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One of the most common classes of agricultural insecticides is the organophosphorus pesticides (OPs). OPs exert their toxicity through inhibition of brain acetylcholinesterase (AChE), leading to hyperactivity of the nervous system. There is growing concern that exposure to low levels of OPs induces negative outcomes in developing children. The chemical most commonly linked to these issues is chlorpyrifos (CPF).

We previously observed a susceptibility difference to CPF between juvenile mice and rats. The basis for this difference is unknown but may be due to differences in detoxification mechanisms and enzyme affinities for CPF's active metabolite (CPF-oxon). In the blood and liver, carboxylesterase (CES) and cholinesterase (ChE) enzymes act as alternative binding sites which removes the CPF-oxon from circulation.

To investigate this toxicity difference, 10 day old rat and mice pups were exposed daily for 7 days to corn oil or a range of CPF concentrations via oral gavage. Pups were sacrificed on day 16 and brain, blood, and liver were collected.

Exposure resulted in greater inhibition of brain AChE in juvenile rats than in mice. Regardless of species, CES activity was significantly higher than ChE activity in both tissues. Juvenile rats had slightly higher levels of CES activity in the liver than juvenile mice, however the level of inhibition was also higher in rats, so it is not a key location for protection. In serum, juvenile mice had an 8-fold higher CES activity than juvenile rats, and a CPF dosage that almost eliminated CES activity in rats only resulted in 24% inhibition in mice, making serum CES a key protective enzyme.

Because CES plays an important role in CPF toxicity protection, the difference in affinity for CPF-oxon between species could also play a role in susceptibility differences. Enzyme affinity was determined by the enzyme's IC50 (the oxon concentration needed to inhibit 50% of the total enzyme activity). Juvenile rat CES enzymes reached their IC50 point at a lower CPF-oxon concentration than juvenile mice in both the liver and serum. It appeared there was a higher enzyme affinity in juvenile rats than in mice which suggests that saturation of CES occurs more rapidly in juvenile rats, resulting in more CPF reaching the brain of rats to inhibit AChE.

Finally, the A-esterase enzyme, PON1, was studied to determine if it also played a role in the susceptibility difference, however, there was no statistically significant difference between the species.

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INTRODUCTION

One of the most common classes of agricultural pesticides is the organophosphorus insecticides (OPs). Earlier studies have reported a link between exposure to OPs and negative impacts on the brain of developing children (Eskenazi et al. 1999; Eskenazi et al. 2007; Rauh et al. 2011; Rauh et al. 2006; Rauh et al. 2012). Preclinical studies using rodents have also reported impacts on behavior including deficits in memory, coordination, anxiety, and social behavior (Aldridge et al. 2005; Carr et al. 2020; Carr et al. 2017; Chanda and Pope 1996; Johnson et al. 2009; Levin et al. 2010; Perez-Fernandez et al. 2020a; Perez-Fernandez et al. 2020b; Ricceri et al. 2003; Roegge et al. 2008; Timofeeva et al. 2008). The chemical most linked to these negative impacts in the literature is chlorpyrifos (CPF). After absorption into the body, CPF is metabolized to its active metabolite chlorpyrifos-oxon (CPF-oxon) by cytochrome P450. Following metabolism, CPF-oxon adversely affects the nervous system of animals and humans by inhibiting cholinesterase activity. At high exposure levels, CPF results in significant inhibition of brain acetylcholinesterase (AChE), leading to an accumulation of acetylcholine (ACh) in the neuronal and neuromuscular synaptic junctions. This accumulation results in continual stimulation and hyperactivity of the nervous system.

Most environmental exposures to CPF occur at lower levels with no inhibition of AChE and thus, no hyperactivity of the nervous system suggesting the presence of a non-cholinergic target. With that in mind, our previous studies investigating the neurotoxic effects of developmental CPF exposure utilized dosages of CPF that did not yield any inhibition of brain AChE. These studies utilized juvenile rats as the research model and we have observed that repeated exposure to these low dosages results in altered behavior (Carr et al. 2020; Carr et al. 2017). However, the basis for these changes is unclear.

To further this line of investigation, we had hopes of utilizing transgenic mouse models to further identify the basis for the neurotoxic effects observed following low-level CPF exposure. Thus, we initiated a pilot dose-response study to determine the effects of repeated oral CPF exposure on brain AChE activity in wild-type juvenile mice. As presented in this study, we observed a significant species difference in the inhibition of brain AChE between juvenile mice and juvenile rats with greater AChE inhibition in juvenile rat brain than in juvenile mouse brain. This toxicity difference in juveniles differs from that observed in adults. Published literature has

reported that adult rats (LD_{50} of 82-155 mg/kg) are more resistant to CPF toxicity than adult mice (LD_{50} of 60 mg/kg) (El-Sebae et al. 1978; Gaines 1969; Garcia et al. 2006; Storm et al. 2000). Thus, the basis for the differences in the toxicity of CPF between juvenile rats and mice is not clear.

During exposure, CPF is converted to its active metabolite, CPF-oxon, which is not only a potent inhibitor of brain AChE but a potent inhibitor of other serine esterases such as the peripheral cholinesterases (ChE) and carboxylesterases (CES) in non-target tissues. Peripheral ChE includes both the enzymes AChE and butyrylcholinesterase which are present in rodent and human liver and serum. The inhibition of these enzymes functions as a protective mechanism (Chambers et al. 1990; Clement 1984; Maxwell 1992) by stoichiometrically destroying a portion of the oxon thereby preventing it from reaching the brain to inhibit AChE. CPF-oxon can also be catalytically destroyed by paraoxonase 1 (PON1) which would also reduce the levels of circulating CPF-oxon. It is possible that a species difference exists in these detoxification mechanisms and contributes to the differences in brain AChE inhibition. Therefore, in this study, we measured the levels of these non-target esterases in the liver and serum of juvenile rats and mice and determined the impact that repeated oral CPF exposure has on ChE and CES activity levels.

OBJECTIVE AND EXPERIMENTAL PLAN

Objective:

Previous work has shown that there is a significant toxicity difference between juvenile rats and mice when exposed to the agricultural insecticide chlorpyrifos (CPF). Juvenile mice have lower inhibition of brain acetylcholinesterase than juvenile rats and are more resistant to toxicity when exposed to the same concentration of CPF. This toxicity difference in juveniles differs from that observed in adults, where adult rats are more resistant to toxicity. The objective of this study was to investigate the basis for the species differences in the inhibition of CPF-induced brain AChE in juvenile mice and rats. Our hypothesis is that "*Juvenile mice will have higher concentrations of protective enzymes in their liver and serum than will juvenile rats*".

Experimental Plan:

To accomplish this, we determined the control activities of two major protective enzymes, carboxylesterase (CES) and cholinesterase (ChE), in the blood and liver of non-CPF exposed animals. We also determined the effect of CPF exposure on these enzymes. We determined the sensitivities of CES to inhibition by CPF-oxon. We also determined the liver and serum activity of paraoxonase 1 (PON1) which can hydrolyze organophosphorus pesticides (OPs) without being inhibited. All of these things can play a role in the protection of the organism against OP toxicity.

MATERIALS AND METHODS

Chemicals: Chlorpyrifos (>99%) was a generous gift from DowElanco Chemical Company (currently known as Corteva, Inc., Indianapolis, IN). Chlorpyrifos-oxon and paraoxon (>97%) were synthesized as previously described (Chambers et al. 1990) and were generous gifts of the late Dr. Howard Chambers. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals: Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited, temperature-controlled environment (22 ± 2°C) with a 12-hour dark-light cycle with lights on between 0700 and 1900. Lab Diet rodent chow and tap water were freely available during the experiment. Adult male and female Sprague-Dawley CD rats and adult male and female C57BL/6 mice (Envigo, Indianapolis, IN) were used for breeding. Following parturition, male and female pups within the same litter were assigned to different treatment groups. There were always representative control animals of the same sex present in each litter to match the CPF-treated animals. The day of birth was considered postnatal day 0 (PND0). All procedures were approved by the Mississippi State University Institutional Animal Care and Use Committee.

Animal Treatment: CPF was administered daily by oral gavage to male and female rat and mice pups beginning on PND10 and continuing through PND16 (7 days). This age range is beyond the period of the growth spurt (PND7 and below) in rodents but encompasses a time of significant brain maturation and corresponds to the period following birth in humans (Andersen 2003; Counotte et al. 2011; Tau and Peterson 2010). CPF was dissolved in corn oil and delivered to the back of the throat using a 10-microliter tuberculin syringe equipped with a 1-inch 24-gauge straight intubation needle (Cadence Science, Cranston, RI). Mice pups received either corn oil or 1.0, 2.5, 5.0, 7.5 or 10 mg/kg CPF, whereas rat pups received either corn oil or 0.5, 0.75, or 1.0 mg/kg CPF, both at a volume of 0.5 ml/kg. Each day, body weights were recorded and weight gain was calculated as the difference between the body weights on PND11-16 and the original body weight at the initiation of treatment on PND10. For rats, weight gain data were obtained for 2.5 and 5.0 mg/kg CPF from a previous experiment published by our laboratory (Carr et al. 2011).

Enzyme Assays: Mice and rats were sacrificed 4 hours following the final administration of CPF on PND16 and forebrain (whole brain minus medulla, pons, and cerebellum), serum, and liver were collected, immediately frozen on dry ice, and stored at -80°C until assay. Brain and liver were homogenized in assay buffer (0.05 M Tris-HCl buffer (pH 7.4)) in a glass mortar using a Wheaton motorized grinder and a Teflon pestle.

For determination of forebrain AChE and liver ChE activities, homogenates were diluted in the assay buffer to final concentrations of 1.0 and 0.25 mg/ml, respectively. Activities were measured using a discontinuous spectrophotometric assay using a modification (Chambers et al. 1988) of Ellman et al. (Ellman et al. 1961) using acetylthiocholine as the substrate (1 mM final concentration) and 5,5'-dithiobis (nitrobenzoic acid) as the chromogen. For determination of serum ChE activity, serum was diluted in assay buffer to a final concentration of 7.69 µl/ml. Activity was measured using a continuous spectrophotometric assay modified to a 96-well microplate as described by Ellman et al. (Ellman et al. 1961) using acetylthiocholine as the substrate (1 mM final concentration) and 5,5'-dithiobis (nitrobenzoic acid) as the chromogen. For all ChE assays, additional tubes/wells containing eserine sulfate (10 µM final concentration) were included in the assay to correct for non-enzymatic hydrolysis for each sample.

For determination of liver and serum CES activities, liver homogenates and serum were diluted in the assay buffer to working concentrations of 1.0 mg/ml and 6.66 µl/ml, respectively. Activity was measured using a spectrophotometric assay based on a previously described method (Carr and Chambers 1991) that was modified to a continuous 96-well microplate assay. Briefly, 150 µl of the tissue (working concentration) was preincubated at 37°C for 5 min prior to the addition of prewarmed 150 µl substrate 4-nitrophenol valerate (to obtain a 0.1 mM final concentration). Continuing to incubate at 37°C, the absorbance at 405 nm was read every 20 sec for 5 minutes and the slope was calculated. For each sample, additional wells containing paraoxon (10 µM final concentration) were included in the preincubation part of the assay to correct for non-enzymatic hydrolysis.

For determination of hydrolysis of CPF-oxon by PON1 in liver and serum, liver homogenates and serum were diluted in either the assay buffer or the assay buffer + 1mM EDTA to final concentrations of 1.0 and 0.5 mg/ml, respectively. PON1-mediated hydrolysis of CPF-oxon was determined by measuring the production of 3,5,6-trichloro-2-pyridinol based on a previously

described method (Furlong et al., 1989) but adapted to a continuous assay on a microplate. Briefly, 200 µl of the tissue dilution (+/- EDTA) was preincubated at 37°C for 5 min prior to the addition of the substrate CPF-oxon (0.35 mM final concentration). Continuing to incubate at 37°C, the absorbance at 315 nm was read every 20 sec for 5 minutes and the slope was calculated. For each sample, the wells containing EDTA were to correct for non-enzymatic hydrolysis.

For all tissues, protein concentrations were determined using the BCA reagent (ThermoPierce) with bovine serum albumin standards. Using either the absorbance units (discontinuous assay) or slopes (continuous assays), the specific activity of each enzyme from each sex of each species was determined as nmoles min^{-1} mg $protein^{-1}$

Carboxylesterase IC_{50} Determinations: IC_{50} values assays were determined based on previously published methods (Atterberry et al. 1997; Meek et al. 2021) but were adapted to a 96-well microplate. Tissue concentrations were determined that would yield similar control slope levels. Control liver from rats and mice was homogenized in assay buffer and centrifuged to obtain supernatant which was diluted in assay buffer to obtain final concentrations of 1.0 and 0.5 mg/ml, respectively. Control serum from rats and mice was diluted in assay buffer to obtain final concentrations of 0.25 and 0.025 µl/ml, respectively. Increasing concentrations of either CPF-oxon (5 µl) or the ethanol vehicle were pipetted into different wells of a 96-well plate. Diluted prewarmed tissue (150 µl) was added and incubated at 37°C with for 30 minutes on a shaker. This was followed by the addition of 150 µl of the prewarmed substrate 4-nitrophenol valerate as described above. Absorbance at 405 nm was read every 20 sec for 5 minutes and the slope was calculated. For each replication, additional wells containing paraoxon (10 µM final concentration) were included in the preincubation part of the assay to correct for non-enzymatic hydrolysis. IC_{50} values were calculated as described in Meek et al. (Meek et al. 2021).

Statistical Analysis: Statistical analysis was performed using SAS statistical package (SAS Institute Inc., Cary, NC). The sphericity of the body weight gain data was initially tested by analysis of variance (ANOVA) using the general linear model with a repeated measures paradigm and was not found to violate the assumption of sphericity. Using a repeat measures paradigm, analysis of body weight gain was conducted by ANOVA using the Mixed procedure (Littell et al. 1996). Enzyme activities and IC_{50} values were also analyzed by ANOVA using the

Mixed procedure. All analyses determined significant differences in sex, treatment, and sex × treatment interactions. No sex differences were detected so males and females were pooled for final analysis. Mean separation was performed by least-square means. The criterion for significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

No signs of cholinergic hyperactivity such as lacrimation, diarrhea, or tremors were observed at any time during CPF exposure in either species. In rats, there was a significant effect of treatment on weight gain ($p < 0.0214$) but only the 5.0 mg/kg CPF treatment group was significantly different from controls ($p < 0.0050$). Lower level analysis indicated that these effects occurred on days 12-16 (Figure 1A). This pattern of effect on weight gain is similar to that reported earlier (Carr et al. 2017; Carr et al. 2011; Carr et al. 2014). In contrast, there was no effect on weight gain in any of the treatment groups in mice even though a dosage of 10 mg/kg CPF was administered (Figure 1B).

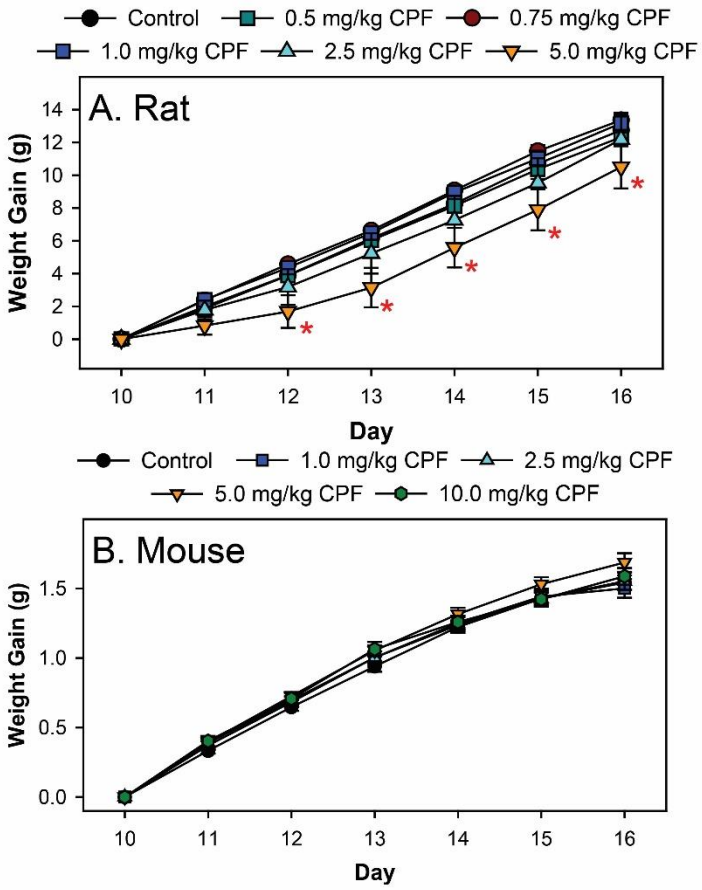

Figure 1. Rates of Weight Gain in Juvenile Rats and Mice. Rat (A) and mouse (B) pups were exposed daily from postnatal day 10 through 16 to either corn oil (control) or various dosages of CPF. Values are expressed as weight ± SE (n = 12-15). Values significantly different from their respective control ($p \leq 0.05$) are indicated with an asterisk (*).

Based on our results, the control activities of AChE in juvenile rat and mouse brains are similar (Table 1). This finding agrees with adult studies comparing rat and mice brain AChE (Johnson and Wallace 1987). In addition, the *in vitro* sensitivities of brain AChE to inhibition by OPs, such as paraoxon and chlorpyrifos-oxon, have been reported to also be similar in *in vitro* adult studies that studied both species (Coban et al. 2016; Johnson and Wallace 1987). However, we observed that repeated daily oral exposure to CPF induced different patterns in the inhibition of brain AChE between juvenile rats and juvenile mice (Figure 2). The lowest dosage of CPF that resulted in significant brain AChE inhibition in juvenile rats was 1.0 mg/kg which yielded 21% inhibition, whereas, this dosage did not result in any inhibition of brain AChE in juvenile mice. The highest dosage of CPF administered to juvenile rats was 5.0 mg/kg which yielded 63% inhibition of brain AChE inhibition. This dosage also yielded significant inhibition of brain AChE in juvenile mice but only at a level of 14%. A higher dosage of CPF (10 mg/kg) was administered in juvenile mice and yielded 50% inhibition. These data indicate that repeated daily oral CPF exposure results in a larger toxicological impact in juvenile rats than in juvenile mice.

Table 1

Specific Activities of Enzymes from Juvenile Mouse and Rat

	Mouse			Rat		
Brain AChE	88.17	±	2.58	89.07	±	2.28
Liver ChE	153.02	±	8.61	69.74	±	3.41*
Serum ChE	5,960.94	±	683.48	2,779.10	±	198.34*
Liver CES	19,249.98	±	745.89	37,804.30	±	674.55*
Serum CES	20,017.29	±	757.44	2,539.30	±	95.31*

Data (nmoles min^{-1} mg $protein^{-1}$) presented as Mean ± SEM. * = Significantly difference between species in row.

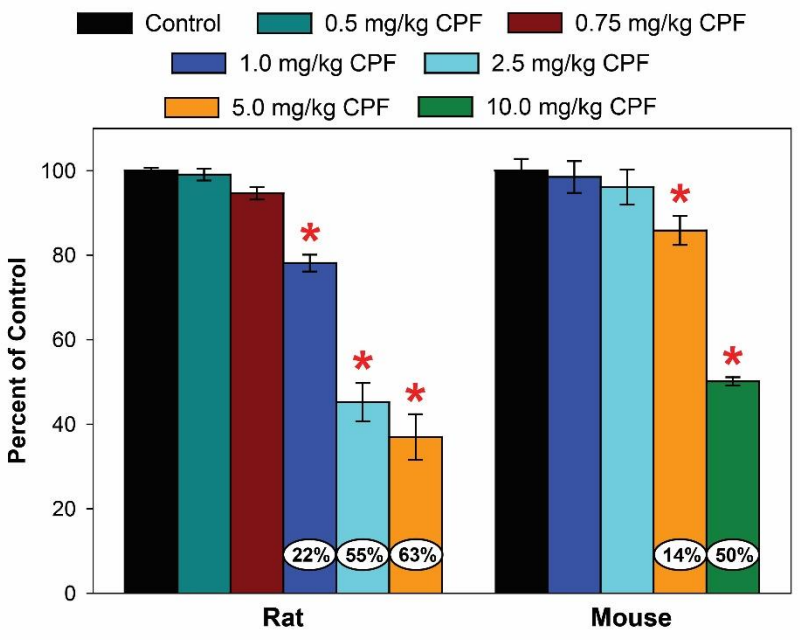

Figure 2. Brain Acetylcholinesterase Inhibition. Percent inhibition of acetylcholinesterase in the brain of rat and mice pups exposed daily from postnatal day 10 through 16 to either corn oil (control) or various dosages of CPF. Values are expressed as mean ± SE (n = 12–14). Percent inhibition for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Values significantly different from their respective control ($p \leq 0.05$) are indicated with an asterisk (*).

The first non-target esterase investigated was the effect of exposure on the activities of liver and serum ChE where repeated daily oral exposure to CPF resulted in a dose-response type pattern in both tissues (Figures 2A and 2B). For both liver and serum ChE, control juvenile mice possessed over 2-fold higher ChE activities than did control juvenile rats (Table 1). These differences in activity levels could have certainly contributed to the increased resistance in juvenile mice. However, at the same exposure level (1.0 mg/kg CPF), the inhibition of liver ChE in juvenile rat liver was 64%, while in juvenile mice, it was only 52%. As with liver ChE, there was a similar percentage inhibition of serum ChE between rats (53%) and mice (42%) at the same level of exposure (1.0 mg/kg). Overall, this suggests that the higher level of liver and serum ChE present in juvenile mice did not offer the juvenile mice a significantly greater level of protection as compared to juvenile rats.

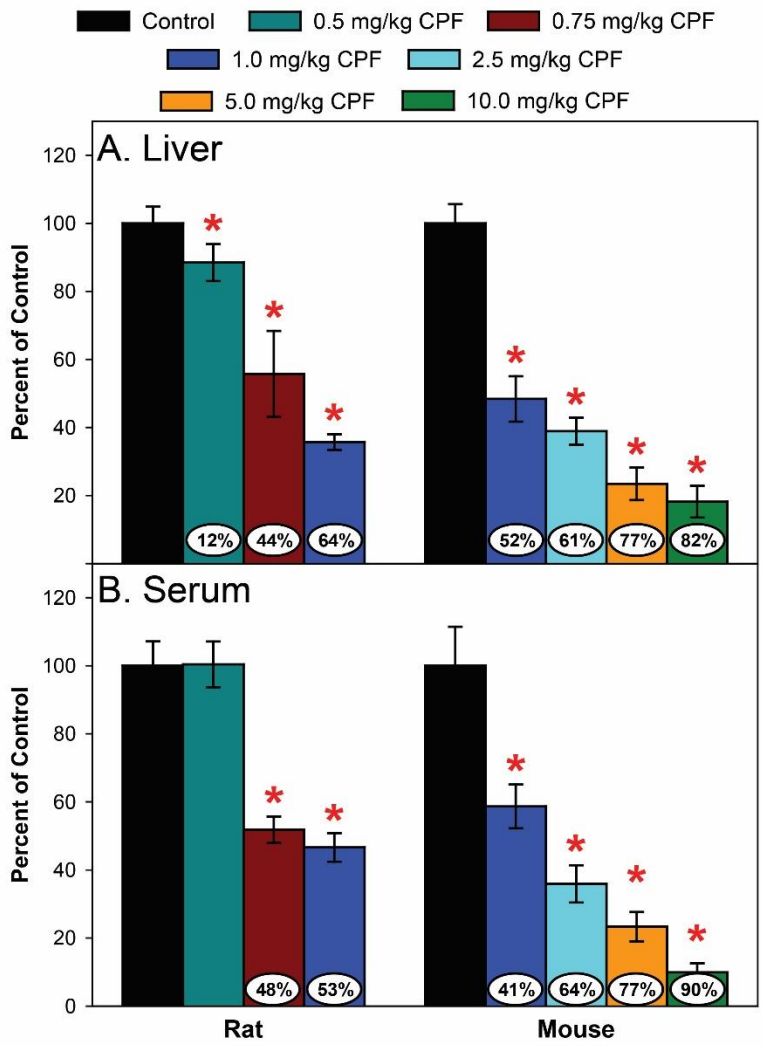

Figure 3. Liver and Serum Cholinesterase Inhibition. Percent inhibition of cholinesterase in the liver (A) and serum (B) of rat and mice pups exposed daily from postnatal day 10 through 16 to either corn oil (control) or various dosages of CPF. Values are expressed as mean ± SE (n = 9-15). Percent inhibition for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Values significantly different from their respective control ($p \leq 0.05$) are indicated with an asterisk (*).

The next non-target esterase investigated was the effect of exposure on the activity of liver CES where repeated daily oral exposure to CPF resulted in a dose-response type pattern (Figure 4A). Interestingly, for liver CES, control juvenile rats had almost a 2-fold higher CES liver activity than did control juvenile mice (Table 1). In addition, the IC_{50} value for rat liver CES was 3.6-fold lower than the same value for mouse liver CES indicating greater sensitivity of rat liver CES (Table 2). Based strictly on activity levels and enzyme sensitivity, if liver CES was playing

the main protective role, juvenile rat liver should be more resistant to CPF exposure since they have twice the amount of enzyme activity. However, even with the higher activity, CES activity in juvenile rat liver was almost maximally inhibited (96%) at 1.0 mg/kg CPF, whereas, CES activity in juvenile mouse liver resulted in only 54% inhibition when exposed to the same dosage of CPF. This maximal inhibition in rats suggests that while liver CES does effectively remove CPF-oxon from circulation, it does not appear to be the key protective enzyme.

The next non-target esterase investigated was the effect of exposure on the activity of serum CES where, as observed with the other enzymes, repeated daily oral exposure to CPF resulted in a dose-response type pattern (Figure 4B). Control juvenile mice had almost an 8-fold higher serum CES activity than did control juvenile mice (Table 1). At the same exposure level (1.0 mg/kg CPF), serum CES activity in juvenile rats was almost maximally inhibited (95%) suggesting that the protective mechanism provided by the serum CES was saturated. However, the same dosage in juvenile mice resulted in only 22% inhibition suggesting that a significant level of enzymes remained active in the serum. It required raising the CPF dosage to 10 mg/kg in juvenile mice to approach saturation of the protective mechanism provided by serum CES. As evidenced by a significantly lower IC_{50} value, rat serum CES was statistically more sensitive to inhibition by CPF-oxon than was mouse serum CES (Table 2). However, the differences in the IC50s between rats and mice were not very large suggesting that this sensitivity difference probably did not play an important role (Table 2).

Table 2

Carboxylesterase IC_{50}s in Liver and Serum of Juvenile Mouse and Rat

	Mouse	Rat
Liver	3.36 ± 0.16	0.93 ± 0.05*
Serum	10.75 ± 0.19	8.79 ± 0.32*

Data (nM CPF-oxon) presented as Mean ± SEM. * = Significantly different from other species in row.

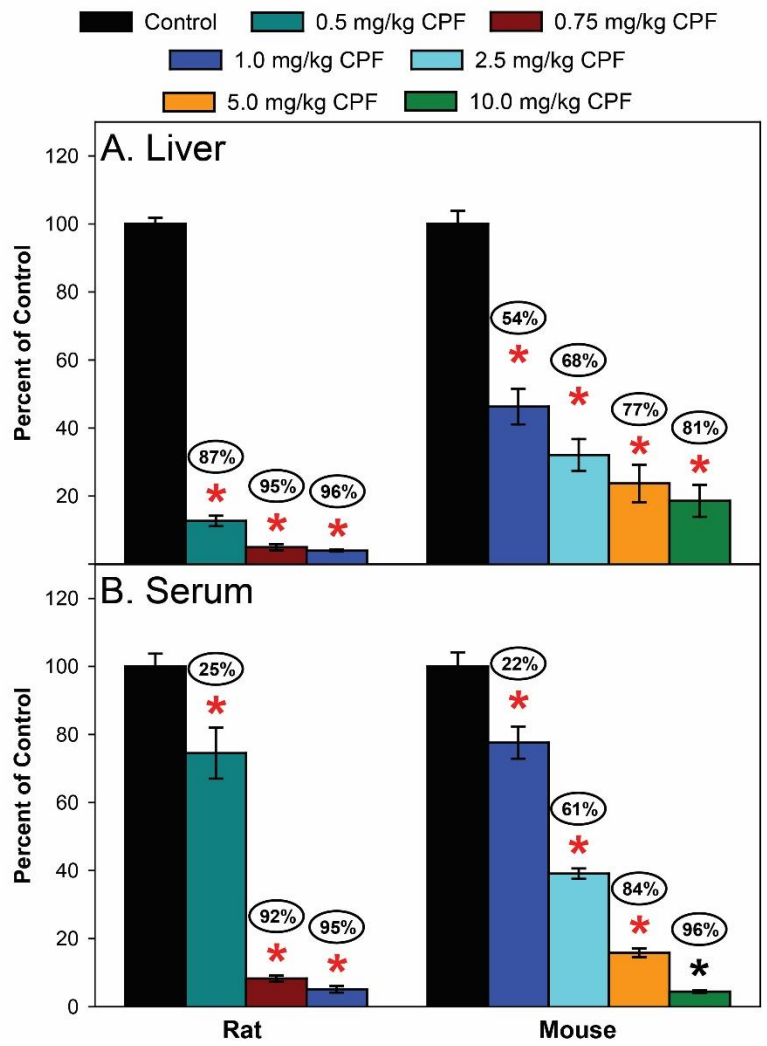

Figure 4. Liver and Serum Carboxylesterase Inhibition. Percent inhibition of carboxylesterase in the liver (A) and serum (B) of rat and mice pups exposed daily from postnatal day 10 through 16 to either corn oil (control) or various dosages of CPF. Values are expressed as mean ± SE (n = 12-15). Percent inhibition for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Values significantly different from their respective control ($p \leq 0.05$) are indicated with an asterisk (*).

In addition to the esterases, another potential protective enzyme that could have played a role in the species difference is paraoxonase 1 (PON1) which catalytically hydrolyzes organophosphates such as CPF-oxon. However, the activity of PON1 in both liver and serum was similar between species suggesting that it did not play a role in the differences in sensitivity to CPF exposure (Table 3).

Table 3

Chlorpyrifos-oxon Hydrolysis by PON1 in Liver and Serum of Juvenile Mouse and Rat

	Mouse	Rat
Liver	627.62 ± 36.35	626.31 ± 19.87
Serum	811.02 ± 46.91	814.45 ± 28.73

Data (nmoles min^{-1} mg $protein^{-1}$) presented as Mean ± SEM. * = Significantly different from other species in row.

Of the protective enzymes investigated, it appears that the differences in serum CES activity may play the most significant role in the toxicity differences between juvenile rats and mice during CPF exposure. Following oral exposure, a toxicant is absorbed by the intestine and enters the blood stream via the hepatic portal vein which takes it to the liver. Normally, the liver is considered the main point of metabolism. However, Poet et al. (2003) reported that metabolism of CPF occurs in the intestine and, at low-dose oral exposures (such as those used in this study), that metabolism would contribute significantly to the overall activation of CPF. If a large amount of the CPF is converted to CPF-oxon in the intestine, the release of CPF-oxon into the circulation would initially occur in the hepatic portal vein where it would have the first opportunity to be bound by CES. This would allow elimination of any CPF-oxon produced by the intestine prior to delivery to the liver. This would be beneficial to the species with the higher level of serum CES.

Hypothetically, let's consider what occurred in rats and mice exposed to the same dosage of CPF (1.0 mg/kg). In rats, the CPF-oxon entering the hepatic portal vein saturated the serum CES thereby allowing CPF/CPF-oxon to reach the liver. Once in the liver, the CPF-oxon inhibited CES at that location, the CPF was converted to CPF-oxon, and together these saturated the liver CES. This allows more CPF/CPF-oxon to enter general circulation and reach the brain. In mice, most of the CPF-oxon produced in the intestine is bound by serum CES such that mostly CPF reaches the liver. The CPF reaching the liver is converted to CPF-oxon but that amount is not sufficient to saturate the liver CES thus lower levels of CPF-oxon escape into the general circulation and are able to reach the brain.

CONCLUSIONS AND FUTURE DIRECTIONS

The basis for the difference between juvenile rats and mice is still unclear. It is well known that adult rodents have higher levels of CES in their blood which is an important factor in why juveniles are more susceptible than adults to OP compounds such as CPF (Atterberry et al. 1997; Moser et al. 1998). It has also been reported that adult mice have only 1.8-fold more CES activity in their blood than do adult rats (Rudakova et al. 2011). It is possible that mice may simply develop faster than rats and reach adult levels of blood CES activity much earlier than do rats. However, other factors may play a role in the different susceptibility of juvenile mice and rats to CPF exposure. These include species differences in the cytochrome P450-mediated activation and detoxification of CPF in the liver and/or intestine and differences in the intestinal PON1-mediated detoxication of CPF-oxon.

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