Caenorhabditis elegans generates biologically relevant levels of genotoxic metabolites from aflatoxin B_1 but not benzo[a]pyrene *in vivo*

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Running head: CYP1 enzymes and genotoxicity in *C. elegans*

Abstract

There is relatively little information regarding the critical xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in *Caenorhabditis elegans*, despite this organism's increasing use as a model in toxicology and pharmacology. We carried out experiments to elucidate the capacity of *C. elegans* to metabolically activate important promutagens via CYPs. Phylogenetic comparisons confirmed an earlier report indicating a lack of CYP1 family enzymes in *C. elegans*. Exposure to aflatoxin B_1 (AFB_1), which is metabolized in mammals by CYP1, CYP2, and CYP3 family enzymes, resulted in significant DNA damage in *C. elegans*. However, exposure to benzo[a]pyrene (BaP), which is metabolized in mammals by CYP1 family enzymes only, produced no detectable damage. To further test whether BaP exposure caused DNA damage, the toxicities of AFB_1 and BaP were compared in nucleotide excision repair-deficient (*xpa-1*) and -proficient (N2) strains of *C. elegans.* Exposure to AFB_1 inhibited growth more in *xpa-1* than N2 nematodes, but the growth-inhibitory effects of BaP were indistinguishable in the two strains. Finally, a CYP-NADPH reductase- deficient strain (*emb-8*) of *C. elegans* was found to be more resistant to the growth inhibitory effect of AFB_1 exposure than N2, confirming that the AFB_1-mediated growth inhibition resulted from CYP-mediated metabolism. Together, these results indicate that *C. elegans* lacks biologically significant CYP1 family-mediated enzymatic metabolism of xenobiotics. Interestingly, we also found that *xpa-1* nematodes were slightly more sensitive to chlorpyrifos than were wild-type. Our results highlight the importance of considering differences between xenobiotic metabolism in *C. elegans* and mammals when using this alternative model in pharmaceutical and toxicological research.

Keywords: *Caenorhabditis elegans,* cytochrome P450, aflatoxin B_1, benzo[a]pyrene, genotoxicity, nucleotide excision repair

Introduction

The nematode *Caenorhabditis elegans* is emerging as an important model in pharmacology and toxicology (Leung *et al.*, 2008; Peterson *et al.*, 2008). *C. elegans* is similar to higher eukaryotes in many molecular and cellular pathways (Kaletta and Hengartner, 2006) and offers unique advantages over conventional mammalian models, including the ease of maintenance, short life cycle, genetic manipulability, and high-throughput capability. *C. elegans*-based assays are increasingly used to evaluate potential toxicity of different stressors in humans (Boyd *et al.* 2010*;* Dengg and van Meel, 2004; Rajini *et al.*, 2008; Sprando *et al.*, 2009) and mechanisms of toxicity after chemical exposures (Cui *et al.*, 2007; Donohoe *et al.*, 2006; Valmas and Ebert, 2006).

A limitation associated with using *C. elegans* as a model in toxicology is incomplete understanding of its response to human mutagens. The DNA damage response appears to be generally similar in *C. elegans* and higher eukaryotes (Leung *et al.*, 2008; O'Neil and Rose, 2005; Stergiou and Hengartner, 2004), and some direct-acting DNA-damaging agents that have been commonly used in *C. elegans* produce comparable responses to those observed in mammals (Ahringer, 2006; Anderson, 1995; Greber *et al.*, 2003; Hartman *et al.*, 1995; Ishiguro *et al.*, 2001; Meyer *et al.*, 2007; Stewart *et al.*, 1991). However, activation-dependent mutagens (i.e. promutagens) have not been well studied in *C. elegans* and might produce different responses in *C. elegans* and mammalian models due to differences in xenobiotic metabolism (Lindblom and Dodd, 2006). In particular, Gotoh (1998) provided phylogenetic evidence that *C. elegans* lacked CYP1 family genes that are responsible for the activation of many promutagens.

Aflatoxin B_1 (AFB_1) and benzo[a]pyrene (BaP) are two commonly used model promutagens. AFB_1 is a naturally occurring mycotoxin found in foods such as corn, peanuts,

various other nuts, and cottonseed (Groopman *et al.*, 2005). It remains an important environmental carcinogen in many developing countries (Vineis and Xun, 2009). BaP is a model carcinogenic polycyclic aromatic hydrocarbon (PAH). PAHs are environmental carcinogens that occur at high and increasing levels in the environment and result from incomplete combustion of organic compounds including fossil fuels, wood, cigarette smoke, and burnt food (Van Metre and Mahler, 2005). AFB_1 and BaP share a similar general mechanism of mutagenesis, requiring metabolic activation by cytochrome P450 (CYP) enzymes to form epoxide metabolites. The electrophilic epoxides in turn bind to DNA molecules, resulting in bulky, DNA helix-distorting DNA lesions that are repaired by nucleotide excision repair (NER) in the nuclear genome. However, a key difference between AFB_1- and BaP-induced DNA damage in mammals is that while AFB_1 is activated in mammals by CYP1, CYP2, and CYP3 family enzymes, BaP is activated only by CYP1 family enzymes.

Our objective was to investigate the potential role of CYPs in the genotoxicity and metabolism of AFB_1 and BaP in *C. elegans*. We took three complementary approaches. First, we generated a phylogenetic tree of CYPs in *C. elegans* and other species. Second, we quantified DNA damage caused by exposure to AFB_1 and BaP using a quantitative PCR (QPCR)-based assay. Chlorpyrifos (CPF, an organophosphate pesticide) and β-naphthoflavone (BNF, a non-carcinogenic PAH) were also evaluated. Our third approach was to investigate the genotoxicity of AFB_1 and BaP exposure in *C. elegans* using genetic approaches. In the first genetic experiment, we assessed the metabolic activation of AFB_1 and BaP in *C. elegans in vivo* by comparing the relative susceptibility of DNA adduct repair-deficient (*xpa-1*) and -proficient (N2) strains to AFB_1 and BaP exposure. In the second genetic experiment, we evaluated the importance of the CYP system in AFB_1 activation by comparing the relative susceptibility of

CYP-NADPH reductase deficient (*emb-8*) and wild-type (N2) strains to AFB_1 exposure. The results suggested that: (1) *C. elegans* lacks CYP1 family enzymes; (2) AFB_1, but not BaP, produced a biologically significant level of DNA adducts; and (3) the CYP system played an important role in activating AFB_1 in *C. elegans*. This important difference between the xenobiotic metabolism of *C. elegans* and higher eukaryotes needs to be taken into account when using this alternative model in pharmaceutical and toxicological research.

Materials and Methods

Phylogenetic analysis. Gene models in publically available nematode genomes were searched using Hmmer (v2.3.2: Eddy, 1998). Amino acid sequences were aligned using Muscle (v3.6: Edgar, 2004) and automatically masked based on the alignment quality score assigned by Muscle. A maximum likelihood phylogenetic tree was constructed with RAxML using the WAG model of amino acid substitution and a gamma distribution of rate categories (Stamatakis, 2006). Previously unnamed nematode CYPs in *C. briggsae* were assigned names by the Cytochrome P450 Nomenclature Committee and are available at the Cytochrome P450 homepage (Nelson, 2009); CYPs in *M. incognita,* and *B. malayi* have not been formally named yet.

C. elegans *culture.* The wild-type N2 (Bristol), *emb-8* (CYP-NADPH reductase-deficient MJ69), and *glp-1* (germline-deficient JK1107) strains of *C. elegans* were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota). *xpa-1* (NER-deficient strain RB864) was previously outcrossed 3 times (Meyer et al., 2007). Populations of *C. elegans* were maintained on K agar plates seeded with OP50 bacteria (Lewis and Fleming, 1995) at 20° C unless otherwise stated. Semi-synchronized populations of nematodes were obtained by bleach-sodium hydroxide isolation of eggs (Lewis and Fleming, 1995). L1 growth-arrested (starved) larvae were obtained by hatching eggs in complete K-medium (Boyd *et al.*, 2009) overnight with

shaking (Lewis and Fleming, 1995). All transfers were made by washing nematodes off of agar plates and rinsing in K medium (Williams and Dusenbery, 1990) after centrifugation at 2000 *g* for 2 min.

Chemical exposures. AFB_1, BaP, CPF, and BNF (Sigma Chemical Co., St Louis, MO) were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions. Three hundred *glp-1* adults were dispensed into each well of a 12-well plate. Each well contained a mixture of 990 µL complete K-medium, 10 µL stock solution dissolved in DMSO, and OP50. 1% DMSO was found not to affect nematode growth or reproduction (data not shown). The exposure concentrations were selected based on preliminary lethality assays (data not shown) or solubility, such that the highest concentration was either that which first showed mortality, or the highest achievable based on solubility if lethality could not be reached. This was the case for AFB_1 and BaP, which had solubility limits of ~100 µM in complete K-medium with 1% DMSO. *C. elegans* showed normal behavior at all concentrations of AFB_1, BaP, and BNF and lower concentrations of CPF, but were paralyzed at 100 µM of CPF.

QPCR-based DNA damage assay. Nuclear DNA damage was evaluated using a QPCR-based method (Meyer *et al.*, 2007) as adapted for use in a small number of individual nematodes (Boyd et al., 2010; Hunter et al., 2010). This assay defines the control samples as undamaged and generates a lesion frequency in experimental samples based on a decrease in amplification efficiency relative to the control samples, and has previously been used to detect BaP-induced DNA damage (Jung *et al.*, 2009a; Jung *et al.*, 2009b). Two nuclear genome targets (*unc-2* and small nuclear, 9316 and 225 nt, respectively; Meyer *et al.*, 2007) were amplified. The amount of long PCR product provides a measurement of lesion frequency, while the amount of short PCR product provides normalization to DNA template amount. Lesion calculations were performed as

described previously (Ayala-Torres *et al.*, 2000; Meyer, 2010). Nematodes were sampled after 48 h exposures. These experiments were carried out using a temperature-sensitive mutant strain (*glp-1*) in which maintenance at 25° C blocks germline proliferation and therefore blocks cell division, since outside of the germ line, no cell divisions occur in adult *C. elegans* (Sulston, 1988). Since young adult *C. elegans* have a rapidly proliferating germ line, DNA damage caused by chemical exposure could be readily "diluted" by the new DNA produced by dividing germ cells, confounding measurements of DNA damage (Meyer *et al.*, 2007). Six adults were pooled for each biological replicate and four biological replicates were taken per treatment. A total of eight biological replicates per treatment were used in the analysis.

Growth assay. Two genetic experiments were carried out to investigate (1) the effects of AFB_1, BaP, CPF, and BNF on NER-deficient (*xpa-1*) and -proficient (N2) strains of *C. elegans*; and (2) the effect of AFB_1 on CYP-NADPH reductase-deficient (*emb-8*) and wild-type (N2) strains of *C. elegans*. The growth of *C. elegans* was assessed essentially as previously described (Smith *et al.*, 2009). In both experiments, growth inhibition was measured as an indicator of chemical-induced genotoxicity, since *xpa-1* larval growth is dramatically impaired by DNA damage that requires NER proteins for removal (Astin *et al.*, 2008).

In the first growth assay, L1 N2 and *xpa-1* nematodes were transferred to the sample cup of the COPAS Biosort (Union Biometrica Inc., Somerville, MA, USA) and diluted to approximately 1 nematode/μL. Fifty L1s were then added to each well of a 96-well plate, containing a total volume of 50 μL complete K-medium, OP50, and chemical stock solution. *C. elegans* cohorts were incubated for 48h at 20° C and then size measurements of individual nematodes were acquired with the COPAS Biosort ReFLEx as previously described (Boyd *et al.*, 2009).

The second growth assay was conducted using L1 N2 and *emb-8* nematodes. The nematodes were hatched overnight at 15° C and then transferred to unseeded 100 mm K agar plates containing solvent control (1 % *v:v* DMSO), 30 uM AFB_1, and 100 uM AFB_1 and incubated at 23° C for two days. The MJ69 strain carries a temperature-sensitive mutation in the *emb-8* gene such that the phenotype is essentially normal at 15° C but CYP-NADPH reductase activity is impaired at and above 23° C (Kulas *et al.*, 2008). The animals were then transferred to seeded K agar plates, incubated at 15° C for two days, and photographed using a Nikon Eclipse E600 camera (Tokyo, Japan). The length of the nematode was determined using Lucia 5 (Laboratory Imaging, Prague, Czech). Two separate experiments were conducted, and the results combined.

Statistical analysis. All data were analyzed with Statview© for Windows (Version 5.0.1, SAS Institute Inc., Cary, NC). DNA damage data were assessed using an initial 2- or 3-way analysis of variance (ANOVA on exposure level and time-point, as well as presence/absence of bacteria in the case of the AFB_1 exposure) with a Bonferroni correction for 5 multiple comparisons (4 chemicals plus presence/absence of bacteria for AFB_1). Post-hoc analysis was carried out using Fisher's Protected Least Significant Differences (FPLSD) test. Growth data were not normally distributed (as assessed by the Kolmogorov-Smirnov Normality Test) and so were analyzed using Mann-Whitney *U* or Kruskal Wallis tests followed by Bonferroni corrections for multiple comparisons. p-values < 0.05 (after Bonferroni corrections) were considered significant. Box plots indicate 10th, 25th, 50th, 75th, and 90th percentiles, plus outliers.

Results

Lack of gene sequence-based evidence for CYP1 family CYPs in C. elegans. Previous investigations have found no evidence for CYP1 family genes in non-chordates (Goldstone *et al.*, 2007). Our investigation of the CYP complements of the four nematode genomes reported here (*C. elegans, Caenorhabditis briggsae, Meloidogyne incognita,* and *Brugia malayi*) support the fact that CYP1s are not present in the nematode genomes. A phylogenetic tree of the CYP complements of the four nematodes demonstrates that CYP1 genes are not present, although a large number of CYP2-like (Clan 2) genes are present and expressed in *C. elegans* (Fig. 1). Many CYP2 genes in vertebrates are xenobiotic (drug) metabolizing genes, and at least one (CYP2S1) is inducible via the important xenobiotic-responsive transcription factor aryl hydrocarbon receptor (AHR; Saarikoski *et al.*, 2005).

AFB_1 exposure results in DNA damage. To empirically test the prediction of our phylogenetic analysis, we measured DNA damage after exposure to promutagens requiring (BaP) and not requiring (AFB_1) CYP1-like activity for activation, using a QPCR assay (Hunter *et al.*, 2010)**.** This assay detects any DNA lesions that significantly inhibit the progression of the DNA polymerase used in the PCR reaction. AFB_1 exposure resulted in concentration-dependent DNA damage (p = 0. 0007 for main effect of concentration, 2-factor ANOVA) in *C. elegans*. Damage was detectable after exposures of 30 and 100 μM AFB_1. BaP, BNF, and CPF exposure did not result in any detectable DNA damage (p = 0.615, 0.161, and 0.454, respectively, for the effect of concentration) (Fig. 2). The limit of detection of the QPCR assays is approximately 1 lesion per 10^5 bases (Hunter *et al.*, 2010).

In order to determine whether the OP50 strain of *E. coli* (i.e. the *C. elegans* food source) might be responsible for the production of carcinogenic AFB_1 metabolites in our experimental system, we repeated AFB_1 exposure without adding bacteria to the exposure medium (Fig. 2).

The exclusion of bacteria did not abrogate the induction of DNA damage ($p = 0.0005$ for main effect of concentration, 2-factor ANOVA on OP50-fed nematodes only), indicating that *C. elegans* was responsible for metabolizing AFB_1 to the activated form. In fact, exposure without bacteria actually resulted in a slightly greater level of DNA damage than exposure with bacteria ($p = 0.039$ for interaction of presence of bacteria and concentration, 3-factor ANOVA).

DNA repair-deficient nematodes are more sensitive than wild-type to the growth inhibitory effects of AFB_1 and CPF, but not BaP or BNF. It remained possible that BaP, BNF, or CPF caused DNA damage at a level not detected by QPCR but nonetheless biologically relevant. To test this possibility, we employed the *xpa-1* strain. The *xpa-1* strain carries a large deletion in the nematode homologue of the xeroderma pigmentosum group A gene, which is required for NER (Berneburg and Lehmann, 2001). Many structurally dissimilar environmental genotoxins, including PAHs such as BaP, mycotoxins such as AFB_1, and ultraviolet C radiation can produce helix-distorting DNA lesions that are removed by NER (Hanawalt, 2002; Sancar and Reardon, 2004). *xpa-1* nematodes are exquisitely sensitive to DNA damage that is repaired by the NER pathway (Astin *et al.*, 2008; Boyd *et al.*, 2010a; Hartman and Herman, 1982; Meyer *et al.*, 2007). In particular, larval growth of *xpa-1* nematodes is highly sensitive to such DNA damage (Astin *et al.*, 2008). Therefore, if any of these chemicals cause biologically significant helix-distorting DNA damage, *xpa-1* nematodes would show more growth inhibition than N2.

Exposure levels of AFB_1, BaP, BNF, and CPF that would lead to larval growth inhibition in the wild-type N2 strain were identified first. BNF caused the strongest growth inhibitory effects (Fig. 3 and Suppl. Fig. 2), causing a >40% size reduction as compared to controls at the concentration of 1 μM (based on comparison of median values). The length of nematodes as measured by time of flight (TOF) is shown in Figure 3; their optical density (extinction; EXT) is

shown in Supplemental Figure 2, and detailed statistical information is presented in Supplemental Table 1. Exposures to AFB_1, CPF, and BaP resulted in a similar growth inhibitory effect at the concentrations of 3, 3, and 10 μM, respectively.

As shown in Figure 3, exposure to AFB_1 and CPF resulted in a greater growth inhibition in *xpa-1* as compared to N2. BaP and BNF resulted in comparable responses in N2 and *xpa-1* ($p > 0.05$ for N2 vs. *xpa-1* at all concentrations for all three chemicals). Since larval growth inhibition is a very sensitive indicator of DNA damage in *xpa-1* nematodes, and sensitivity to DNA damage is the only phenotype documented in *xpa-1* nematodes (Boyd *et al.*, 2010a), these results suggest that AFB_1 and CPF but not BaP or BNF produced DNA damage (of the type repaired by NER) at a biologically significant level in *C. elegans*.

AFB_1-mediated larval growth inhibition is partially rescued in nematodes deficient in CYP-NADPH reductase activity. We hypothesized that AFB_1 activation to a genotoxic form was CYP-mediated based on the presence of CYP2 and CYP3 family homologues in *C. elegans*. To test this hypothesis directly, we compared the effect of AFB_1 toxicity in N2 and *emb-8* nematodes. *emb-8* nematodes carry a point mutation in the gene coding for CYP-NADPH reductase (Rappleye *et al.*, 2003) resulting in temperature-sensitive disruption of function. Since AFB_1 activation via CYP catalytic activity requires CYP-NADPH reductase, *emb-8* mutants are deficient in CYP activity at the non-permissive temperature (Kulas *et al.*, 2008). Exposure to AFB_1 resulted in less growth inhibition in the *emb-8* than the N2 strain (Fig. 4), confirming a role for CYP enzymes in AFB_1 toxicity. AFB_1 inhibited growth in both strains ($p < 0.0001$ and $p = 0.0006$ for N2 and *emb-8*, respectively, Kruskal Wallis test). However, while *emb-8* nematodes were somewhat smaller than N2 under control conditions (*emb-8* median ~86% of N2; p =

0.0002, Mann-Whitney U test), they were larger after exposure to 100 μM AFB_1 (*emb-8* median ~140% of N2; p = 0.0007). There was no difference in size at 30 μM AFB_1 (p = 0.1376).

Discussion

C. elegans *appears to lack CYP1 family enzymes and the corresponding ability to enzymatically activate the procarcinogen BaP*. Cytochrome P450s play critical roles in normal metabolism as well as in xenobiotic metabolism. Our phylogenetic analysis suggests that while *C. elegans* has a large number of CYPs (83), it lacks family 1 genes. Our molecular and genetic experiments indicated that BaP, an environmentally important and well-studied promutagenic PAH, is not activated to a DNA-reactive form at biologically significant rates in *C. elegans*, indicating that *C. elegans* lacks a CYP capable of this CYP1-like activity.

A previous study by Gotoh (1998) also failed to identify CYP1 family homologues in *C. elegans*. However, Chakrapani *et al*. (2008) suggested that *C. elegans* contains a CYP1A2 homolog, and found that this gene (*cyp-14A3*) was induced by both BaP and (to a lesser extent) BNF. In addition, Schäfer et al. (2009) showed that *cyp-14A3* and related genes were able to hydroxylate PCB-52. Finally, improved and much-expanded sequence data have become available for *C. elegans* and other nematode and non-nematode species. Therefore, we carried out additional phylogenetic analyses, but still failed to identify any CYP1 family genes in *C. elegans*. Nematodes have other Clan 2 genes, including the CYP2-like CYP14, CYP33, CYP34, and CYP35 families (Abad *et al.*, 2008; Gotoh, 1998). In particular *C. elegans* CYP35 genes are responsive to a variety of xenobiotic stressors (Menzel *et al.*, 2001; Menzel *et al.*, 2005; Reichert and Menzel, 2005), and a number of other CYPs have been shown via microarray to be induced by PCB52 (Menzel *et al.*, 2007), including members of families CYP13, CYP14, CYP25, CYP29, CYP33, CYP34, and CYP37.

The promutagen AFB_1 causes DNA damage detectable by QPCR analysis in C. elegans*, but BaP does not.* AFB_1 and BaP are both promutagens that require metabolic activation before reacting with DNA. AFB_1 and BaP are similar in size and structure, both requiring addition of an epoxy group to become DNA-reactive (Suppl. Fig. 1). The electrophilic epoxy metabolites attack the nucleophilic centers of the DNA molecule, such as the ring nitrogen (i.e. N7) of guanine. The resultant large DNA adducts, often referred to as "bulky lesions," distort the DNA helix and can interfere with DNA transcription and replication. Some can also detach along with the adducted base from the DNA strand, resulting in abasic sites. While the metabolic activation of both AFB_1 and BaP in mammals requires CYP-mediated hydroxylation, different CYP family members are involved. The activation of AFB_1, for instance, can be carried out by mammalian CYP1A2, CYP2A6, CYP2B6, and CYP3A4 (Egner *et al.*, 2003; Mace *et al.*, 1997). In contrast, the activation of BaP (and other PAHs) in mammals is mainly catalyzed by CYP1 family enzymes, especially CYP1B1 and CYP1A1 (Shimada, 2006; Shimada and Fujii-Kuriyama, 2004).

Our results indicate that *C. elegans* can metabolize AFB_1 into DNA-binding metabolites, and that this activation is CYP-dependent. We have previously observed that *xpa-1* nematodes are more sensitive than N2 to AFB_1-induced growth inhibition (Meyer *et al.*, In press), and here extend that result with more extensive growth analysis, direct measurements of DNA damage, and genetic data indicating that the AFB_1 activation is CYP-mediated. In contrast, *C. elegans* cannot activate BaP, at least not sufficiently to lead to DNA damage detectable by the QPCR assay. While it is impossible to entirely rule out the possibility that some low amount of BaP-metabolizing capacity exists in *C. elegans*, the lack of a growth inhibitory effect in the *xpa-1* strain indicates that any such capacity that might exist is too small to be biologically relevant for *C. elegans*. A similar apparent lack of effect of BaP was previously observed by Miller and

Hartman (Miller and Hartman, 1998) working with the independently-isolated *rad-3* (allelic to *xpa-1*: Astin *et al.*, 2008) strain, as well as with additional radiation-sensitive strains of *C. elegans*.

Since BNF is not a carcinogenic PAH, it was not surprising that BNF exposure resulted in no detectable DNA damage or differential inhibition of growth in *xpa-1* nematodes. We did not detect statistically significant DNA damage after CPF exposure by QPCR analysis, but the *xpa-1* nematodes were somewhat more sensitive than wild-type to CPF-induced growth inhibition (although the difference was quantitatively less than for AFB_1). There is evidence that exposure to CPF may result in oxidative DNA damage under some circumstances (Crumpton *et al.*, 2000); our results support the likelihood that high concentrations of CPF (close to those that caused paralysis in our experiments) can cause DNA damage. It is unclear why *xpa-1* growth was more inhibited than N2 growth by CPF, despite a lack of detectable DNA damage as assessed by QPCR. We have previously shown that *xpa-1* nematodes have very few if any phenotypes in unstressed conditions, yet are highly sensitive to DNA damage (Boyd *et al.*, 2010a). It is conceivable, however, that there is a phenotype that can only be observed after exposure to a neurotoxin. Neurodegeneration is one of relatively few phenotypes observed in NER-deficient humans, and there is evidence that this may result at least in part from unusual types of oxidative DNA damage that are only repaired by NER (Brooks, 2008). Other potential explanations for the discrepancy would be if the growth assay is more sensitive than the QPCR assay, or if chlorpyrifos causes a type of DNA damage that the QPCR assay detects inefficiently (Meyer, 2010).

Comparative biology of CYP1 family activity and PAH metabolism in C. elegans. Some invertebrates do metabolize common vertebrate CYP1 family substrates such as BaP, although

typically relatively slowly compared to vertebrates (den Besten, 1998; Jorgensen *et al.*, 2005; Little *et al.*, 1985; Mcelroy, 1990); many others do not (James and Boyle, 1998; Lee, 1998; Rewitz *et al.*, 2006). While BNF and BaP were both shown to induce some CYPs in *C. elegans* (Menzel *et al.*, 2001), *C. elegans* would appear to be among the invertebrates that do not metabolize BaP. Another important difference between *C. elegans* (and many other invertebrates) and higher eukaryotes is that *C. elegans* homologs of the AHR do not bind to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or BNF (Butler *et al.*, 2001; Powell-Coffman *et al.*, 1998). Thus, the CYP induction and growth inhibition resulting from these two chemicals is presumably AHR-independent. The physiological significance of the AHR pathway in *C. elegans* is currently relatively poorly understood, although there is evidence that it plays a role in developmental neurobiology (Huang *et al.*, 2004; Qin and Powell-Coffman, 2004; Qin *et al.*, 2006). Similarly, the gene regulatory pathways controlling CYP expression in *C. elegans* will be an important area of future research both from the perspective of using *C. elegans* as a model organism, and to understand the evolution and function of the *C. elegans* response to environmental cues (Braendle *et al.*, 2008).

Toxicity of AFB_1, BaP, CPF, and BNF in C. elegans. BNF was the most potent growth inhibitor in our study, and BaP the least. That finding appears to contradict the observation of (Menzel *et al.*, 2001) in which the EC_{10} values of BaP and BNF in a reproductive assay were 1 and 18 μM, respectively. We carried out preliminary studies to test the effect of AFB_1, BaP, CPF, and BNF on reproduction using published methods (Boyd *et al.* 2010), and found a similar order of reproductive toxicity as for growth inhibition (BNF>AFB_1≈CPF>BaP, with *xpa-1* more sensitive than N2 only to AFB_1). Therefore, the difference between our rank order and that of Menzel *et al*.'s presumably results from differences in experimental procedures.

Although BaP exposure did not result in detectable DNA adducts in *C. elegans*, it did inhibit the growth of *C. elegans*. This likely occurred via a nongenotoxic mechanism since *xpa-1* nematodes were no more sensitive than wild-type. One possibility is that BaP caused narcosis (Di Toro *et al.*, 2000; Schultz, 1989), although we do not have data to indicate either how much BaP is taken up by *C. elegans*, or at what level BaP causes narcosis in this species. The presumably very slow metabolism of BaP in *C. elegans* increases the likelihood of this possibility. Another possibility is altered gene expression. Menzel et al. (2001), for instance, reported that BaP can induce CYP35 expression in *C. elegans* at 1 μM. While the functional consequences of CYP35 (and other gene) induction requires further investigation, it is possible that it may interfere with developmental processes in *C. elegans*; PAHs are potent developmental toxicants in some species, and not all act via AHR agonism (Billiard *et al.*, 2008).

Similarly, the mechanism of toxicity of BNF in *C. elegans* is unclear since it presumably does not act via AHR agonism, the best-described mode of action of this chemical. Like BaP, it may also act through altered gene transcription. It affects expression of CYPs and many other genes in *C. elegans* and other invertebrates (Reichert and Menzel, 2005; Watanabe et al., 2008).

Implications and conclusions. We identified an important difference in chemical mutagenesis between the model organism *C. elegans* and vertebrates, resulting from differences in CYP-mediated xenobiotic metabolism. While both AFB_1 and BaP are routinely used in mammalian models in cancer research, exposure to AFB_1 but not BaP resulted in detectable DNA damage through metabolic activation in *C. elegans*. Our results suggest that CYP1 family-like enzymatic activities in general are lacking in *C. elegans*. If so, this will result in altered pharmacokinetics and toxicokinetics for many important xenobiotics, causing either more or less toxicity as compared to most vertebrates due to decreased clearance and/or decreased metabolic

activation. This finding highlights the importance of considering xenobiotic metabolism in the interpretation of toxicological data from this alternative model.

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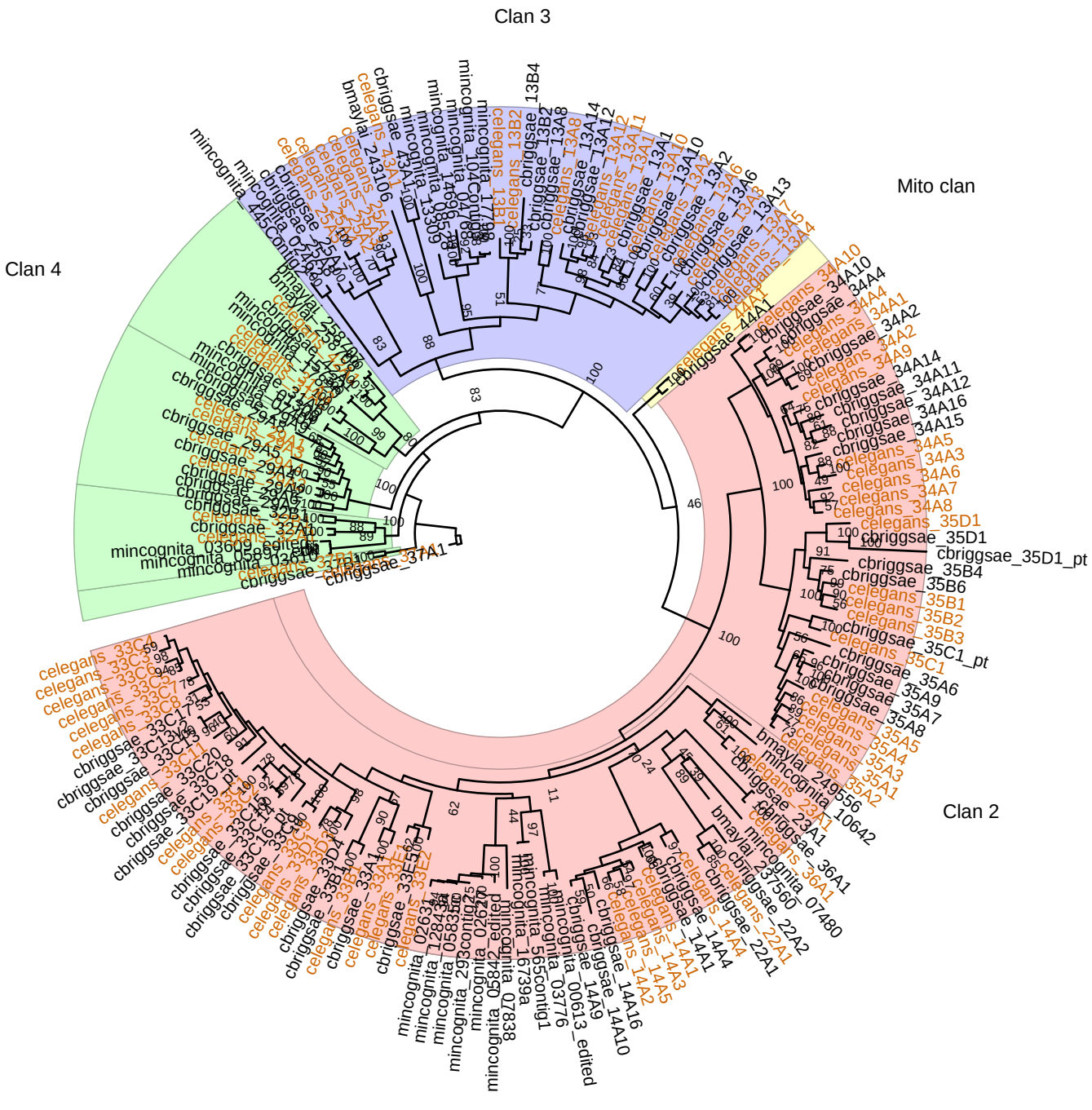
Figure Legends

Figure 1. Maximum likelihood phylogeny of CYPs from four nematode genomes, including the free-living *Caenorhabditis elegans* (yellow) and *C. briggsae* (black), and the parasitic *Meloidogyne incognita* (red) and *Brugia malayi* (blue). The CYP Clan 2 genes, related to vertebrate xenobiotic-metabolizing CYP2s, are highlighted in yellow. Values at node points are bootstrap values (100 replicate bootstraps, randomly seeded).

Figure 2. DNA damage is caused by exposure to aflatoxin B_1 (with and without bacteria), but not benzo[a]pyrene, β-naphthoflavone, or chlorpyrifos in *C. elegans.* Young adult *glp-1* nematodes were exposed for 48 h in liquid medium and sampled at 24 and 48 h (total n = 8 nematodes per concentration per chemical per time point). AFB_1 exposure in *C. elegans* resulted in concentration-dependent DNA damage ($p < 0.001$, main effect of concentration in 2-factor ANOVA); concentrations at which the AFB_1-induced DNA damage measured was significantly different from controls ($p < 0.05$ by FPLSD) are indicated by asterisks. BaP, BNF, and CPF exposure did not result in a detectable level of DNA damage ($p = 0.615$, 0.161, and 0.454 respectively). The experiment was carried out twice (n=4 each) and the results combined.

Figure 3. Aflatoxin B_1 and chlorpyrifos inhibited growth more in a DNA repair-deficient strain (*xpa-1*, white) than in the wild-type (N2, black) strain of *C. elegans*. Exposure to benzo[a]pyrene and β-naphthoflavone inhibited growth of both strains to a statistically indistinguishable degree. n =25-143 nematodes per concentration per strain per chemical; results include three separate (pooled) experiments. See Supplemental Table 1 for statistical details. Size measurements were taken on day two after feeding began, and are presented here as length (time of flight) measurements. For optical density-based growth measurements, see Supplemental Figure 2.

Figure 4. Aflatoxin B_1 inhibited the growth of a cytochrome P450 NADPH reductase-deficient strain (*emb-8*, white) less effectively than growth of wild-type (N2, black) *C. elegans* ($p = 0.0002$, 0.1376, and 0.0007, strain comparisons at 0, 30, and 100 µM AFB_1 by Mann-Whitney *U* test). n = 17-24 nematodes, two separate biological experiments pooled.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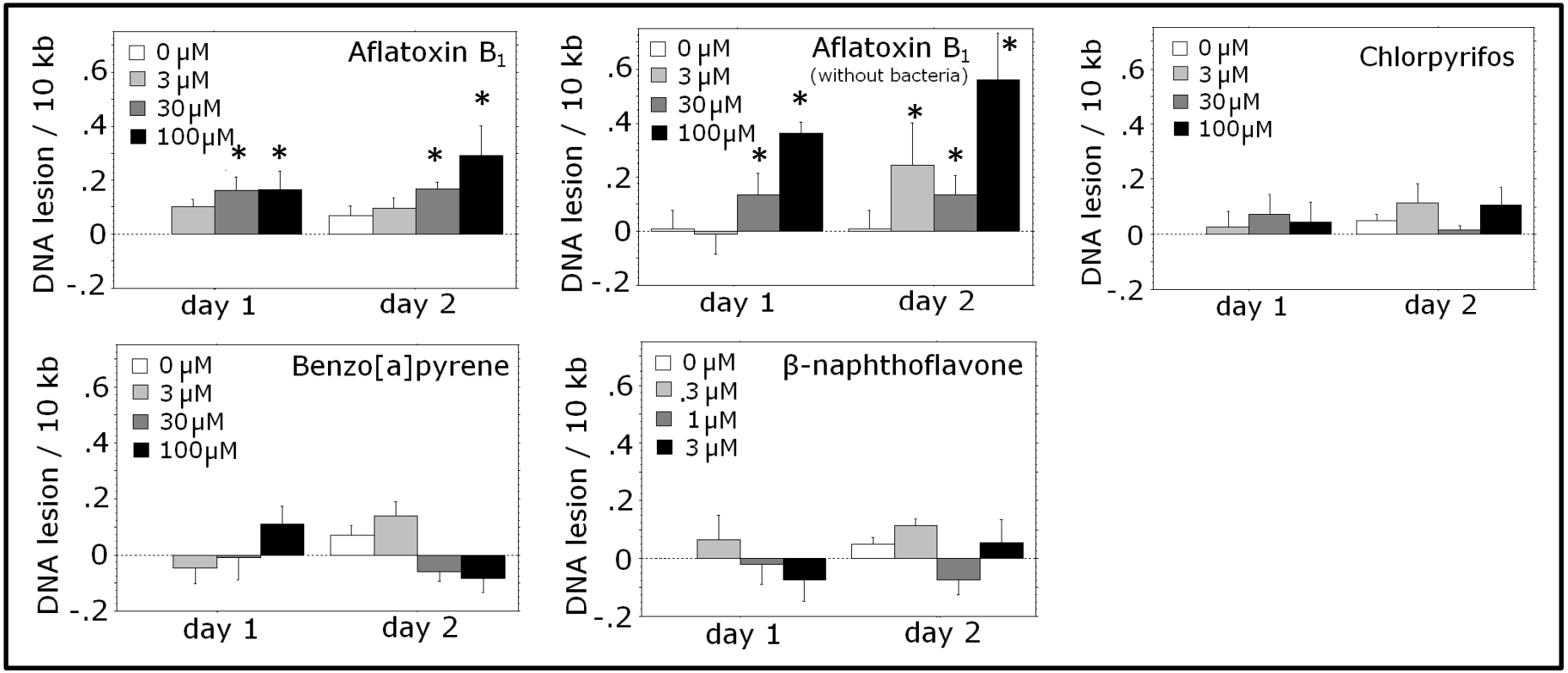
Aflatoxin B1
0 µM
3 µM
30 µM
100µM
DNA lesion / 10 kb
.6
.4
.2
0
-.2
day 1
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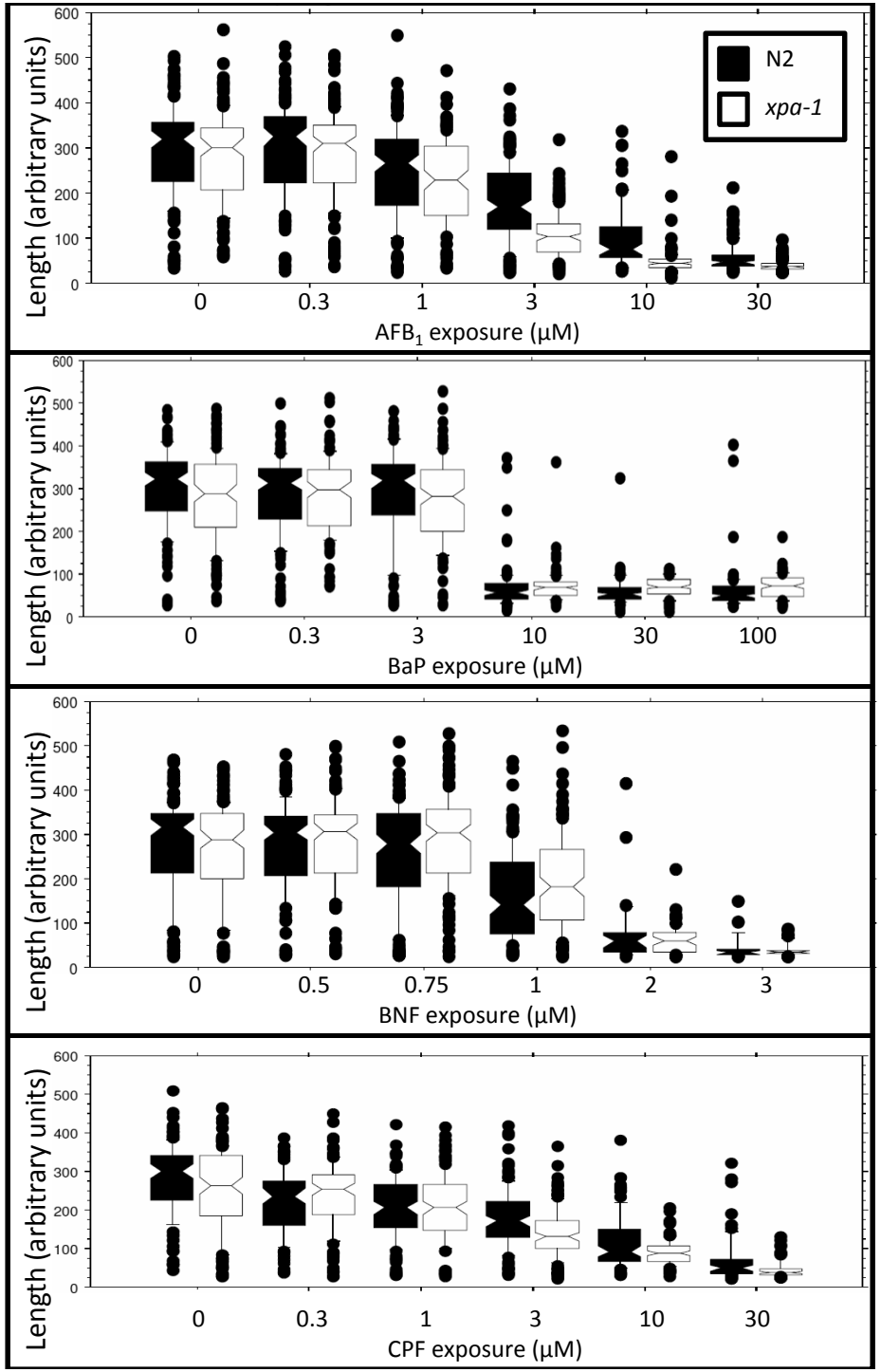
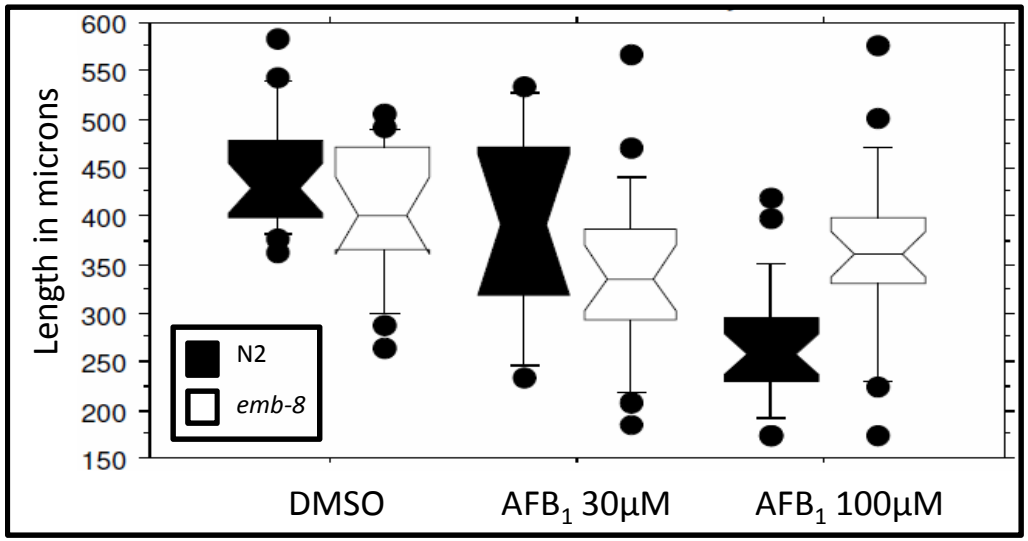
Aflatoxin B1
(without bacteria)
Chlorpyrifos
Benzo[a]pyrene
β-naphthoflavone
.3 µM
1 µM


N2
xpa-1
Length (arbitrary units)
AFB1 exposure (µM)
BaP exposure (µM)
BNF exposure (µM)
CPF exposure (µM)


Length in microns
600
550
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N2
emb-8
DMSO
AFB1 30µM
AFB1 100µM