-naphthol, 2-naphthol glucoside, xanthurenic acid and $MgCl_2$ were from Sigma Chemical Co. (U.S.). 1-naphthol glucoside was from Serva (U.S.), and 5-ethyl-phenyl-barbituric acid (phenobarbital) was obtained from Fluka (U.S.). Xanthurenic acid 8-glucoside was chemically synthesized as described by Real and Ferré (1988).

***Drosophila* cultures.** The organism used was *D. melanogaster,* strain Oregon-R (wild type) and was reared at 25 ± 1°C. When individuals of synchronized age were needed, flies were set to oviposit on a medium consisting of agar and water, for no longer than 5 h. The recently oviposited eggs were transferred either to liquid or solid medium. The solid medium (30 ml), consisting of corn flour (5.2%), agar-agar (0.8%), sugar (4.3%), water (86%), methyl p-hydroxybenzoate (0.3%), ethanol (0.8%), propionic acid (0.4%) and live yeast (2.1%), was dispensed in 250-ml glass bottles. Overcrowding was avoided in the cultures by placing up to 100 larvae per bottle.

When a large number of insects was needed, eggs were placed in 30 × 20 × 10 cm plastic containers with 500 ml of liquid medium. The composition of the liquid medium was yeast (28.8%), sugar (14.4%), propionic acid (0.06%), phosphoric acid (0.28%), and water (56.5%). After pupation, individuals were transferred to bottles with solid standard medium.

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For induction experiments, larvae (72 ± 12 h after the emergence of the egg) were transferred to bottles with standard solid medium containing 0.5, 1, and 2 mg/ml of phenobarbital. After 24 h of treatment, they were collected and stored at −20°C, until needed. Larvae of the same age also were transferred to liquid standard medium containing 0.7 mg/ml of phenobarbital. Larvae exposed to the inducer from 1 to 24 h were collected and also stored at −20°C.

When induced adults were required, flies of 24 ± 12 h from puparium emergence, starved for 3 h, were transferred to bottles with standard solid medium containing 1 mg/ml of phenobarbital. After 4 d in this medium, they were collected and stored at −20°C.

Protein extracts and enzyme assays. Homogenates were prepared with an electric blender in 600 µl of 0.1 M Tris-HCl buffer (pH 7.1) per 100 individuals (larvae or adult flies) in an ice-cold bath, and then centrifuged for 30 min at 20,000 × *g* at 4°C. Supernatant aliquots (up to 0.5 ml) were applied to Sephadex G-25 columns (5 × 1 cm) equilibrated with the homogenizing buffer, and the columns were centrifuged for 2 min at 1000 × *g* (Neal and Florini 1973). An additional 0.2 ml of buffer was added to the columns, and centrifugation was repeated. The eluates were combined and directly used for the enzyme assays.

Xanthurenic acid:UDP-glucosyltransferase assay. The standard reaction mixture contained, in a final volume of 0.25 ml, 1.6 mM xanthurenic acid, 3.2 mM UDPG, 16 mM $MgCl_2$, 200 mM Tris HCl (pH 7.1), and 40 µl of enzyme extract (in an equivalent amount to 200 to 450 µg of protein). Incubation was carried out for 2 h at 37°C, and the reaction was finally terminated by adding 20 µl of 40% $HClO_4$. Proteins and insoluble material were removed by centrifugation at 12,500 × *g* for 3 min. The supernatant was filtered and used for HPLC analysis of xanthurenic acid 8-glucoside under the conditions described in HPLC section. Controls were run in the absence of UDPG or enzyme fraction.

In the enzyme assays of FPLC fractions the reaction mixture contained, in a final volume of 0.25 ml, 1.6 mM xanthurenic acid, 3.2 mM UDPG, 16mM $MgCl_2$, 200 mM Tris-HCl (pH 7.1), and 100 µl of FPLC fraction.

1-naphthol and 2-naphthol:UDP-glucosyltransferase assay. The standard reaction mixtures contained, in a final volume of 0.25 ml, 0.28 mM 1-naphthol or 2-naphthol, 6.4 mM UDPG, 16 mM $MgCl_2$, 8 mM Tris-HCl buffer (pH 7.1), 100 mM glycine-KOH buffer (pH 10.5), and 20 µl of enzyme extract (in an equivalent amount to 100 to 225 µg of protein). The reaction mixtures were incubated for 20 min at 43°C, and the reaction was terminated by the addition of 20 µl of 40% $HClO_4$. Precipitated proteins were removed by centrifugation at 12,500 × *g* for 3 min.

In the enzyme assays of FPLC fractions the reaction mixtures contained, in a final volume of 0.25 ml, 0.28 mM 2-naphthol, 6.4 mM UDPG, 16 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 7.1), 200 mM glycine-KOH buffer (pH 10.5) and 100 µl of FPLC fraction.

HPLC. The reaction products of xanthurenic acid:UDP-glucosyltransferase and 1-naphthol and 2-naphthol:UDP-glucosyltransferase assays were analyzed by HPLC. The HPLC consisted of a Merck-Hitachi L6200 pump, a Merck AS-2000 automatic injector on a reverse phase column (Spherisorb ODS 50 DS, 4.5 × 250 mm, 5 µm) at 0.9 ml/min flow rate. All HPLC analyses were performed at room temperature.

In xanthurenic acid:UDP-glucosyltransferase assays, the mobile phase was 10 mM potassium phosphate (pH 7.8) with 10% methanol and when 1-naphthol and

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2-naphthol were used as substrates, the mobile phase was 10 mM potassium phosphate (pH 7.8) with 28% acetonitrile.

The fluorescence of the glucosides of xanthurenic acid, 1-naphthol and 2-naphthol was monitored with a Merck-Hitachi F-1050 fluorometric detector (excitation 360 nm, emission 425 nm for xanthurenic acid 8-glucoside; excitation 287 nm, emission 335 nm for 1-naphthol glucoside; excitation 283 nm, emission 341 nm for 2-naphthol glucoside).

Determination of the protein content. The protein content of the crude homogenates was measured according to Bradford (1976) using bovine serum albumin as standard.

FPLC chromatofocusing. UDP-glucosyltransferase was partially purified by FPLC. The liquid chromatograph consisted of a FPLC set from Pharmacia with a GP-250 controller, two P-500 pumps, a V-7 injection valve and a mixer. The protein extracts (0.5 ml, approximately equivalent to 4 mg of solubilized protein in 0.025 M bis TrisHCl buffer, pH 6.3) were applied, through a 0.5 ml loop, to a Mono P HR 5/5 from Pharmacia, which had been previously equilibrated with 0.025 M bis Tris HCl buffer (pH 6.3). The column was eluted with an elution buffer (pH 4.0) containing 10% of Polybuffer 74 according to the instructions of the supplier. Chromatography was conducted at room temperature. The flow rate was 1 ml/min; 0.5 ml fractions were collected and cooled down in iced water after measuring their pH. The chromatographic separation of proteins was monitored by UV absorbance at 280 nm with a L-4250 detector from Merck and a Spectra Physics SP 4290 recording integrator. Proteins with pI under the pH range of the gradient were retained in the column and were eluted with 0.5 ml of 1 M NaCl. The first five consecutive fractions (0.5 ml) were collected for their subsequent analysis.

Results and Discussion

UDP-glucosyltransferase activity towards the endogenous substrate xanthurenic acid and the exogenous compounds 1-naphthol and 2-naphthol has been reported in *D. melanogaster* (Real and Ferré 1990, Real et al. 1991). Inducibility of xanthurenic acid UDP-glucosyltransferase activity by phenobarbital in *D. sordidula* adults also has been previously reported (Real and Ferré 1990). Our results show inducibility of xanthurenic acid, 1-naphthol, and 2-naphthol UDP-glucosyltransferase activities in *D. melanogaster* adults, too.

Drosophila melanogaster adults treated with 1 mg/ml phenobarbital showed a 3.5-fold increase in total UDP-glucosyltransferase activity toward xanthurenic acid and 2-fold increase toward 1-naphthol and 2-naphthol we also observed (Table 1).

No data concerning induction of UDP-glucosyltransferase activity in *D. melanogaster* larvae have been previously reported. In order to analyze the inducibility of UDP-glucosyltransferase activities at this developmental stage, larvae were reared on two different standard media. On the solid medium, maximum level of induction of UDP-glucosyltransferase activity was achieved for all three substrates when larvae were treated with 1 mg/ml of phenobarbital for 24 h (Fig. 1). However, when larvae were reared on the liquid medium, for the same 24 h inducer exposure, 220% induction of xanthurenic acid:UDP-glucosyltransferase activity was observed and repression of the activity toward the exogenous substrates was noted (only around 40% of the activity in the absence of phenobarbital). Further evaluation showed an additional peak of induction for all three substrates after 2.5 h of phenobarbital treatment (Fig. 2).

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Table 1. Mean (±SE) UDP-glucosyltransferase activity in *Drosophila melanogaster* adults treated with phenobarbital*

Treatment	Xanthurenic acid:UDP-glycosyltransferase activity		1-naphthol:UDP-glucosyltransferase activity		2-naphthol:UDP-glucosyltransferase activity	
	pmol/h/individual	% induction	pmol/h/individual	% induction	pmol/h/individual	% induction
None (control)	25.38 ± 2.20		2.56 ± 0.01		1.50 ± 0.04	
Phenobarbital (1 mg/ml)	89.12 ± 3.32	351	5.17 ± 0.20	202	3.19 ± 0.04	213

* Induction percentages of enzyme activities refer to untreated adults (control).

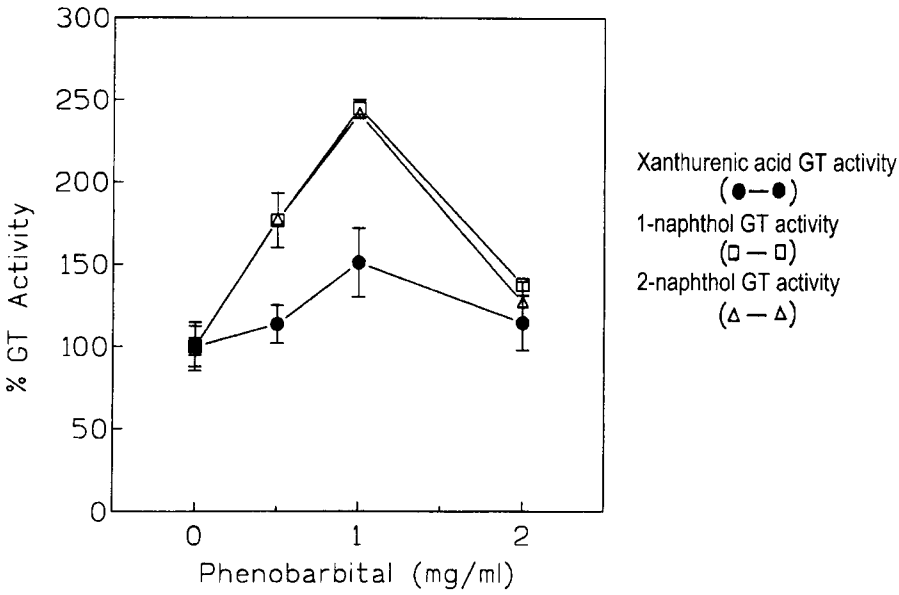

Fig. 1. Induction percentages of UDP-glucosyltransferase (GT) activity in 72 ± 12 h *Drosophila melanogaster* larvae treated for 24 h with different concentrations of phenobarbital in a standard solid medium. (Error bars are the standard error of three replicates).

The conclusion can be drawn that, in larvae, different maximum levels of induction of UDP-glucosyltransferase activities were obtained, depending on the medium and the time of exposure to the inducer. For the exogenous substrates, a 2.5-fold maximum increase of the activity was found after 24 h of phenobarbital treatment on solid medium and also after 2.5 h of phenobarbital exposure on liquid medium. A 2.0-fold increase of UDP-glucosyltransferase activity toward the endogenous substrate was observed after a 24-h phenobarbital treatment on liquid medium, but only an approxi-

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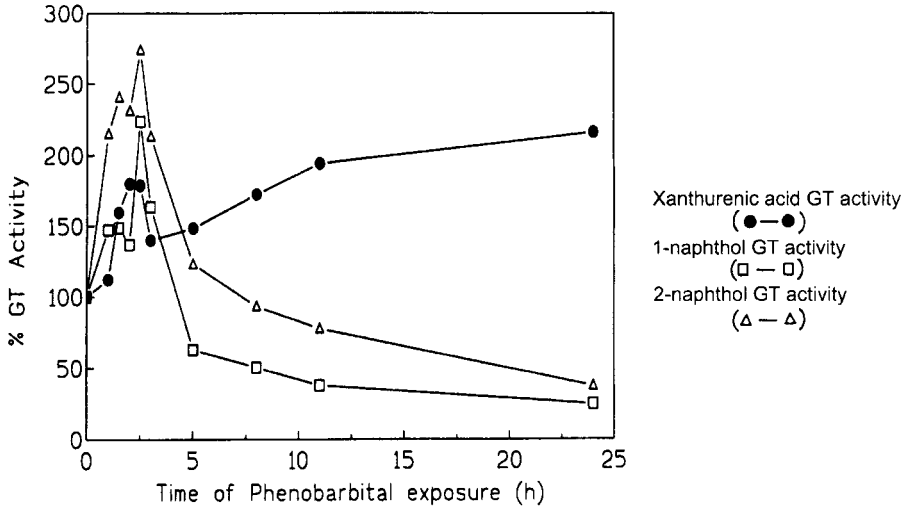

Fig. 2. Induction percentages of UDP-glucosyltransferase (GT) activity as a function of the phenobarbital (0.7 mg/ml) pre-treatment time in 72 ± 12 h *Drosophila melanogaster* larvae reared on standard liquid medium.

mate 1.5-fold increase was occurred when larvae were treated for 2.5 h on this medium or for 24 h on solid medium.

Given the differences in the induction percentages of UDP-glucosyltransferase activities toward the endogenous and exogenous substrates, protein extracts prepared from larvae exposed to phenobarbital for 2.5 h and 24 h were subjected to FPLC chromatofocusing with the intention to establish the presence of different isoenzymes after phenobarbital treatment. The collected fractions were analyzed to determine the presence of UDP-glucosyltransferase activity using the endogenous metabolite (xanthurenic acid) and one of the exogenous compounds (2-naphthol) as a substrate. When larvae were treated for 2.5 h with phenobarbital, fractions showing UDP-glucosyltransferase activity toward 2-naphthol (fractions of pHs > 6.00) and toward xanthurenic acid (pH 6.15 and 5.00) were found (Fig. 3a). However, after 24 h of inducer exposure, only one peak of activitity specific for xanthurenic acid (pI 5.00) was detected (Fig. 3b).

Our results demonstrate that using an endogenous metabolite (xanthurenic acid) and two exogenous compounds (1-naphthol and 2-naphthol) as substrates, the inducibility of UDP-glucosyltransferase activity in *D. melanogaster* larvae and adults occurs. The maximum induction percentage in adults was achieved for xanthurenic acid:UDP-glucosyltransferase activity (Table 1), while in larvae, UDP-glucosyltransferase activity toward the exogenous substrates was mainly induced (Fig. 1, 2). The higher inducibility of xanthurenic acid:UDP-glucosyltransferase activity in adults may be due to the fact that xanthurenic acid metabolic pathway in relation to eye pigment synthesis in adult flies is specifically active in this developmental stage.

Regarding phenobarbital induction in larvae, different induction levels were attained depending on the rearing medium. As is shown in Fig. 1 and 2, for all three

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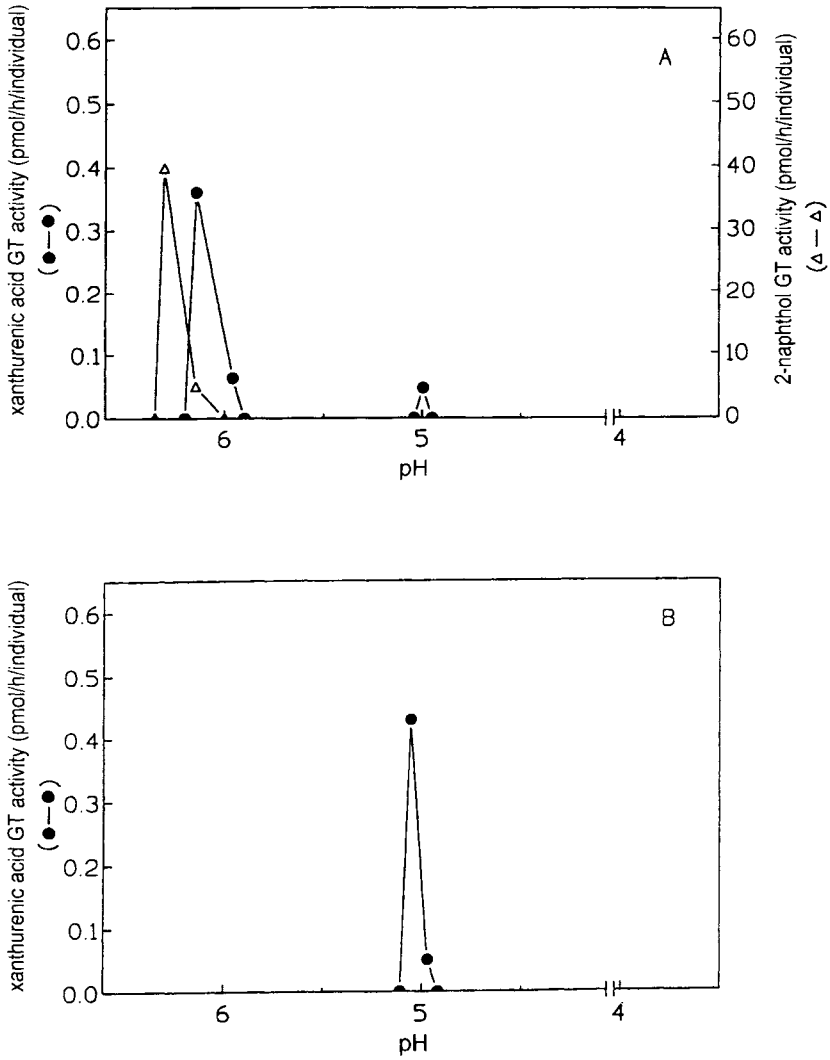

Fig. 3. UDP-glucosyltransferase (GT) activity profiles after chromatofocusing elution of *Drosophila melanogaster* larvae and adults pre-treated with phenobarbital. (A), 4-day-old *Drosophila melanogaster* larvae pre-treated for 2.5 h with 0.7 mg/ml of phenobarbital on standard liquid medium; (B), 4-day-old *Drosophila melanogaster* larvae pre-treated for 24 h with 0.7 mg/ml of phenobarbital on standard liquid medium.

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substrates, the induction percentages after 2.5 h of phenobarbital treatment on liquid medium were similar to the ones obtained after 24 h of inducer exposure on solid medium. This indicates a direct relationship between phenobarbital intake and larval feeding behavior, determined by the composition and physical consistency of the medium. The capability of UDP-glucosyltransferase activity of being selectively induced could enable insects to respond swiftly to fluctuations in the supply of nutrients, allowing them to better adapt to the environment due to their ability to switch from metabolizing one substrate to another. The observed differences show that, although the response to pre-treatment with inducers is evidently under genetic control, it could also influence the insect's metabolic capacity.

On the other hand, our results showed a clear relationship between the profile of the total UDP-glucosyltransferase activities observed (Fig. 2) and the detected isoenzymes (Fig. 3). Increasing phenobarbital exposure leads to a higher induction of total xanthurenic acid:UDP-glucosyltransferase activity and a dramatic decrease of total 2-naphthol:UDP-glucosyltransferase activity (Fig. 2). This variation can be correlated to the increase of the isoenzyme of pI 5.00, specific to xanthurenic acid, and the disappearance of isoenzymes of pI > 6.00, detected after 2 h 30 min of inducer exposure (Fig. 3a), which cannot be found after 24 h of larvae treatment (Fig. 3b).

Finally, the distinctive response to induction of UDP-glucosyltransferase activity towards the endogenous and exogenous substrates is noteworthy, as it indicates the existence of metabolic requirements that may need alternative detoxification strategies. The differential induction of UDP-glucosyltransferases demonstrated in this paper indicates, for the first time, a straightforward approach to the characterization of genes encoding these proteins which have not yet been identified.

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