Published in final edited form as:

Toxicology. 2020 January 15; 429: 152327. doi:10.1016/j.tox.2019.152327.

Gestational and Perinatal Exposure to Diazinon Causes Long-lasting Neurobehavioral Consequences in the Rat

Andrew Hawkey^{1,2}, Erica Phippen^{1,2}, Hannah White^{1,2}, Joseph Kim^{1,2}, Eva Greengrove^{1,2}, Bruny Kenou^{1,2}, Zade Holloway^{1,2}, Edward D. Levin^{1,2}

¹Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC 27710 USA

²Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710 USA

Abstract

Diazinon is a widely-used organophosphate pesticide. Pulsatile exposure to diazinon during neonatal development has previously been shown cause long-term neurobehavioral impairments in rats. However, the effects of chronic low concentration exposures during perinatal development remain unclear. This experiment evaluated such effects in Sprague-Dawley rats by implanting osmotic pumps in breeder females prior to conception (N=13–15 litters per condition) which then delivered chronic, zero order kinetic low-level infusions of 0, 114 or 228 ug/day of diazinon throughout pregnancy. One male and one female from each litter was assessed with a battery of behavioral tests that continued from four weeks of age into adulthood. Litter was used as the unit of variance for the analysis of variance test of significance, with sex as a within litter factor. Diazinon treatment condition was the between subjects factor and time or sessions were repeated measures. Chronic diazinon exposure from pre-mating until the neonatal period caused a significant ($p<0.05$) increase in percent of time spent on the open arms of the elevated plus maze, an index of risk-taking behavior. Gestational and lactational diazinon exposure also caused a significant ($p<0.05$) degree of hyperactivity in the Figure-8 apparatus during adolescence, specifically affecting the early part of the hour-long test session. This effect had dissipated by the time the rats reached adulthood. Diazinon exposure also caused a significant impairment in novel object recognition, a test of cognitive function. Offspring exposed to 228 ug/day diazinon ($p<0.05$) showed significantly less preference for the novel vs. familiar object than controls during the first five minutes of the novel object recognition test.

Correspondence: Edward D. Levin, Ph.D., Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Box 104790, Durham, NC 27710, USA, Phone: 1-919-681-6273; Fax: 1-919-681-3416, edlevin@duke.edu.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Behavioral Teratogenesis; Diazinon; Development

1 Introduction

Diazinon (DZN) is a widely used organophosphate (OP) insecticide with substantial potential for environmental contamination and adverse effects on human health. The efficacy of OPs in pest control have been principally linked to inhibition of the enzyme acetylcholinesterase (Fukuto, 1990), a mechanism that is well preserved in off-target species, including vertebrates like rodents and humans. Organophosphate insecticides may also produce toxic effects through other mechanisms, such as other serine hydrolases, oxidative stress, neuro-inflammation and epigenetic mechanisms (Mangas, 2017; Mostafalou, 2018; Pope, 2005). These non-cholinesterase mechanisms may then allow OPs to produce toxicity at low doses with minimal impact on cholinesterase function. Epidemiological studies of populations with insecticide exposures suggest that early life exposure to organophosphates may have long-term effects on the brain and enhance the risk for psychiatric and neurodevelopmental issues later in life (Bouchard, 2011; Engel, 2011; Munoz-Quezada, 2013; Rauh, 2012). In human populations, it can be difficult to determine which specific compounds or risk factors are responsible for these effects, particularly later in life, so work with animal models has been conducted to evaluate the potential risk that specific compounds, like DZN, may pose to the developing nervous system.

Animal models, particularly rodents, suggest that DZN has substantial neurotoxic potential during development. In rodent models, it has been demonstrated that even brief exposures to DZN at doses below the threshold for cholinesterase inhibition can produce persistent effects. For example, daily injections of rat pups with 0.5–2 mg/kg/day DZN from postnatal day (PND) 1–4 can result in persistent changes in cell densities in the brain (Slotkin et al., 2008a) impaired cholinergic function, reduced numbers of nicotinic acetylcholine receptors (Slotkin, et al., 2008a) and modulations of serotonergic receptors and transporters (Slotkin et al., 2008b; Slotkin et al., 2006). Parallel behavioral studies have further shown persistent and diverse behavioral effects in adolescence and adulthood, including learning deficits, impaired prepulse inhibition, and disruptions of reward, anxiety-like behaviors and fear responsivity (Roegge, 2008; Timofeeva et al., 2008a). Similar brief neonatal exposure models in rats or mice have corroborated the potential neurotoxicity of DZN and shown additional effects, such as impairments in glutamatergic function (Win-Shwe, 2013), passive avoidance learning (Vatanparast, 2013) and novel object recognition (Win-Shwe, 2013).

Although prior studies have been valuable in showing that brief DZN exposure may have lasting effects on neural functions and behavior, there are still substantial gaps in this literature. Limited data are available to suggest how exposures across perinatal development may differ from the brief pulsatile neonatal exposures described in previous studies. However, existing data suggests that the inclusion of earlier exposures may produce unique effects. For example, Vatanparast and colleagues (2013) compared the effects of neonatal DZN exposure (1 mg/kg/day on PND 1–4) with a similar exposure during the latter portion

of gestation (gestational day 15–18). Both exposures disrupted later passive avoidance learning, although these exposures led to qualitatively different effects, whereby gestational exposures preferentially affected female offspring and neonatal exposures preferentially affected males. This parallels previous work with another prominent OP, chlorpyrifos, which has been demonstrated to produce differential effects across varying critical windows of early development (Levin et al., 2002; Levin et al., 2001). Based on these findings, it is hypothesized that DZN exposures in gestational and perinatal development would produce persistent neurobehavioral effects, similar to neonatal exposures, but that the nature of those effects may be quite distinct.

The current study was conducted to evaluate developmental and behavioral endpoints in offspring with chronic exposure to DZN (114–228 ug/day, corresponding to an initial dose of 0.5–1.0 mg/kg/day) across gestational and perinatal development. These “low” exposures were selected to fall below the threshold for overt toxicity in the dams or pups, and to approach the threshold for cholinesterase inhibition (Slotkin et al., 2006). Exposure was delivered via osmotic minipump implanted subcutaneously in female breeders prior to conception, allowing DZN to be delivered at a constant low rate throughout conception, gestation and post-parturition. As the life of the osmotic pumps extended beyond birth (8–11 days), additional oral DZN exposure continued through the neonatal period via the dam’s milk. Gestational exposure in the rat overlaps key neurodevelopmental events which take place in the first and second trimesters of human development, while lactational/neonatal exposures in the rat overlap key neurodevelopmental events of the human third trimester (Semple, 2013). Offspring were monitored for neonatal health and growth, then assessed in a behavioral battery containing tests of locomotor, affective and cognitive functions from adolescence into early adulthood.

2 Methods

2.1 Subjects and Housing

Subjects in the present study consisted of Sprague Dawley (Charles Rivers Labs, Raleigh, NC, USA) and their offspring. All animals were maintained on a 12:12 reversed day-night cycle with ad libitum access to food and water, except in select behavioral procedures which required food restriction to motivate feeding behaviors (*see below*). Developmental monitoring and behavioral testing were conducted under low-ambient lighting conditions during the dark phase of the reversed light cycle (800hr–1700hr). All procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Duke University.

2.2 Exposures and Behavioral Testing

Adult female Sprague-Dawley rats were anaesthetized using ketamine (60 mg/kg) and dexdormitor (15 mg/kg) and implanted with Alzet osmotic infusion pumps (2ML4, Durect Inc, Cupertino, CA, USA). Diazinon (purity 98.8%, Chem Service Inc. West Chester, PA) was dissolved in dimethyl sulfoxide (DMSO) and used to fill the pump reservoirs. Fluid concentrations were calculated based on the rat weight at the time of implantation in order to achieve the following doses: the DMSO vehicle control (100%), 0.5 mg/kg/day or 1.0

mg/kg/day of DZN which translated to daily release of either 0, 114 ug/day ($\pm$ 3.7ug) or 228 ug/day ($\pm$ 5.1ug) of DZN respectively (See Table 1). Average weights at the time of surgery were as follow: control (227.0g $\pm$ 6.6), low DZN (227.1g $\pm$ 7.3), and high DZN (228.4g $\pm$ 5.1). Each rat was implanted with a pump. After a recovery period of 3 days, a 5 day mating period began. As per the manufacturer, the osmotic pumps are rated to deliver a consistent volume for 4 weeks (28 days), although it actually takes ~35 days for the reservoir to be completely exhausted. Across the breeding pairs, the dates of insemination varied, as did the dates when the reservoir was exhausted. So, the exposure period for the dams began 3–7 days prior to fertilization and ended 8–11 days after birth.

Organophosphates like DZN are lipophilic and readily found in mammalian milk, allowing maternal exposure to be transferred to neonates during nursing (see review, (Salama, 2017), albeit in smaller amounts per body weight than the dam experienced (Mattsson, 2000).

At weaning, 1 male and 1 female were randomly selected from each litter for behavioral testing. Remaining pups from the litters were separately housed and used for neurochemical analysis, which is presented elsewhere (Slotkin et al., 2019). Behavioral animals acclimated to their new housing for one week after weaning (3 weeks of age), then began the behavioral battery at 4 weeks of age and continued throughout adolescence into adulthood, as we have used previously to identify persisting behavioral effects of low chronic gestational toxicant exposure (Cauley, 2018). The behavioral battery included the following tests:

2.3 Elevated Plus Maze (week 4)

The rats were tested on the elevated plus maze (Med Associates, St Albans, VT, USA) to assess their anxiety like behavior vs. risk-taking like behavior. The maze was made of black Plexiglas and measured 142 cm $\times$ 104 cm $\times$ 76 cm high and consisted of two arms with 15 cm high enclosed walls and two open arms with low 2 cm railings. Rats generally avoid open spaces, preferring to explore closed areas near protective walls. Each rat was assessed individually on the elevated plus maze for a single five-min session. The number of center crossings was recorded to assess the amount of locomotor movement from arm to arm, and the duration of time spent in the open arms was timed. The percent time the rat spent in the open vs. enclosed arms of the maze was then calculated to reflect the preference or avoidance of the open arms.

2.3 Figure-8 Locomotor Activity Apparatus (5 weeks of age and adult)

Locomotor activity was assessed in an enclosed maze in the shape of a figure-8. The Figure-8 apparatus consisted of a continuous alley that measured 10 cm $\times$ 10 cm, with the entire maze measuring 70 cm $\times$ 42 cm. Animals were allowed to freely explore and locomotor activity was assessed by the crossing of eight photobeams located at equal points in the alley. Each locomotor test session lasted 1 h, and photobeam breaks were tallied in 5 min blocks across the session. Subjects were tested in the Figure-8 maze at two time points, 5 weeks of age and following the end of attention testing in adulthood.

2.4 Novelty Suppressed Feeding (6 weeks of age)

To assess fear responsivity, the offspring rats were tested for suppression of feeding in a novel environment. The rats had food restriction for 24 h prior to the novelty suppressed

feeding test. A novel environment consisted of a plastic rectangular cage (different from the home cage) placed in the middle of a brightly lit testing room. There was no cage top or bedding in the cage. Twelve standard rat-chow pellets were weighed before testing and then spread across the floor of the cage in 4 rows of 3 pellets each. The sessions were 10 min long and the latency for the rat to begin eating, the duration of eating and the bouts of eating were recorded. Eating was defined as the act of chewing the food and not merely sniffing, holding, or carrying the food around in the mouth. The food pellets which remained after the test session were weighed to determine the amount of food eaten. A composite score representing the latency to eat, time spent eating and amount of food eaten was also generated (percent of control mean).

2.5 Novel Object Recognition (7 weeks of age)

To test attention and memory in a low-motivational state recognition of a novel vs. familiar object was determined. Tests were conducted in opaque plastic enclosures measuring 70 cm × 41 cm × 33 cm. Objects consisted of plastic, glass, or ceramic material and were randomized for each animal. Animals were first habituated to the apparatus in two consecutive 10 min sessions over the course of two days. Testing began on day 3 with a 10 min “information” session in which two identical objects (A/A) were placed in the cage for the animal to explore. The A/A session was then followed by a 1 h delay period spent in the animal’s home cage. The animal was then placed back in the enclosure with one object from the A/A session and with another, dissimilar, “novel” object (A/B session). The spatial placement of the objects (left/right) was randomized for each animal. The time spent actively exploring each object was recorded. For data analysis, the time spent investigating each object was measured across two five minute blocks which split the session in two. This was done to detect within-session familiarization effects, because the novel object may become more familiar, and therefore less discriminable from the familiar object, over the course of the session. Between sessions, both the novel and familiar objects were wiped clean with a solution of 10% acetic acid in order to avoid odor recognition cues by the rats.

2.6. 16-Arm Radial Maze (8–11 weeks of age)

To index spatial learning and spatial memory the offspring rats were tested in 16-arm radial mazes. The mazes were black painted wood with a central platform and 16 arms placed radially from the central platform. The center was 50 cm in diameter, and each arm was 10 cm across × 60 cm long. A food cup was 2 cm from the end of each arm. Visual cues (cardboard shapes) were placed on the walls of the testing room to facilitate spatial orientation. Each rat was habituated in the maze in two 10 min sessions in which they were placed on the central platform inside a large, round, opaque cylinder with halves of sugar-coated cereal (Froot Loops®; Kellogg’s Inc, Battle Creek MI, USA) available to consume. Food cups of twelve of the arms of the maze were baited at the beginning of each session to test working memory performance and the remaining four arms were always left unbaited to test reference memory. The baited arms of the maze for each rat remained constant throughout series of testing sessions but the choice of arms baited was randomized among the different rats. Each trial began by placing the rat on the central platform inside the opaque cylinder for 10 s. Then, the cylinder was lifted and the rat was allowed to move freely. Each session lasted ten min or until the rat had entered all twelve baited arms,

whichever came first. Each rat was trained for 18 sessions and working and reference memory errors were assessed. Working memory errors were defined as repeated entries into a baited arm, and reference memory errors were defined as entry into one of the arms that was never baited. Latency was calculated as the total session time divided by the number of arm entries. After each session, the maze was cleaned with a damp cloth.

2.7 Operant Visual Signal Detection Task (11+ weeks of age)

To assess attention, the operant visual detection task was used. Briefly, each rat inside an operant chamber was trained to press one of two retractable levers in response to a visual cue-light that was illuminated for a duration of 500 ms. If the cue-light became illuminated (“signal” trial), the animal needed to press one of the two levers to receive a 20 mg food pellet reward. If the cue-light did not illuminate (“blank” trial) the animal needed to press the opposite lever in the chamber to receive the reward. The choice of “signal” and “blank” levers was randomized among the rats. If the rat made no response within 5 s of insertion of the response levers into the chamber, both levers retracted and a response “failure” was recorded. Each “signal” and “blank” pair was considered one test trial, and each test session consisted of 240 trials.

2.8 Data Analysis

The dependent measures for each test were evaluated for statistical significance by the analysis of variance. Litter was the unit of variance for treatment effects. DZN treatments were between subjects factors. Sex was a within litter factor and repeated measures for each test (e.g., test period, trial and error type) were within subjects factors. Interactions $p < 0.10$ were followed up by tests of the simple main effects within the interaction as recommended by Snedecor and Cochran (1967). Main effects or post hoc tests were performed, as needed based upon the omnibus ANOVA, using Dunnett’s method for multiple comparisons, unless specified otherwise. The cut-off for statistical significance was $p < 0.05$, two-tailed.

3 Results

3.1 Birth Data and Body Weight

Maternal exposure to DZN at 114–228 ug/day was not found to cause significant changes in maternal weight gain, litter size, sex-distribution, anogenital distance of the offspring, birth weight or later weight gain of the offspring (*data not shown*). However, increases in maternal weight across gestation resulted in a reduction in the relative dose of the DZN being delivered (see Table 1). Zero-order pharmacokinetic delivery of 0, 114 or 228 ug/day of DZN was selected to produce initial doses of 0, 0.5 and 1mg/kg/day DZN respectively. By the time of birth, these doses were reduced to 0, 0.35 and 0.74 mg/kg/day.

3.2 Elevated Plus Maze

With percent open arm time there was a significant main effect of maternal DZN exposure ($F(2,39) = 3.75$, $p < 0.05$) with post hoc Dunnett’s tests showing that the offspring of dams exposed to 228 ug/day DZN had a significantly elevated percent time in the open arms relative to control while the 114 ug/day DZN group did not have a significant effect (Fig. 1).

No sex-related effects were seen with present open arm time. No significant effects were seen with the number of center crosses (*data not shown*).

3.3 Locomotor Activity in the Figure-8 Apparatus

During adolescence, locomotor activity was assessed in the maternal DZN and control offspring in the Figure-8 apparatus. Figure 2a shows the typical habituation curve over the one-hour session for each of the treatment groups. The analysis showed an interaction of DZN $\times$ session block ($F(22,429) = 1.48, p < 0.08$) that prompted further analyses of DZN effects over the course of the test session. Dunnett's tests of the simple main effects of DZN at each time block showed significant ($p < 0.05$) degrees of hyperactivity in the offspring of dams exposed to 114 ug/day DZN on session blocks 1–4. The 228 ug/day DZN group showed hyperactivity relative to controls ($p < 0.05$) during the fourth block (Fig. 2). Figure 2b shows the effects of DZN on adolescent activity in male and female offspring. Females showed a more pervasive effect following maternal 114 ug/day DZN exposure, specifically showing significant ($p < 0.05$) hyperactivity in session blocks 1–4. Males in the 114 ug/day group showed significant ($p < 0.05$) hyperactivity only during session block 6.

The rats were re-tested for locomotor activity in the Figure-8 apparatus as adults, just after the attention test. The analysis showed an interaction of DZN $\times$ session block ($F(22,429) = 1.45, p < 0.09$) that prompted further analyses of DZN effects over the course of the test session. However, none of the tests of the simple main effects of maternal DZN exposure were found to be significant in any of the session blocks.

3.4 Novelty Suppressed Feeding

No significant effects of DZN exposure were seen with this measure of fear response (*data not shown*). None of the four measures: latency to begin eating, number of bouts of feeding, duration of feeding and amount of food eaten was significantly affected by maternal DZN exposure. There were significant main effects of sex for duration of feeding ($F(1,39) = 19.86, p < 0.0005$, male = 211.6 ± 12.4 , female = 149.8 ± 8.7) and amount of food eaten ($F(1,39) = 9.04, p < 0.005$, male = 1.76 ± 0.15 , female = 1.19 ± 0.12) and a nearly significant ($F(1,39) = 4.03, p < 0.06$, male = $91.26.7$, female 112.0 ± 9.1) effect of sex on response latency.

3.5: Novel Object Recognition

As expected, during the first five minutes of the session there was a significant main effect of novel vs. familiar object investigation ($F(1,22) = 31.00, p < 0.0005$). The main effect of maternal DZN exposure was not significant but the interaction of DZN $\times$ novel vs. familiar object ($F(2,22) = 3.12, p < 0.07$) prompted tests of the simple main effect of DZN on the novel-familiar difference. There was a reduction in the difference between novel and familiar object investigation in the offspring of female rats exposed to 228 ug/day of DZN. Dunnett's tests showed that maternal exposure to 228 ug/day DZN caused a significant ($p < 0.05$) decrease in the discrimination between the novel and familiar objects among the offspring relative to controls (Fig. 3). No treatment effects were observed in the second five minutes of the testing session.

3.6 16-Arm Radial Maze

No significant effects of maternal DZN exposure were seen with radial-arm maze choice accuracy or response latency (*data not shown*). The radial-arm maze test did perform as expected with significant improvement on the working memory component with training. There was a significant ($F(3,108) = 5.08$, $p < 0.005$) main effect of session block with a significant ($p < 0.05$) improvement from sessions 1–3 to 10–12. As typical in other studies, the males (10.28 ± 0.40) had significantly ($F(1,36) = 8.80$, $p < 0.01$) fewer error than females (11.64 ± 0.37). No significant effects of DZN were seen with either reference memory errors or response latency (seconds/arm entry).

3.7 Operant Visual Signal Detection Attention Task

No significant effects of maternal DZN exposure on percent correct performance in the attention task were seen (*data not shown*). The characteristic significant ($F(1,38) = 202.93$, $p < 0.0005$) improvement in percent correct from the first block of three sessions ($84.36 \pm 0.51\%$) to the second block of three sessions ($90.4 \pm 0.47\%$) of training was seen, indicating that the test was operating as designed. Also validating the test was the typical finding that the percent correct for correct rejection ($86.14 \pm 0.59\%$) was significantly higher than for percent hit ($82.97 \pm 0.74\%$). Finally, as typically seen on this task, response accuracy was high across all subjects, and a sex difference was detected in percent correct performance. Males ($88.67 \pm 0.46\%$) had slightly, but significantly ($F(1,38) = 15.11$, $p < 0.0005$) higher percent correct performance than females ($86.18 \pm 0.60\%$), though a mean difference of ~2.5% may not be biologically significant for this test.

4 Discussion

Chronic DZN exposure during perinatal development caused selective neurobehavioral impairments in rat offspring that persisted into adolescence and young adulthood. Exposures (0, 114 or 228 ug/day DZN) were delivered via osmotic minipumps implanted into the rat dams, allowing for maternal exposure from preconception until over a week post-parturition. Offspring exposure was chronic through gestation, and a degree of exposure was extended into the neonatal period via the dam's milk. This exposure period covers equivalent anatomical and nervous system development from conception and to birth in humans (Semple, 2013). These levels of exposure were selected to be low, meaning that they were not sufficient to produce observable health effects, including effects on maternal body weight and general health, as well as neonatal number, weight, morphology and survival. Correspondingly, a parallel analysis with siblings of these rats showed that these exposures were not sufficient to produce measurable cholinesterase inhibition on the day of birth (Slotkin et al., 2019). Although the dams and offspring showed no overt signs of toxicity, multiple aspects of offspring behavior were altered. These impairments were specific to certain functions including locomotor activity, anxiety-like avoidance and object recognition. Other aspects of neurobehavioral function were unaffected, including fear-like responding, spatial learning and memory, and signal detection.

In weanling animals (4 weeks of age), offspring of dams exposed to higher levels of DZN (228 ug/day) showed a greater preference for the open arms in the elevated plus maze

relative to controls. This test is typically used to assay anxiety-like behaviors in rodents based on a species-typical preference for areas that are dark or close to walls (Walf, 2007). As rodents are prey species, this avoidance of open spaces is generally understood to reduce the risk of predation. Consistent with this, control offspring spent a minority of their time in the open arms, accounting for ~30% of the session length. By contrast, offspring of dams exposed to 228 ug/day of DZN approached 50% preference for the open arms, indicating a lack of aversion to open spaces. This boldness may suggest an underlying deficit in the anxiety-like functions that typically suppress open area exploration and the risk of predation. This boldness is quite different from the effects of neonatal DZN exposure on elevated plus maze performance, which Roegge and colleagues (2008) reported as a male-specific reduction in open arm exploration, interpreted as an enhancement in the anxiety-like response. The present findings are more consistent with findings from a different avoidance task. Vatanparast and colleagues (2013) found that DZN exposure, either gestational or neonatal, sex-dependently impaired learned avoidance in the passive avoidance test, leading to shorter latencies to explore an area associatively-paired with shock and a greater exploration of that area. Taken together, these data suggest that DZN exposures early in life can modulate later risk-avoidance, but that the nature of those effects may be heavily dependent upon more specific aspects of the exposure, such as the critical windows impacted.

In addition to avoidance behaviors, perinatal DZN exposure was also found to cause significant locomotor hyperactivity in adolescence. Female offspring of dams exposed to 114 ug/day DZN also showed significantly higher activity levels early in the session. This pattern became attenuated by the fifth 5-min block of the session. Males showed a much less pronounced trend in the first half of the session which only reached significance in block 6. Overall, maternal exposure to 228 ug/day DZN produced a less robust effect on activity than the lower dose treatment, with elevations in activity only reaching significance in time block 4, despite very similar values on blocks 2 and 3. No effects of maternal exposure at 228 ug/day reached significance when the data was split by sex. The selectivity of the effect in the lower DZN concentration in females may indicate a non-monotonic effect, in which an increase in dose may meet thresholds for additional neurotoxic or compensatory effects which attenuate effects like hyperactivity. Such a pattern is not uncommon for environmental toxicants (Zoeller, 2015). Such a reversal effect is most plausible in the first time block (initial activity), although activity levels in the two DZN-exposed groups were quite similar in nearly all other time blocks. Given that seemingly minor mean differences between treatment groups still accounted for group differences in statistical significance, it may be more accurate to interpret the treatment-dependency as showing that the DZN treatment effect in this range is quite close to the threshold for detection in this test, with present sample size and variability.

Although adolescent offspring were sensitive to DZN-effects on locomotor activity, this effect appeared to be reversible over time. In the short term, this was evident through the within-session analysis of activity in the figure-8 maze. All animals generally habituated to the apparatus and became less active with extended exposure. Maternal exposure at 114 ug/day led to a high level of initial activity in females which reached control levels of activity by mid-session, indicating that the habituation functions in these animals were

highly functioning and effectively counteracted that hyperactivity effect, given sufficient time. The transient aspect is partially in agreement with findings observed by Timofeeva and colleagues (2008a), whereby neonatal DZN exposure led hyperactivity on the initial trials of a T-maze task which was subsequently attenuated. However, locomotor hyperactivity appears to be a more prominent aspect of the perinatal model, as neonatal DZN administrations did not affect activity in the figure-8 maze. In addition to habituation, it was noted that the hyperactivity effects observed in adolescence were reversible across development, as they did not persist into adulthood. This bears a resemblance to hyperactivity effects in the clinical literature which appear less stable across development than other neurobehavioral symptoms and frequently dissipate as patients mature (Martel, 2016; Willoughby, 2003). Taken together, the attenuation of DZN-induced hyperactivity suggests that these phenotypes may be most relevant to younger individuals with early life exposure to DZN and that interventions utilizing the natural flexibility of these responses may be beneficial.

With respect to early exposure to other OPs, rodent studies have shown somewhat mixed potential for OPs to modulate activity. For instance, studies of developmental exposure to chlorpyrifos (see review, Burke et al, 2017) have found evidence for hyperactivity (e.g. Ricceri, 2006), hypoactivity (Dam, 2000, Lee, 2015), or no effect (e.g. Carr, 2001). Studies with other compounds, such as parathion, malathion, and sumithion have similarly shown variable effects on activity (e.g. Lehotzky, 1989, N'Go, 2013, Timofeeva et al, 2008b). These prior studies have relied on a range of methods, time points and dose ranges for exposure, any of which may contribute to these mixed results. In light of this literature though, the present data lend support to the hypothesis that developing neural structures involved in activity regulation are sensitive to disruption by early OP exposures. This may also lend further support to epidemiological data connecting early OP exposures to symptoms of attention deficit hyperactivity disorder (ADHD) (Muñoz-Quezada, 2013).

The final behavioral effect observed in this study was on the novel object recognition test. Offspring of dams exposed to DZN at 228 ug/day showed a reduced preference between novel and familiar objects. In this test, a preference to investigate the novel object can be used as an indicator of memory for the familiar object (Antunes, 2012). Given that these animals show control levels of performance on the radial-arm maze, a test of spatial learning, working memory and reference memory, it does not appear that these subjects have a global deficit in learning and memory. Rather, there appears to be a more specific deficit in recognition memory, a subtle process that updates the salience of an object after its first presentation and allows future discriminations based on familiarity. This finding is similar to an effect observed by Win-Shwe and colleagues (2013), who reported that neonatal DZN exposure (0.5–5mg/kg/day, PND 8–11) in mice resulted in disruptions in novel object recognition in adolescence and adulthood. However, the specificity of this finding to recognition, but not other forms of learning and memory is different from neonatal effects previously explored in rats. Timofeeva and colleagues (2008a) reported that neonatal DZN (0.5mg/kg/day, PND1–4) led to a slower rate of learning in the radial arm maze. This suggests that the neonatal period may represent a more critical period for this particular effect and that DZN-exposed milk consumption during the same time period may not recapitulate this effect. Taken together, the potential for early DZN exposures to induce

cognitive and learning deficits is concerning, and should be examined more closely as a potential target for the development of pro-cognitive pharmacotherapies.

In the present study, it was found that females were uniquely impacted by early DZN exposure, in that they showed an additional behavioral effect which was not detected in males. Maternal DZN exposure at 228 ug/day led to female offspring that were significantly hyperactive relative to controls across the first four time-blocks of the session in the figure-8 maze. The finding that females may be more impacted by early DZN exposure than males is in contrast with earlier findings with neonatal DZN exposure models, where it appeared that that males, rather than females, were uniquely affected. More specifically, neonatal DZN exposure led to male-specific effects on anxiety-like responses, fear-like responses, prepulse inhibition and passive avoidance (Roegge, 2008; Timofeeva et al., 2008a; Vatanparast, 2013), while sparing activity in the figure-8 maze (Timofeeva et al., 2008a). At the neurochemical level, Slotkin et al (2008b) also observed that neonatal DZN selectively increased in serotonergic 5HT1A receptors among males but not females, further suggesting that males may be uniquely sensitive to certain effects of DZN exposure. However, the present data complements other studies of gestational DZN exposure in highlighting the sensitivity of females during that developmental period. Vatanparast and colleagues (2013) examined the effects of gestational injections of DZN (1 mg/kg/day DZN, GD 15–18) on behavior in adulthood and observed a female-specific deficit in passive avoidance performance not observed in males. Neurochemical assays run in parallel with the present study similarly indicated that females are particularly sensitive to perinatal DZN exposures. In this analysis, Slotkin and colleagues (2019) found perinatal DZN-induced reductions in presynaptic acetylcholine function, nicotinic acetylcholine receptors, and the serotonergic receptor 5HT1A. These effects were broadly observed in male and female offspring, but in each case, the females appeared to be more sensitive than males. This generally supports the practice of comparing male and female offspring within developmental neurotoxicity testing and further suggests that the susceptibility of male and female offspring to DZN exposure depends on the age of exposure, with unique female sensitivity in perinatal development and unique male sensitivity in neonatal development.

While the doses included in the present study are comparable to those reported in prior studies (scaled to 0.5–1 mg/kg/day DZN at pump implantation), it should be acknowledged that the “low concentrations” used in this animal model are not a direct model of “low levels” of environmental levels of exposure. The selection of concentrations below the threshold for observable health or reproductive effects provides a model of physiologically significant exposures that are likely to go without detection or intervention, due to a lack of clear indications of toxicity. As such, they demonstrate which neurological and functional systems are most likely to be impacted in subtle ways or in high risk subpopulations, such as those with point sources of contamination (e.g. occupational exposures) or high individual sensitivity to DZN. The scaling of these exposures for rats, rather than replicating human exposure exposures, also allows these models to compensate for inter-species differences in organophosphate metabolism and sensitivity (e.g. Timchalk et al., 2002, Vidair, 2004).

Overall, the present data suggests that early exposure to DZN may have meaningful and persistent effects on behavior. However, certain limitations in the design must be

acknowledged, as they may impact how these findings relate to others in the prior literature. One prominent limitation concerns the doses experienced by the offspring over the course of gestation and neonatal development. While the osmotic pumps are effective in producing a consistent DZN output over 5 weeks, the relative dose in the dam, and therefore the pups, is subject to change based on weight gain across pregnancy. Therefore, a given level of exposure (ug/day) may be represented by one number, calculated per initial body weight, but the internal dose of the animal is progressively reduced across time. This shift is quantifiable based on the dam's weight, but will vary across litters and therefore may contribute to the within-group variability in a given study. The advantage to osmotic pumps is that unlike injections or gavage, the implanted pumps require minimal handling and stress over the course of pregnancy, but the inability to modulate the dose day by day is a trade-off with this methodology. Unfortunately, gestational kinetic data is not available for this study, so certain aspects of the exposure remain to be described, including the relative doses of parent and metabolite compounds in the maternal blood stream and verification of changes in internal dose in the dam and pups across gestation. Although osmotic pumps offer a promising way to deliver drug in a low stress model, future studies will be needed to directly assess the dosimetry characteristics of osmotic pump delivery in gestation. Additionally, future studies using this methodology will need to include dosimetry assays so that they can be meaningfully compared with similar studies using other methods of exposure.

With respect to milk-delivery of drug to neonatal rats, the relative dosage of DZN in the pups was not verified in the present study, therefore the exact level of exposure during this period is not known. Pharmacokinetic studies of chlorpyrifos exposure via milk suggest that the dose received by a neonate may to be considerably lower than that of the dam (5mg/kg/day in the dam vs. 0.12mg/kg/day in pups) (Mattsson, 2000). As a result, it is not clear whether the DZN exposure experienced by each neonate is sufficient to impact the results in this study. Future testing with osmotic pump delivery should verify the internal concentration of drug due to milk-exposure and/or supplement the neonates with drug to better model the effects of DZN on key developmental events which occur in neonatal rats and third-trimester humans. A final limitation is the lack of a sham (or no-vehicle) control group. Lipophilic compounds like DZN require a solvent, in this case DMSO, in order to be effectively delivered. The slow infusion of concentrated DZN solution allowed the level of DMSO exposure to remain low (e.g. 11uL/kg/day per 200 gram rat), at levels well below those previously shown to be neurotoxic in rodents (Hanslick, 2009), however the presence of the solvent does present a unique confound which was not directly addressed in this study.

In summary, the present study detected multiple persistent consequences of perinatal DZN exposure in behavior later in life at low levels of exposure, with minimal cholinesterase inhibition. These behavioral changes spanned affective, locomotor and cognitive functions, and consisted of disruptions in open area avoidance in the elevated plus maze, brief hyperactivity in the figure-8 maze and impaired object recognition. The brief hyperactivity effect was most apparent in offspring of dams with the lower level of DZN exposure and was most apparent among female offspring. These varied behavioral changes corresponded with underlying effects on key neurotransmitter systems (Slotkin et al., 2019) and included deficits in presynaptic acetylcholine activity, reductions in nicotinic acetylcholine receptors and reductions in serotonergic 5HT1A receptors. These effects were more pronounced in

cortical and hippocampal tissues than other brain regions and were more pronounced in females than males. The neurobehavioral consequences of gestational and perinatal DZN exposure complement, but remain distinct from, the consequences of specific exposures during the third-trimester equivalent of brain development, modeled through neonatal exposures in rodents. These data underscore the potential risk posed by OP insecticides like DZN on the developing nervous system and further highlight the need for new and effective methods to identify and treat the long-term effects on neurotoxic OP exposures.

Acknowledgement

This research was sponsored by the Duke University Superfund Center (ES010356).

Abbreviations

DMSO	Dimethyl Sulfoxide
DZN	Diazinon
GD	Gestational day
OP	Organophosphate
PND	Postnatal day

References

- Antunes M, & Biala G (2012). The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive Processing*, 13(2), 93–110. [PubMed: 22160349]
- Bouchard MF, Chevrier J, Harley KG, Kogut K, Vedar M, Calderon N, Trujillo C, Johnson C, Bradman A, Barr DB & Eskenazi B (2011). Prenatal exposure to organophosphate pesticides and IQ in 7-year-old children. *Environmental Health Perspectives*, 119(8), 1189–1195. [PubMed: 21507776]
- Carr RL, Chambers HW, Guarisco JA, Richardson JR, Tang J, & Chambers JE (2001). Effects of repeated oral postnatal exposure to chlorpyrifos on open-field behavior in juvenile rats. *Toxicological Sciences*, 59(2), 260–267. [PubMed: 11158719]
- Cauley M, Hall BJ, Abreu-Villaça Y, Junaid S, White H, Kiany A, Slotkin TA and Levin ED (2018). Critical developmental periods for effects of low-level tobacco smoke exposure on behavioral performance. *Neurotoxicology*, 68, 81–87. [PubMed: 30036564]
- Dam K, Seidler FJ, & Slotkin TA (2000). Chlorpyrifos exposure during a critical neonatal period elicits gender-selective deficits in the development of coordination skills and locomotor activity. *Developmental Brain Research*, 121(2), 179–187. [PubMed: 10876030]
- Engel SM, Wetmur J, Chen J, Zhu C, Barr DB, Canfield RL, & Wolff MS (2011). Prenatal exposure to organophosphates, paraoxonase 1, and cognitive development in childhood. *Environmental Health Perspectives*, 119(8), 1182–1188. [PubMed: 21507778]
- Fukuto TR (1990). Mechanism of action of organophosphorus and carbamate insecticides. *Environmental Health Perspectives*, 87, 245–254. [PubMed: 2176588]
- Hanslick JL, Lau K, Noguchi KK, Olney JW, Zorumski CF, Mennerick S, & Farber NB (2009). Dimethyl sulfoxide (DMSO) produces widespread apoptosis in the developing central nervous system. *Neurobiology of Disease*, 34(1), 1–10. [PubMed: 19100327]
- Lee I, Eriksson P, Fredriksson A, Buratovic S, & Viberg H. (2015). Developmental neurotoxic effects of two pesticides: Behavior and biomolecular studies on chlorpyrifos and carbaryl. *Toxicology and applied pharmacology*, 288(3), 429–438. [PubMed: 26314619]

- Lehotzky K, Szeberenyi MJ, & Kiss A (1989). Behavioral consequences of prenatal exposure to the organophosphate insecticide sumithion. *Neurotoxicology and Teratology*, 11(3), 321–324. [PubMed: 2755428]
- Levin ED, Addy N, Baruah A, Elias A, Christopher NC, Seidler FJ, & Slotkin TA (2002). Prenatal chlorpyrifos exposure in rats causes persistent behavioral alterations. *Neurotoxicology and Teratology*, 24(6), 733–741. [PubMed: 12460655]
- Levin ED, Addy N, Nakajima A, Christopher NC, Seidler FJ, & Slotkin TA (2001). Persistent behavioral consequences of neonatal chlorpyrifos exposure in rats. *Developmental Brain Research*, 130(1), 83–89. [PubMed: 11557096]
- Mangas I, Estevez J, Vilanova E, & França TC (2017). New insights on molecular interactions of organophosphorus pesticides with esterases. *Toxicology*, 376, 30–43. [PubMed: 27311923]
- Martel MM, Levinson CA, Langer JK, & Nigg JT (2016). A network analysis of developmental change in ADHD symptom structure from preschool to adulthood. *Clinical Psychological Science*, 4(6), 988–1001. [PubMed: 28083448]
- Mattsson JL, Maurissen JP, Nolan RJ, & Brzak KA (2000). Lack of differential sensitivity to cholinesterase inhibition in fetuses and neonates compared to dams treated perinatally with chlorpyrifos. *Toxicological Sciences*, 53(2), 438–446. [PubMed: 10696792]
- Mostafalou S, & Abdollahi M (2018). The link of organophosphorus pesticides with neurodegenerative and neurodevelopmental diseases based on evidence and mechanisms. *Toxicology*, 409, 44–52. [PubMed: 30053494]
- Muñoz-Quezada MT, Lucero BA, Barr DB, Steenland K, Levy K, Ryan PB, Iglesias V, Alvarado S, Concha C, Rojas E & Vega C (2013). Neurodevelopmental effects in children associated with exposure to organophosphate pesticides: a systematic review. *Neurotoxicology*, 39, 158–168. [PubMed: 24121005]
- N’Go PK, Azzaoui FZ, Ahami AOT, Soro PR, Najimi M, & Chigr F (2013). Developmental effects of Malathion exposure on locomotor activity and anxiety-like behavior in Wistar rat. *Health*, 5(3), 603–611.
- Pope C, Karanth S, & Liu J (2005). Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. *Environmental Toxicology and Pharmacology*, 19(3), 433–446. [PubMed: 21783509]
- Rauh VA, Perera FP, Horton MK, Whyatt RM, Bansal R, Hao X, Liu J, Barr DB, Slotkin TA & Peterson BS (2012). Brain anomalies in children exposed prenatally to a common organophosphate pesticide. *Proceedings of the National Academy of Sciences*, 109(20), 7871–7876.
- Ricceri L, Venerosi A, Capone F, Cometa MF, Lorenzini P, Fortuna S, & Calamandrei G (2006). Developmental neurotoxicity of organophosphorous pesticides: fetal and neonatal exposure to chlorpyrifos alters sex-specific behaviors at adulthood in mice. *Toxicological Sciences*, 93(1), 105–113. [PubMed: 16760416]
- Roegge CS, Timofeeva OA, Seidler FJ, Slotkin TA, & Levin ED (2008). Developmental diazinon neurotoxicity in rats: later effects on emotional response. *Brain Research Bulletin*, 75(1), 166–172. [PubMed: 18158111]
- Salama AK (2017). Lactational Exposure to Pesticides: A Review. *Toxicology Open Access*, 9(3).
- Semple BD, Blomgren K, Gimlin K, Ferriero DM, & Noble-Haeusslein LJ (2013). Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Progress in Neurobiology*, 106, 1–16. [PubMed: 23583307]
- Slotkin TA, Bodwell BE, Levin ED, & Seidler FJ (2008a). Neonatal exposure to low doses of diazinon: long-term effects on neural cell development and acetylcholine systems. *Environmental Health Perspectives*, 116(3), 340–348. [PubMed: 18335101]
- Slotkin TA, Ryde IT, Levin ED, & Seidler FJ. (2008b). Developmental neurotoxicity of low dose diazinon exposure of neonatal rats: effects on serotonin systems in adolescence and adulthood. *Brain Research Bulletin*, 75(5), 640–647. [PubMed: 18355640]
- Slotkin TA, Skavicus S, Ko A, Levin ED, & Seidler FJ (2019). Perinatal diazinon exposure compromises the development of acetylcholine and serotonin systems. *Toxicology*, 424, 152240. [PubMed: 31251962]

- Slotkin TA, Tate CA, Ryde IT, Levin ED, & Seidler FJ (2006). Organophosphate insecticides target the serotonergic system in developing rat brain regions: disparate effects of diazinon and parathion at doses spanning the threshold for cholinesterase inhibition. *Environmental Health Perspectives*, 114(10), 1542–1546. [PubMed: 17035140]
- Snedecor GW, & Cochran WG. (1967). *Statistical Methods*. IA: Iowa State College Press.
- Timofeeva OA, Roegge CS, Seidler FJ, Slotkin TA, & Levin ED (2008a). Persistent cognitive alterations in rats after early postnatal exposure to low doses of the organophosphate pesticide, diazinon. *Neurotoxicology and Teratology*, 30(1), 38–45. [PubMed: 18096363]
- Timofeeva OA, Sanders D, Seemann K, Yang L, Hermanson D, Regenbogen S, Agoos S, Kallepalli A, Rastogi A, Braddy D & Wells C (2008b). Persistent behavioral alterations in rats neonatally exposed to low doses of the organophosphate pesticide, parathion. *Brain Research Bulletin*, 77(6), 404–411. [PubMed: 18817854]
- Vatanparast J, Naseh M, Baniasadi M, & Haghdoost-Yazdi H. (2013). Developmental exposure to chlorpyrifos and diazinon differentially affect passive avoidance performance and nitric oxide synthase-containing neurons in the basolateral complex of the amygdala. *Brain Research*, 1494, 17–27. [PubMed: 23219576]
- Walf AA, & Frye CA (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols*, 2(2), 322. [PubMed: 17406592]
- Willoughby MT (2003). Developmental course of ADHD symptomatology during the transition from childhood to adolescence: a review with recommendations. *Journal of Child Psychology and Psychiatry*, 44(1), 88–106. [PubMed: 12553414]
- Win-Shwe TT, Nakajima D, Ahmed S, & Fujimaki H. (2013). Impairment of novel object recognition in adulthood after neonatal exposure to diazinon. *Archives of Toxicology*, 87(4), 753–762. [PubMed: 23212306]
- Zoeller RT, & Vandenberg LN (2015). Assessing dose-response relationships for endocrine disrupting chemicals (EDCs): a focus on non-monotonicity. *Environmental Health*, 14(1), 42. [PubMed: 25971795]

Gestational and Perinatal Diazinon Exposure Effects on Elevated Plus Maze: Percent Open Arm Time

Figure 1.

Elevated Plus Maze: percent open arm time (mean $\pm$ sem). Asterisk (*) indicates a significant group difference relative to controls, collapsed across sex.

A

B

Figure 2.

Figure-8 Apparatus: Locomotor activity results are plotted two ways. A. Adolescent mean locomotor activity (mean $\pm$ sem), shown collapsed across sex. Asterisk (*) indicates a significant group difference relative to controls, collapsed across sex. B. Figure-8 Apparatus: Adolescent locomotor activity, shown split by sex (mean $\pm$ sem). Asterisk (*) indicates a significant group difference relative to controls.

Gestational and Perinatal Diazinon Exposure Effects on Novel Object Recognition Test Seconds of Investigation: Min 1-5

Figure 3.

Novel Object Recognition: Novel vs. Familiar investigation, Minutes 1–5 (mean ± sem).

Asterisk (*) indicates a significant group difference in discrimination (novel investigation – familiar investigation) relative to controls, collapsed across sex.

Table 1.
Maternal Dosing and Body Weight.

Dams gained substantial weight across the gestational period, which led to a reduction in the relative dose delivered by the osmotic pumps. Drug received (ng/day DZN) is reported for each group (mean $\pm$ sem), as well as weight and calculated DZN dose at the time of pump implantation and parturition.

	<u>Drug delivery (ng/day)</u>	<u>Maternal Body Weight (in g)</u>		<u>Maternal Dose (mg/kg/day)</u>	
DZN Treatment	<i><u>Constant</u></i>	<i><u>Implantation</u></i>	<i><u>Parturition</u></i>	<i><u>Implantation</u></i>	<i><u>Parturition</u></i>
<i><u>Control</u></i>	0	227.0 $\pm$ 6.6	305.0 $\pm$ 7.5	0	0
<i><u>Low</u></i>	113.5 $\pm$ 3.7	227.1 $\pm$ 7.3	324.5 $\pm$ 6.7	0.50 $\pm$ 0.0	0.35 $\pm$ 0.01
<i><u>High</u></i>	228.4 $\pm$ 5.1	228.4 $\pm$ 5.1	307.5 $\pm$ 6.8	1.00 $\pm$ 0.0	0.74 $\pm$ 0.01