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INDUCTION, SUPPRESSION AND INHIBITION OF MULTIPLE HEPATIC CYTOCHROME P450 ISOZYMES IN THE MALE RAT AND BOBWHITE QUAIL (*COLINUS VIRGINIANUS*) BY ERGOSTEROL BIOSYNTHESIS INHIBITING FUNGICIDES (EBIFs)*

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Abstract—Ergosterol biosynthesis inhibiting fungicides (EBIFs) have complex effects on the hepatic microsomal monooxygenase systems of vertebrate species, having been described as mixed inducers and inhibitors of cytochrome P450. In the current study, we examined the effects of two EBIFs in clinical use, clotrimazole and ketoconazole, and two agricultural EBIFs, propiconazole and vinclozolin, on hepatic monooxygenase activities and P450 apoprotein expression in the male Sprague–Dawley rat and the male bobwhite quail. EBIFs produced Type II binding spectra with hepatic microsomes from both species and were effective inhibitors of methoxyresorufin *O*-demethylase, an activity selective for P450 isozymes in gene family 1. However, the EBIFs varied widely in their effectiveness as inducers of P450 isozymes in gene families 1, 2, 3 and 4, both within the same species and between species. In the rat, clotrimazole was the most effective inducer, increasing expression of CYP 3A isozymes over 450-fold, CYP 2B1/2 30-fold and CYP 1A1/2 12-fold and suppressing expression of CYP 2C11 nearly 70%. By contrast, in the quail, clotrimazole was the least effective inducer. In quail, vinclozolin and propiconazole elevated total P450 content 10- and 7-fold, respectively. The induction response also appeared to be mixed, but in this case consisted of a 5-fold induction of P450s in gene family 1A, a 3-fold induction of P450s in gene family 3A and 4A, and induction of protein(s) from gene family 2, cross-reactive with antisera against rat CYP 2C11 and CYP 2A1. A protein that was cross-reactive with antibodies raised against rat CYP 2B1 was decreased with EBIF treatment. In conclusion, EBIFs have complex patterns of induction, suppression and inhibition of cytochrome P450 isozymes in both mammals and birds, which vary according to both the fungicide and the species.

Key words: cytochrome P450; fungicide; induction; suppression; inhibition

Hepatic microsomal cytochrome P450-dependent monooxygenase is the most important enzyme system involved in the phase I biotransformation of drugs and other xenobiotics [1]. It has been described as a "second immune system" due to its unusually broad substrate specificity and its ability to respond to xenobiotic challenge by allowing compounds to induce their own metabolism [2]. The multiple P450 isozymes have distinct but overlapping substrate specificities and are separate gene products that have been categorized into families and subfamilies with common structural and regulatory elements [3, 4].

In mammalian species, there are four P450 gene families (CYP‖ 1–CYP 4) that are inducible by xenobiotics [5–8]. Much less is known of P450 isozymes in birds, and only CYP family 1 orthologues have been shown to be inducible in all avian species tested [9]. It appears that induction mechanisms differ for each P450 subfamily and, as such, most inducers are specific for P450 isozymes from one family or subfamily.

A number of compounds are unique in their ability to affect a whole range of P450 forms from all the inducible families. The best characterized of these are the EBIFs. These compounds were designed as inhibitors of the fungal enzyme lanosterol 14α-hydroxylase [10], and many, such as propiconazole and vinclozolin, are in widespread agricultural use. Clotrimazole is an EBIF in clinical use as a topical fungicide to treat athletes foot and vaginal yeast infections. Some EBIFs, such as ketoconazole, are very potent inhibitors of steroid biosynthesis [11] and are in clinical use for the treatment of prostate and breast cancer. In addition to inhibition of monooxygenases, EBIFs are also capable of inducing hepatic P450s. The best characterized in mammals is clotrimazole, which has been shown to be a mixed

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‖ Abbreviations: CYP, cytochrome P450; EBIFs, ergosterol biosynthesis inhibiting fungicides; EROD, ethoxyresorufin *O*-deethylase; PROD, pentoxyresorufin *O*-dependentylase; MROD, methoxyresorufin *O*-demethylase; BROD, benzyloxyresorufin *O*-debenzylase; and DMF, dimethylformamide.

inducer of P450s in gene families 3, 2B and 1A in the female rat [12]. In avian species, a number of agricultural EBIFs, such as procloraz and iprodione, have been described as potent P450 inducers [9]. Much less is known about the effects of clinical EBIFs in birds or about the effects of agricultural EBIFs in mammals; in addition, no characterization of the avian induction response has been conducted at the molecular level.

In the current study, the effects of propiconazole, vinclozolin, clotrimazole and ketoconazole on the hepatic cytochrome P450-dependent monooxygenase system of the male rat and bobwhite quail were examined. Monooxygenase activities and P450 apoprotein levels were assessed by western blot analysis for P450 isozymes in gene families CYP 1–CYP 4. Our results revealed induction of P450s in gene families 1A, 2B and 3A by EBIFs in the male rat, with little effect on isozymes in gene family 4A, accompanied by a suppression of the major constitutive P450 CYP 2C11. In contrast, in the quail, induced forms were seen in all four gene families. Evidence for inhibitory complex formation with microsomal cytochrome P450 occurred with all EBIFs in both species and may explain the lack of correlation seen in some cases between monooxygenase activity and apoprotein expression in microsomes from EBIF-treated animals.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (300 g) were purchased from Harlan Industries (Indianapolis, IN). Male bobwhite quail (*Colinus virginianus*) (200 g) were supplied by local Little Rock breeders. Clotrimazole, ketoconazole, NADPH, testosterone, erythromycin, thiobarbituric acid, sodium dithionite, cytochrome *c* and lauric acid were purchased from the Sigma Chemical Co. (St. Louis, MO). Steroid metabolite standards were supplied by Steraloids Inc. (Wilton, NH) and obtained from the MRC Steroid Reference Collection (St. Mary's Hospital, London, U.K.). [^{14}C]Testosterone (50–60 mCi/mmol) was purchased from New England Nuclear (Boston, MA), and [^{14}C]lauric acid (56.6 mCi/mmol) was obtained from Amersham International (Amersham, U.K.). LHP-KDF normal phase, silica gel HPTLC plates were supplied by the Whatman International Ltd. (Maidstone, U.K.). Carbon tetrachloride was from the Aldrich Chemical Co. (Milwaukee, WI). Ethoxyresorufin, pentoxyresorufin and resorufin were purchased from the Pierce Chemical Co. (Rockford, IL), and methoxyresorufin and benzyloxyresorufin were obtained from Molecular Probes Inc. (Eugene, OR). Propiconazole (technical grade 90.9%) was a gift from the CIBA–GEIGY Corp. (Greensboro, NC). Vinclozolin (technical grade 95%) was a gift from the BASF Corp. (Research Triangle Park, NC). Rabbit polyclonal antibodies monospecific for P450 2E1 and P450EtOH$_2$ were generated as described previously [13, 14]; rabbit polyclonal antibodies to rat cytochrome P450s CYP 1A1/2, CYP 2A1, CYP 2B1 and CYP 3A2 [15] were gifts from Dr. Anders Astrom (Karolinska Institute, Sweden); rabbit polyclonal antibodies to rat cytochrome P450 CYP 2C11 [16] were a gift from Dr. Eddie Morgan (Emory University, GA); mouse monoclonal antibodies specific for CYP 2C11 [17] were a gift from Dr. Paul Thomas (Rutgers College of Pharmacy, NJ); and sheep polyclonal antibodies to rat cytochrome P450 CYP 4A1 [18] were a gift from Dr. Gordon Gibson (Surrey University, U.K.). Rabbit anti-sheep IgG was obtained from Boehringer Mannheim (Indianapolis, IN), and ^{125}I goat anti-rabbit IgG (2–15 μCi/μg) was obtained from ICN Biomedicals Inc. (Costa Mesa, CA).

Animals. Groups of 300 g male Sprague–Dawley rats (N = 5) and 200 g male bobwhite quail (N = 6) had *ad lib.* access to food and water and were treated once a day for 3 days with the EBIFs shown in Fig. 1, suspended in corn oil vehicle or with vehicle alone. The compounds were administered by oral gavage at the following concentrations: propiconazole, 400 mg/kg/day; vinclozolin, 400 mg/kg/day; clotrimazole, 100 mg/kg/day; and ketoconazole, 100 mg/kg/day in 3 mL corn oil (rat) or 1 mL corn oil (quail). The animals were killed 48 hr following the final injection of fungicide, and liver microsomes were prepared as described by Chipman *et al.* [19].

Monooxygenase components. Cytochrome P450 and cytochrome b_5 contents of hepatic microsomes were determined spectrophotometrically by the method of Omura and Sato [20]. Cytochrome P450 reductase activities were determined by measurement of the NADPH-dependent reduction of cytochrome *c*, as described by Dignam and Strobel [21], at 37° for rat microsomes and at 42° for quail microsomes, respectively.

Binding spectra. Binding spectra were generated by the addition of 100 μmol fungicide in 10 μL DMF to hepatic microsomes at a concentration of 2 mg/mL in 50 mM potassium phosphate, pH 7.4, in the sample cuvette of a Shimadzu 2101 UVPC dual beam spectrophotometer and read against untreated microsomes at the same concentration in the reference cuvette between 360 and 500 nm.

Monooxygenase activities. All rat monooxygenase activity measurements were conducted at 37°, and all quail activities were conducted at 42°. Alkoxyresorufin *O*-dealkylase activities (EROD, PROD, MROD and BROD) were measured spectrofluorimetrically as described previously [22]. MROD inhibition was measured as above, following a 5-min preincubation with increasing concentrations of EBIFs dissolved in DMF using microsomes to which the same volumes of DMF alone had been added as a control. [^{14}C]Testosterone metabolism was assayed by the HPTLC method of Ronis *et al.* [23]. Erythromycin *N*-demethylase activity was monitored by determining the rate of formaldehyde formation according to the Nash assay [24]. Carbon tetrachloride-dependent lipid peroxidation was measured according to Johansson and Ingelman-Sundberg [25]. [^{14}C]Lauric acid metabolism was assayed by a modification of the method of Salhab *et al.* [26] using 50 μM [^{14}C]lauric acid in 1 mL of 100 mM potassium phosphate, pH 7.4, 1 mM NADPH and terminated after 5 min with 200 μL of 10% sulfuric acid. Substrate and products were extracted in 2 × 3 mL of diethyl ether, which was

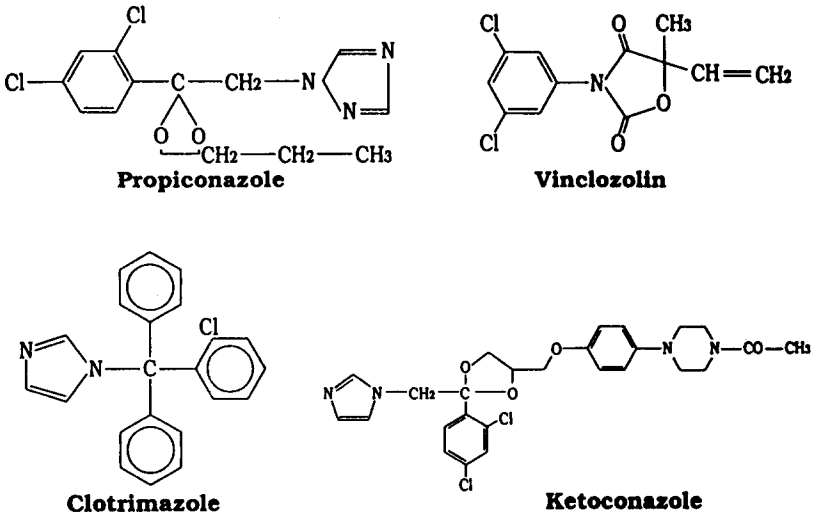

Fig. 1. Structures of the EBIFs used in the present study.

taken to dryness with N_2 (g). Samples were redissolved in 100 μL acetone and spotted on LHP-KDF HPTLC plates. The plates were run twice in the same dimension in a solvent system of 63:35:2 (by vol.) hexane: diethyl ether: acetic acid to produce a good separation of 11- and 12-hydroxy lauric acids and visualized by autoradiography for 48 hr using X-OMAT X-ray film (Kodak). Reaction rates were calculated by densitometric scanning of the autoradiographs.

Western blot analysis. Western analysis was conducted by SDS–PAGE on a Bio-Rad mini-protean II gel system followed by electrophoretic transfer to nitrocellulose [27]. The microsomal protein concentrations used in these experiments were 10 and 20 μg/well for samples isolated from rats and quail, respectively. Blots were incubated overnight at room temperature in 50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.05% Tween 20 (TBS) + 5% milk powder to block non-specific binding. After washing twice with TBS, primary antibodies were incubated at dilutions of 1/1000 except for antibodies to P450EtOH$_2$, which were incubated at 1/100 dilution in TBS + 5% powdered milk overnight at room temperature. Two further washes in TBS were followed by incubation with 1/1000 dilution of [^{125}I]goat anti-rabbit IgG in TBS + 5% powdered milk at room temperature overnight for all antibodies except anti-CYP 4A1. For this antibody, a further step of incubation with 1/1000 rabbit-anti sheep IgG in TBS + 5% powdered milk was required prior to incubation with the [^{125}I]goat anti-rabbit IgG. Following incubation with the radiolabel, the filters were washed overnight at room temperature in 200 mM Tris–HCl, pH 7.5, 800 mM NaCl, 0.2% Tween 20 and subjected to autoradiography using X-OMAT X-ray film (Kodak). Immunoquantitation was achieved by densitometric scanning of the autoradiographs.

Statistics. All data are presented as means ± SEM for N = 5 animals (rat) or N = 6 animals (quail). Statistical significance was calculated using Student's *t*-test with $P < 0.05$ considered significant.

RESULTS

Monooxygenase components. The effects of fungicide treatment on the concentrations of monooxygenase components in hepatic microsomes from rats and quail are summarized in Fig. 2. In the rat, propiconazole, vinclozolin and clotrimazole were equally effective in raising total P450 content 3- to 4-fold ($P < 0.005$). Propiconazole and vinclozolin treatment also significantly elevated the contents of cytochrome b_5 and P450 reductase 2- to 4-fold ($P < 0.05$). In the quail, vinclozolin was the most powerful inducer, increasing P450 content 10-fold ($P < 0.005$). In direct contrast to the rat, clotrimazole was the least effective inducer of quail P450 content, producing a modest 2-fold increase ($P < 0.05$). All the fungicides significantly raised cytochrome b_5 content of quail microsomes ($P < 0.005$), and all except propiconazole significantly increased quail P450 reductase activities ($P < 0.05$).

Spectral interactions. The results of *in vitro* spectral binding studies are shown in Table 1. All the fungicides produced binding spectra in both rat and quail microsomes. The spectra were all Type II in nature with λ_{min} at 385–420 nm and λ_{max} at 415–446 nm [28]. Microsomes from animals treated with fungicides *in vivo* were also compared with control microsomes at the same P450 concentrations to detect the presence of any residual P450/fungicide complex. Such a complex appeared to be present in microsomes from rats treated with clotrimazole, but was not detectable with other treatments.

Alkoxyresorufin metabolism. The effects of fungicide treatment on O-dealkylation of a series of alkoxyresorufins are summarized in Fig. 3. In both rat and quail, propiconazole and vinclozolin were the most effective inducers of this type of monooxygenase activity. In the rat, CYP 1A1/2-

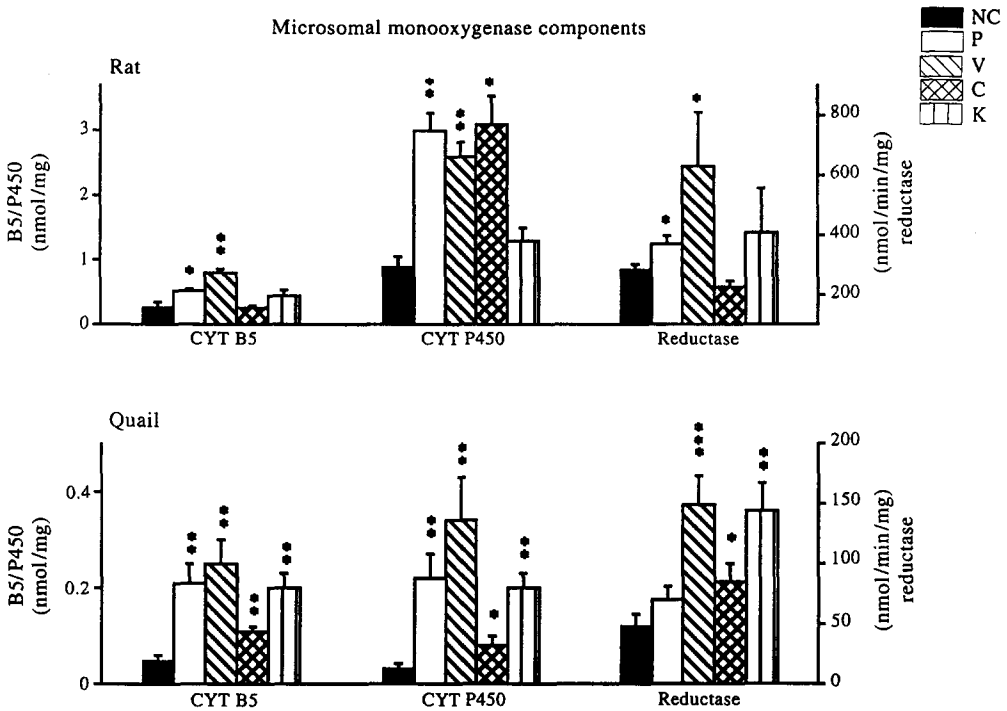

Fig. 2. Concentrations of microsomal monooxygenase components in untreated male rat and quail microsomes and following EBIF treatment. Cytochrome P450 and cytochrome b_5 concentrations were determined spectrally [20]; cytochrome P450 reductase was measured as nmol of cytochrome *c* reduced/min/mg by microsomes in the presence of NADPH [21] at 37° (rat) and 42° (quail). Abbreviations: NC, control; P, propiconazole-treated; V, vinclozolin-treated; C, clotrimazole-treated; and K, ketoconazole-treated. Data are expressed as means ± SEM for rat (N = 5) and quail (N = 6). Key: (*) significant at $P < 0.05$, (**) significant at $P < 0.005$, and (***) significant at $P < 0.0005$.

Table 1. Spectral interactions and inhibition

Treatment	Spectrum	λ_{min} (nm)	λ_{max} (nm)	*In vitro* complex	MROD inhibition IC_{50} (μM)
Rat					
Propiconazole	Type II	397	426	No	55
Vinclozolin	Type II	386	416	No	39
Clotrimazole	Type II	410	430	Yes*	11
Ketoconazole	Type II	398	426	No	10
Quail					
Propiconazole	Type II	400	433	No	8
Vinclozolin	Type II	420	446	No	0.9
Clotrimazole	Type II	410	430	No	1
Ketoconazole	Type II	400	422	No	5

* Difference spectrum of hepatic microsomes from control and clotrimazole-treated rats showed a peak at 432 nm when compared at equivalent concentrations of cytochrome P450.

dependent EROD and CYP 2B1/2-dependent BROD were affected the most, being induced 60- and 150-fold, respectively, by propiconazole and 12- and 300-fold, respectively, by vinclozolin. Propiconazole and vinclozolin also significantly increased CYP 1A2-dependent MROD and CYP2B1/2-dependent PROD 10- to 20-fold and 30- to 40-fold, respectively ($P < 0.005$). In the quail, the degree of induction by these fungicides was considerably lower than in the rat. MROD was affected the most, activity being raised 5- to 6-fold by propiconazole and vinclozolin ($P < 0.005$). EROD was increased 4-fold and PROD and BROD 3- to 4-fold by the same two fungicides ($P < 0.005$). Clotrimazole and ketoconazole had no significant effect on these activities in the quail. *In vitro*,

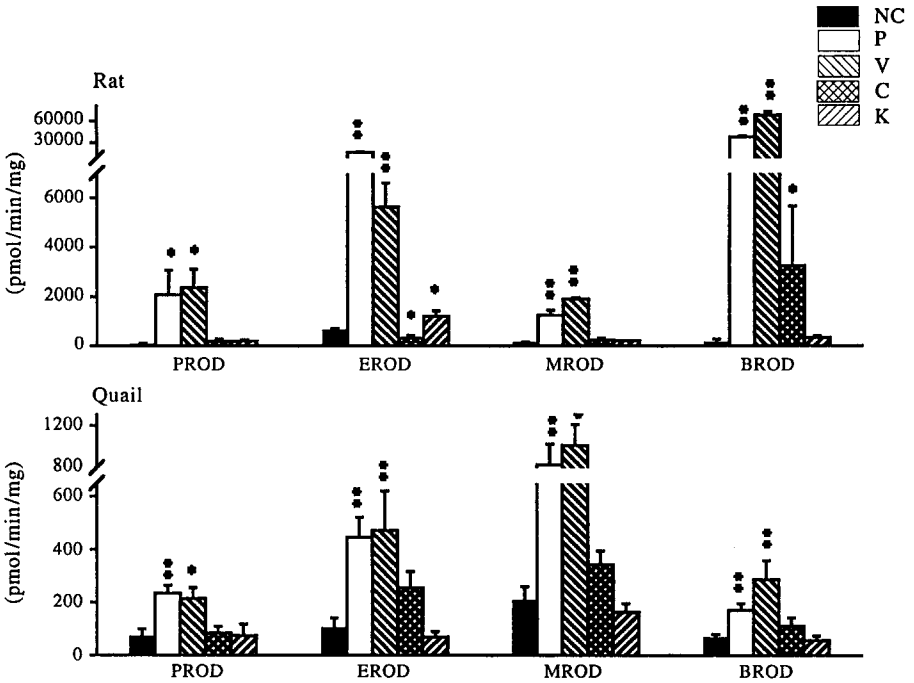

Fig. 3. Metabolism of a series of alkoxyresorufins by untreated male rat and quail microsomes or following EBIF induction. Abbreviations: PROD, pentoxyresorufin *O*-dependentylase; EROD, ethoxyresorufin *O*-deethylase; MROD, methoxyresorufin *O*-demethylase; BROD, benzyloxyresorufin *O*-debenzylase; NC, control; P, propiconazole-treated; V, vinclozolin-treated; C, clotrimazole-treated; and K, ketoconazole-treated. Data are expressed as means ± SEM for rat (N = 5) and quail (N = 6). Key: (*)significant at $P < 0.05$, and (**) significant at $P < 0.005$.

all the fungicides were powerful inhibitors of alkoxyresorufin metabolism. The IC_{50} values for MROD inhibition are shown in Table 1. In general, the EBIFs were about an order of magnitude more effective as inhibitors of quail MROD than rat MROD. No correlation was observed between potency of *in vitro* MROD inhibition and potency of *in vivo* MROD induction.

Testosterone metabolism. In untreated male rat liver, the most abundant hydroxylated testosterone products were 16α- and 2α- produced by CYP 2C11 (Fig. 4). Propiconazole and vinclozolin treatment resulted in increased formation of CYP 3A-dependent 6β-hydroxytestosterone and of two minor hydroxylated products, 16β- and 2β-hydroxytestosterone. This was accompanied by substantial decreases in the formation of 2α-and 16α-hydroxytestosterone. Clotrimazole also induced 16β- and 2β-hydroxytestosterone formation and suppressed formation of 16α- and 2α-hydroxytestosterone; however, significant inhibition of androstenedione, 6β-hydroxytestosterone formation, and CYP 2A1-dependent 7α-hydroxylation also occurred. Ketoconazole treatment had no effect on hepatic testosterone metabolism in the rat. In control quail microsomes (Fig. 5), the major products of testosterone metabolism were androstenedione, 2β- and 6β-hydroxytestosterone. Two unidentified products not found in rat incubations were also formed; one (U1) with an R_f value on HPTLC between testosterone and 2β-hydroxytestosterone (possible 4-androstene-3α,17β-diol) and one (U2) with an R_f value between that of 6β- and 7α-hydroxytestosterone (possibly 6α-hydroxytestosterone). There appears to be no 5α-reductase activity present. Treatment with propiconazole and vinclozolin increased formation of androstenedione, 2β- and 6β-hydroxytestosterone ($P < 0.05$). They also significantly increased the formation of U2 and 16α-hydroxytestosterone ($P < 0.005$). Apart from a small increase in 2β-hydroxytestosterone formation with clotrimazole, clotrimazole and ketoconazole treatment had no significant effects on testosterone metabolism in quail microsomes.

Carbon tetrachloride-dependent lipid peroxidation. In rat hepatic microsomes, reductive dechlorination of carbon tetrachloride is specific for cytochrome P450 CYP 2E1. Fungicide treatment had no effects on this activity in the rat other than a significant suppression in microsomes from ketoconazole-treated animals ($P < 0.005$) (Table 2). By contrast, in quail microsomes the basal activity was 10% of that seen in the rat, and 3- to 4-fold induction was observed following treatment with propiconazole ($P < 0.005$) and vinclozolin.

Erythromycin N-demethylase. CYP 3A-dependent erythromycin *N*-demethylase activity was induced 5- to 6-fold by propiconazole ($P < 0.005$) and clotrimazole in the rat (Table 2). In the quail,

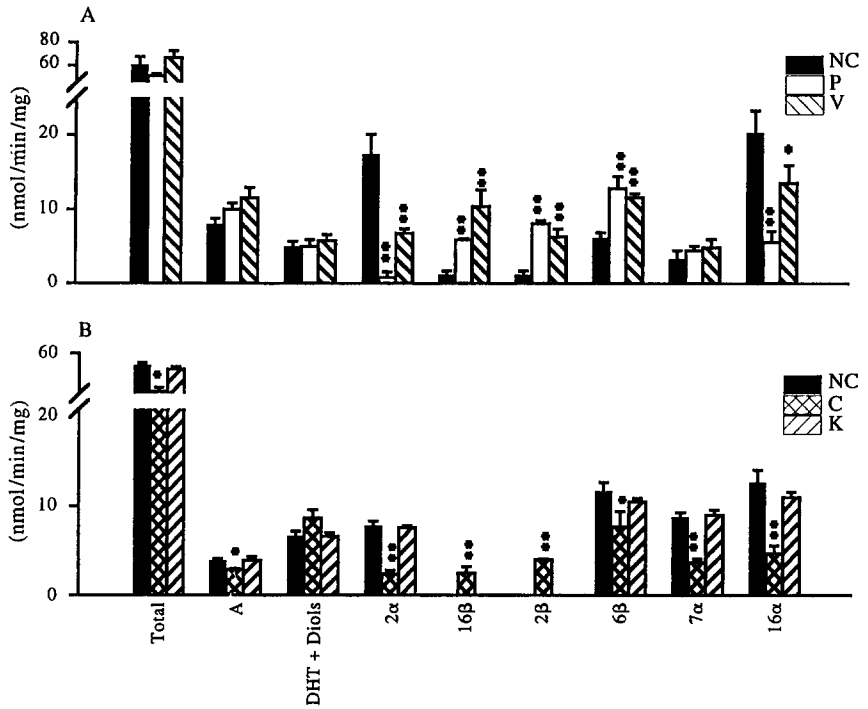

Fig. 4. Metabolism of testosterone by male rat microsomes following EBIF induction. (4A) NC, control; P, propiconazole-treated; and V, vinclozolin-treated. (4B) NC, control; C, clotrimazole-treated; and K, ketoconazole-treated. Abbreviations: A, androstenedione; DHT, dihydrotestosterone; Diols ($3\alpha(\beta)$ 5α-androstanediols); 2α, 2α-hydroxytestosterone; 16β, 16β-hydroxytestosterone; 2β, 2β-hydroxytestosterone; 6β, 6β-hydroxytestosterone; 7α, 7α-hydroxytestosterone; and 16α, 16α-hydroxytestosterone. Data are expressed as means ± SEMS of duplicate assays for N = 5 animals/group. Key: (*) significant at $P < 0.05$, (**) significant at $P < 0.005$.

significant ($P < 0.05$) but much smaller increases were observed following treatment with all the fungicides except clotrimazole.

Metabolism of lauric acid. Lauric acid was metabolized to three products in untreated rat microsomes. In the current HPTLC system, lauric acid itself had an R_f value of 0.69. The two major products, 12-hydroxy (ω hydroxy) lauric acid (catalyzed by CYP 4A isozymes) and 11-hydroxy (ω-1 hydroxy) lauric acid, had R_f values of 0.12 and 0.15, respectively. A third unidentified minor product (X) had an R_f value of 0.2. Untreated quail microsomes catalyzed lauric acid ω hydroxylation only. EBIF treatment had no effect on ω hydroxylation in the rat (Table 2). However, vinclozolin significantly increased ω-1 hydroxylation ($P < 0.05$), and all four fungicides elevated formation of X. In contrast, in the quail treatment with all the fungicides except ketoconazole induced the formation of all three products ($P < 0.05$). Vinclozolin was the best inducer of ω hydroxylation (4-fold).

Western blot analysis. Results of western blot analysis are shown in Fig. 6 for the rat and in Fig. 7 for the quail. Data produced by densitometric scanning of the western blots are presented in Table 3, although in the absence of purified rat and quail P450 isozymes to quantitate against, these data can only be considered semi-quantitative. In the rat, the greatest induction response was observed with a polyclonal antibody raised against rat CYP 3A2. Multiple CYP 3A forms are present in rat with antibody cross-reactivity and identical molecular weights; thus, the cross-reactive species are identified collectively as CYP 3A. Clotrimazole induced CYP 3A over 450-fold. A similar, greater than 200-fold induction of CYP 3A was observed in microsomes from propiconazole-treated animals ($P < 0.005$). Although vinclozolin and ketoconazole treatment also significantly increased CYP 3A expression ($P < 0.05$), they were less potent by two orders of magnitude. In contrast, propiconazole, vinclozolin and clotrimazole were equally good inducers of an apoprotein band recognized by an antibody raised against rat CYP 2B1 (30-fold) and two apoproteins recognized by an antibody against rat CYP 1A1 (8- to 12-fold) ($P < 0.005$). Ketoconazole treatment had no significant effect on expression of CYP 2B1 cross-reactivity, but produced some elevation of CYP 1A1 cross-reactive apoproteins. The two inducible apoprotein bands recognized with the anti-CYP 1A1 antibody are CYP 1A1 (upper band) and CYP 1A2 (lower band). These identities have been confirmed by using the antibody to blot microsomes prepared from rats treated with the CYP 1A-inducers 3-methylcholanthrene, isosafrole and Arochlor 1254

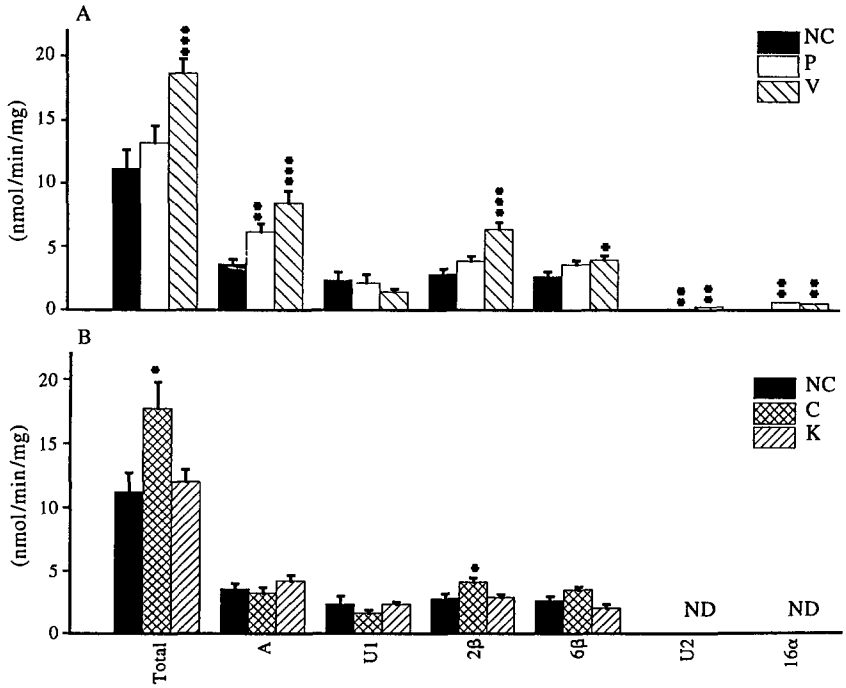

Fig. 5. Metabolism of testosterone by male bobwhite quail microsomes following EBIF induction. (5A) NC, control; P, propiconazole-treated; and V, vinclozolin-treated. (5B) NC, control; C, clotrimazole-treated, and K, ketoconazole-treated. Abbreviations: A, androstenedione; 2β, 2β-hydroxytestosterone; 6β, 6β-hydroxytestosterone; 16α, 16α-hydroxytestosterone; and U1 and U2, unknown metabolites. Data are expressed as means ± SEM of duplicate assays for N = 6 animals/group. Key: (*) significant at $P < 0.05$, (**) significant at $P < 0.005$, and (***) significant at $P < 0.0005$. ND = not detectable.

Table 2. Induction of monooxygenase activities by EBIF treatment in male rat and bobwhite quail

Treatment	CCl_4-Dependent lipid peroxidation	Erythromycin *N*-demethylase	Lauric acid		
			ω-OH	(ω-1)-OH	*X*
Rat					
Control	0.16 ± 0.05	1.5 ± 0.16	2.6 ± 0.4	1.2 ± 0.2	0.2 ± 0.02
Propiconazole	0.18 ± 0.03	5.7 ± 0.35*	3.7 ± 0.4	1.8 ± 0.3	1.5 ± 0.4†
Vinclozolin	0.13 ± 0.05	2.1 ± 0.52	3.8 ± 0.5	2.3 ± 0.2†	0.8 ± 0.2†
Clotrimazole	0.09 ± 0.05	5.1 ± 1.40	2.2 ± 0.3	1.0 ± 0.3	0.8 ± 0.2†
Ketoconazole	0.03 ± 0.02*	1.6 ± 0.20	2.0 ± 0.3	1.2 ± 0.3	0.5 ± 0.1†
Quail					
Control	0.015 ± 0.005	1.3 ± 0.10	1.4 ± 0.1	<0.1	<0.1
Propiconazole	0.035 ± 0.005*	1.8 ± 0.07*	3.9 ± 0.7†	1.0 ± 0.2†	0.4 ± 0.1†
Vinclozolin	0.048 ± 0.014	1.9 ± 0.08*	4.1 ± 0.6*	1.4 ± 0.2†	0.6 ± 0.1†
Clotrimazole	0.014 ± 0.005	1.2 ± 0.1	2.1 ± 0.3	0.3 ± 0.1†	0.2 ± 0.1†
Ketoconazole	0.010 + 0.004	1.7 + 0.1†	1.3 + 0.2	<0.1	<0.1

Data are presented as means ± SEM (nmol/mg/min) for N = 5 (rat), N = 6 (quail).
*,† Significantly at: different from control *$P < 0.005$, and †$P < 0.05$.

(unpublished). Vinclozolin and ketoconazole had no significant effects on expression of the major rat ethanol-inducible P450 CYP 2E1, whereas propiconazole and clotrimazole produced an approximate 50% decrease in cross-reactivity. A second ethanol-inducible form, $P450EtOH_2$, suggested to be in gene family 2C [14], was induced 4- and 8-fold by propiconazole and vinclozolin, respectively ($P < 0.005$). An apoprotein band cross-reactive with mouse monoclonal antibodies monospecific for rat CYP 2C11 was suppressed by propiconazole, vinclozolin and clotrimazole treatment by as much

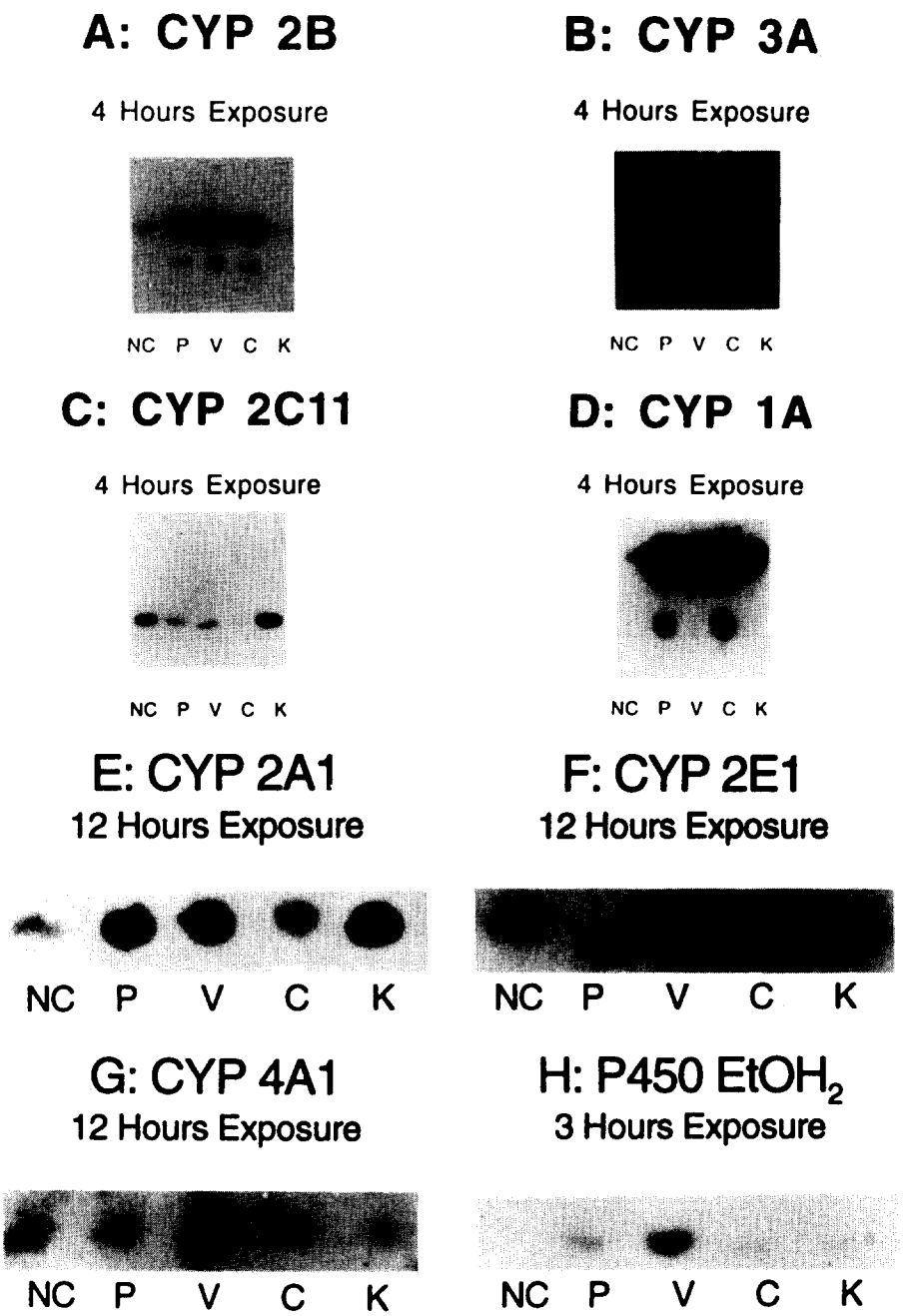

Fig. 6. Western blot analysis of hepatic microsomes from untreated or EBIF-induced male rats using antibodies raised against rat P450 isozymes CYP 2B1, CYP 3A2, CYP 2C11, CYP 1A1, CYP 2A1, CYP 2E1, P450EtOH$_2$ and CYP 4A1. Data are presented as 3- to 12-hr autoradiographs after labeling with [^{125}I]-goat anti-rabbit IgG. Key: NC, control; P, propiconazole-treated; V, vinclozolin-treated; C, clotrimazole-treated; and K, ketoconazole-treated. Samples were loaded at 10 μg microsomal protein/well. Representative blots from N = 5 animals/group are shown.

as 60–70%, whereas ketoconazole was without significant effect. A small increase was observed in CYP 2A1 expression with all the fungicides except clotrimazole ($P < 0.05$), and a 50% suppression of CYP 4A1 cross-reactivity was observed only with ketoconazole.

In the quail, the largest effects on P450 apoprotein expression were observed in propiconazole- and vinclozolin-treated birds. These compounds produced a 4- to 6-fold increase in the two members of P450 gene family 1 recognized in quail microsomes by polyclonal antibodies to rat CYP 1A1 and caused

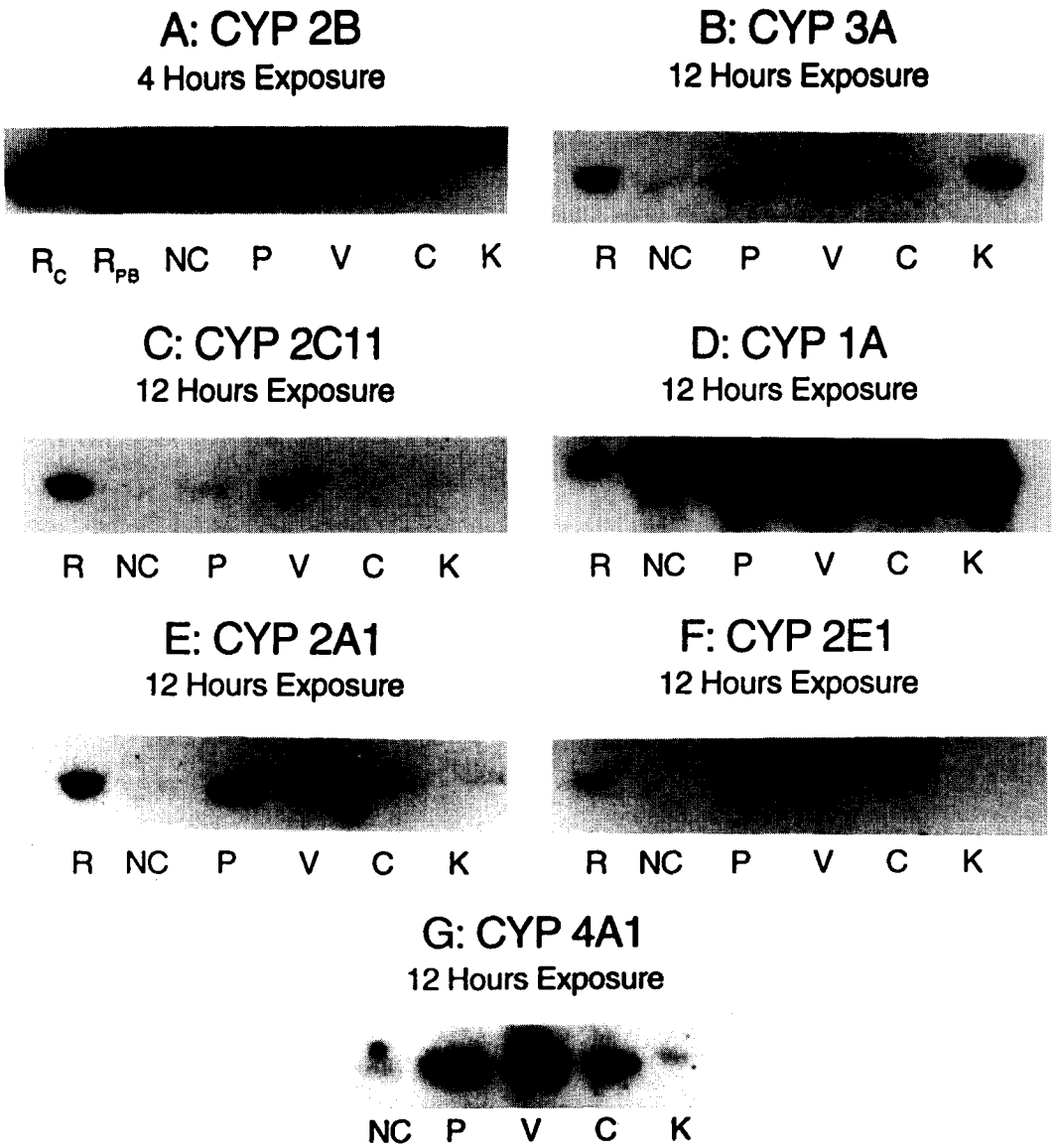

Fig. 7. Western blot analysis of hepatic microsomes from untreated or EBIF-induced male quail using antibodies raised against rat P450 isozymes CYP 2B1, CYP 3A2, CYP 2C11, CYP 1A1, CYP 2A1, CYP 2E1 and CYP 4A1. Data are presented as 4- to 12-hr autoradiographs after labeling with [^{125}I]-goat anti-rabbit IgG. Key: R (R_c), untreated rat liver microsomes, 50 μg/well; R_{pB} rat liver microsomes prepared from phenobarbital-induced animals [5], 5 μg/well; NC, control; P, propiconazole-treated; V, vinclozolin-treated; C, clotrimazole-treated; and K, ketoconazole-treated. Samples were loaded at 20 μg protein/well. Representative blots from N = 6 animals/group are shown.

the appearance of apoprotein(s) recognized by rabbit polyclonal antibodies directed against rat CYP 2C11 and CYP 2A1. Interestingly, no bands were recognized in quail microsomes from any treatment using the mouse monoclonal antibody monospecific for rat CYP 2C11. A small increase was observed in CYP 3A cross-reactive apoprotein (3- to 4-fold) and a 3-fold increase was observed with a CYP 4A1 cross-reactive apoprotein. Ketoconazole induced quail CYP 1A apoproteins 3-fold ($P < 0.05$) and was the best inducer of the CYP 3A cross-reactive protein. In contrast, clotrimazole, the best rat inducer, had little effect in quail other than producing a 3-fold induction of rat CYP 4A1 cross-reactive apoprotein ($P < 0.05$).

DISCUSSION

The above results confirm previous studies such as those of Hostetler *et al.* [12], which showed that in the rat EBIFs represent an important class of agents capable of simultaneously inducing a number

Table 3. Immunoquantitation of P450 induction in rat and quail hepatic microsomes by EBIFs

	Treatment				
	Control	Propiconazole	Vinclozolin	Clotrimazole	Ketoconazole
Rat					
CYP 1A1/2	1.0 ± 0.04	9.5 ± 2.0*	7.8 ± 2.9*	11.7 ± 3.0*	6.4 ± 4.1
CYP 2A1	1.0 ± 0.1	1.5 ± 0.1†	1.6 ± 0.1†	0.8 ± 0.1	1.5 ± 0.02†
CYP 2B1/2	1.0 ± 0.3	26.5 ± 6.2*	32.6 ± 7.1*	30.4 ± 3.5*	3.5 ± 1.0
CYP 2C11	1.0 ± 0.3	0.3 ± 0.03†	0.4 ± 0.2	0.3 ± 0.2†	1.5 ± 0.3
$P450EtOH_2$	1.0 ± 0.2	3.6 ± 0.1*	8.5 ± 0.1*	1.2 ± 0.4	1.1 ± 0.1
CYP 2E1	1.0 ± 0.2	0.4 ± 0.1†	0.7 ± 0.1	0.4 ± 0.1†	0.9 ± 0.2
CYP 3A	1.0 ± 0.6	236 ± 22*	8.2 ± 2.5†	465 ± 125*	4.3 ± 0.5†
CYP 4A1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.3	1.0 ± 0.4	0.5 ± 0.2
Quail‡					
CYP 1A1/2	1.0 ± 0.2	5.8 ± 2.0†	4.5 ± 1.3†	1.7 ± 0.4	2.8 ± 0.5†
CYP 2A1	ND	+++	+++	ND	ND
CYP 2B1/2	1.0 ± 0.2	0.3 ± 0.03†	0.3 ± 0.03†	0.2 ± 0.03†	0.4 ± 0.04
CYP 2C11	ND	+	++	ND	ND
$P450EtOH_2$	ND	ND	ND	ND	ND
CYP 2E1	ND	ND	ND	ND	ND
CYP 3A	1.0 ± 0.2	3.5 ± 1.0†	3.7 ± 1.1†	1.7 ± 0.2	4.2 ± 0.3*
CYP 4A1	1.0 ± 0.3	3.4 ± 1.6†	2.9 ± 1.1†	2.9 ± 1.2†	1.6 ± 0.1

* Values (arbitrary absorbance density units) are based on densitometric scanning of autoradiographs, relative to control = 1.0, and are expressed as means ± SEM for N = 5 (rat), N = 6 (quail).

*,† Significantly different from control at: *$P < 0.005$, and †$P < 0.05$.

‡ Cross-reactivity with antibodies to rat hepatic isozymes. ND: not detectable. The single (+), double (++) and triple (+++) crosses indicate little, moderate or appreciable cross-reactivity, respectively, of a P450 isozyme not present in control microsomes.

of different subfamilies of hepatic P450s. To our knowledge, this is the first report that similar induction of multiple P450 families occurs in avian species and the first characterization of EBIF induction of avian cytochrome P450 isozymes at the molecular level. In addition, this is the first report that this capacity to act as a mixed inducer does not appear to be restricted to N-substituted imidazoles such as propiconazole, clotrimazole and ketoconazole, but is also shared by *N*-(3,5-dichlorophenyl) dicarboximide fungicides such as vinclozolin.

Spectral interactions. All the fungicides displayed Type II binding spectra with rat and quail microsomes *in vitro*, characteristic of the interaction of nitrogenous ligands with the heme of cytochrome P450 [28]. There was spectral evidence indicating that microsomes prepared from rats 48 hr following clotrimazole treatment contained large quantities of fungicide metabolite–P450 complex, whereas with all other sets of treated microsomes it appeared that the majority of fungicide had been cleared prior to the animals being killed.

Induction and suppression of rat hepatic cytochrome P450 isozymes. The effects of EBIFs on a wide variety of P450 isozymes in the rat and bobwhite quail have been surveyed through the use of isozyme selective substrates. Metabolism of the alkoxyresorufins provide data on expression of CYP 1A and 2B isozymes; carbon tetrachloride is selective for CYP 2E1 in the rat, erythromycin is selective for CYP 3A isozymes, while lauric acid ω is selective for CYP 4A1. In this respect, testosterone is an excellent indicator substrate for analysis of cytochrome P450 expression, since it undergoes stereoselective hydroxylations catalyzed by a wide variety of different P450 isozymes and reduction via the steroid 5α-reductase/3$\alpha(\beta)$-hydroxysteroid dehydrogenase pathway. All EBIFs, except ketoconazole, were potent P450 inducers in the rat. Isozymes in three major gene families, 1, 2 and 3, and their associated monooxygenase activities were affected. As reported previously [12], clotrimazole was found to be the most potent inducer of CYP 3A apoprotein yet described with an increase of over 450-fold. A much smaller increase in CYP 3A-dependent monooxygenase activities (erythromycin *N*-demethylase, and testosterone 6β- and 2β-hydroxylase) was observed than might be expected based on the increase in CYP 3A apoprotein. The presence of a fungicide metabolite–P450 complex spectrally in microsomes from clotrimazole-treated animals suggests that a large part of the induced P450 is unavailable for catalysis. A biphasic inhibition followed by induction of monooxygenase activities has been described previously following clotrimazole treatment *in vivo* [29]. This phenomenon may also underlie the induction of CYP 1A1/2 and CYP 2B1/2 apoproteins by clotrimazole treatment unaccompanied by significant effects on alkoxyresorufin O-dealkylation activities. A much better correlation between apoprotein and monooxygenase activity induction was observed following propiconazole or vinclozolin treatment where no spectrally detectable fungicide/P450 complexes were present. Induction of CYP 1A1/2, CYP 2B1/2 and CYP 3A isozymes was accompanied by substantial suppression of expression of the major male specific constitutive cytochrome P450 CYP 2C11. In this case, reductions

in apoprotein expression were paralleled by decreases in the male specific 2α- and 16α-hydroxylation of testosterone. Effects of the fungicides on other constitutive rat P450 isozymes were mixed. Only small effects were observed on expression of CYP 2A1, CYP 2E1 or CYP 4A1 apoproteins with any EBIF. However, a new ethanol-inducible P450 from gene family 2C ($P450EtOH_2$), which appears to be related to CYP 2C7 [14], was selectively induced by vinclozolin up to 8-fold.

Induction of quail hepatic cytochrome P450 isozymes. In the quail, propiconazole, vinclozolin and ketoconazole were good P450 inducers, whereas clotrimazole, the most potent rat inducer, produced few effects. Although western blot cross-reactivity studies across species lines require cautious interpretation, it is generally well accepted that proteins in the P450 gene family 1 are structurally highly conserved in lower vertebrates and display cross-reactivity with antibodies raised against rat CYP 1A1 [9, 30–32]. Two P450 isozymes were recognized in western blots of quail microsomes by polyclonal antibodies directed against rat CYP 1A1 and were induced most effectively by propiconazole and vinclozolin (5- to 6-fold). This was accompanied by the induction of alkoxyresorufin metabolism in the following order of potency: MROD > EROD > BROD > PROD. EROD induction by EBIFs in the bobwhite quail is consistent with reports in Japanese quail and other avian species [9]. Although alkoxyresorufin activities are highly selective for CYP 1A1 (EROD), 1A2 (MROD) and 2B1 (PROD/BROD) in the rat, similar specificity has not been demonstrated for purified P450 isozymes in the quail. Indeed, a protein in quail microsomes cross-reactive with antibodies to rat CYP 2B1 (presumably the quail orthologue of chick CYP 2H) [33, 34] was suppressed by EBIF treatment even though PROD and BROD activities increased. The extent to which quail 1A isozymes and quail family 2 enzymes, other than the CYP 2B1 cross-reactive form, catalyze alkoxyresorufin metabolism awaits their purification and use in reconstitution experiments. At least one protein recognized by antibodies raised against rat P450 gene family 2 proteins was induced in EBIF-treated quail microsomes. A protein band recognized by antibodies directed against rat CYP 2A1 and one recognized by rabbit polyclonal antibodies directed against rat CYP 2C11 (but not monoclonal antibodies against CYP 2C11) appeared in propiconazole- and vinclozolin-induced microsomes. The relationship of the induced CYP 2 isozyme(s) to purified CYP 2H and other avian family 2 forms [33, 34] awaits purification studies. This form(s) may be responsible for the increased carbon tetrachloride reduction seen in microsomes from propiconazole- and vinclozolin-treated quail, since no form cross-reactive with rat CYP 2E1 antibodies was detectable. It is also possible that the induction of aldrin epoxidase reported in avian species following EBIF treatment [9] is due to this form(s), but this activity was not measured in the current study. A single protein band cross-reactive with antibodies directed against rat CYP 3A2 was present in quail microsomes. In contrast to the rat, propiconazole, vinclozolin and ketoconazole induced this protein a modest 3-fold, whereas clotrimazole, the best rat CYP 3A inducer, was completely without effect. Induction of this apoprotein is probably associated with the significant increases observed in erythromycin *N*-demethylase and testosterone 2β- and 6β-hydroxylase seen in EBIF-treated quail microsomes. Also, in direct contrast to the rat, propiconazole, vinclozolin and clotrimazole induced a protein cross-reactive with antibodies to rat CYP 4A1 in quail microsomes 3-fold. This was accompanied by similar increases in lauric acid ω-hydroxylation. Thus, EBIFs were capable of inducing isozymes in all four inducible P450 gene families in the quail.

Induction mechanisms. It is clear from the data presented here that each EBIF appears to affect a unique spectrum of isozymes within a single mammalian species and has a completely different spectrum of effects in avian species. Even though these compounds were administered at very high doses, the specificity of responses argues against a generalized induction mechanism affecting overall P450 synthesis due to some transient overload of the drug-metabolizing system [35]. Although both propiconazole and vinclozolin were technical grade with some 91–95% purity and the effects of minor contaminants on the P450 system cannot be ruled out, the induction responses appear consistent with those seen with the pure EBIFs clotrimazole and ketoconazole. It is possible that the induction mechanism may involve the inhibition of production of endogenous intracellular regulators, as has been proposed for lovostatin, another inducer of isozymes from multiple P450 gene families [36].

Toxicological implications. Since EBIFs are in widespread clinical and agricultural usage, the complex patterns of inhibition and induction of hepatic cytochrome P450 isozymes produced by these compounds in mammals and birds may have potentially significant toxicological consequences. This is particularly true in the area of drug and chemical interactions, since CYP 3A and 2B forms are involved in the metabolism of many clinically utilized drugs and many patients are on multiple medications. Similarly, fungicide treatment of crops followed by spraying with insecticides such as malathion, which undergo cytochrome P450-dependent activation to toxic products, might produce toxic responses in inadvertently exposed wildlife species such as birds. This type of toxic interaction between fungicides and insecticides has been demonstrated in red-legged partridge with the fungicide prochloraz and malathion [37] and in our own laboratory in bobwhite quail with vinclozolin and malathion [38].

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