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Perinatal behavioral and immunological effects
of prenatal diisopropyl fluorophosphate exposure
in domestic fowl

By

Michael Raymond Baker

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Co-majors: Psychology; Neuroscience
Major Professor: Richard A. Hughes

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Ames, Iowa
1997
Graduate College
Iowa State University

This is to certify that the Doctoral dissertation of

Michael Raymond Baker

Has met the dissertation requirements

of Iowa State University

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Major Professor

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For the Co-major Program

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Signature was redacted for privacy.
For the Graduate College
This dissertation is dedicated to my parents,
Betty and Raymond.
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ABSTRACT

Experiment 1: Chick embryos received a single DFP exposure (0, 50, 100, 200, 400, 800, 1600, 3200 μg/kg) on embryogenesis day (E) 11. DFP was fatal to all embryos at 400 μg/kg and above, but did not affect hatch success at lower doses. Prenatal DFP reduced hatch weight and increased weight by posthatch day (PHD) 14. Prenatal DFP produced sex- and dose-dependent changes in distress vocalizations (DVs), but not activity on PHD 1. DFP increased tonic immobility duration (TI) on PHD 14. DFP increased lipopolysaccharide-induced lymphocyte proliferation in blood harvested on PHD 16 in females.

Experiment 2: Daily DFP (0, 50, 100, or 200 μg/kg) on E 10-12 reduced hatch success. DFP reduced hatch weights in males, but not females. Prenatal DFP, 200 μg/kg, increased weight by PHD 14. DFP did not affect DVs, but increased activity in males at 50 μg/kg and females at 100 μg/kg on PHD 1. DFP increased TI in females, but not males, on PHD 14. DFP decreased pokeweed mitogen-induced and increased lipopolysaccharide-induced lymphocyte proliferation in blood and thymus (respectively) harvested on PHD 16.

Experiment 3: Daily DFP (0, 100, or 200 μg/kg) on E 10-12 reduced hatch success, but did not affect weight at hatch or PHD 14. Chicks received 0.0, 0.5, or 1.5 mg/kg scopolamine 30 min before behavioral tests. Prenatal DFP
(100 μg/kg) decreased DVs and 1.5 mg/kg scopolamine reversed the effect on PHD 1; there were no activity effects.

Scopolamine, 0.5 mg/kg reduced TI except at 200 μg/kg DFP on PHD 14.

Experiment 4: Daily DFP (0, 150, or 200 μg/kg) on E 10-12 did not affect hatch success or hatch weight, but increased weight by PHD 14. Chicks received 0.0, 0.25, or 0.5 mg/kg physostigmine 30 min before behavioral tests. On PHD 1, 0.25 physostigmine increased DVs in chicks at 150 μg/kg DFP; activity was not affected. DFP increased TI in females, but not males, and PHY increased TI in females at 0 μg/kg DFP on PHD 18.

These data demonstrate perinatal consequences of prenatal DFP that endure into the third week posthatch and are sensitive to cholinergic manipulation.
INTRODUCTION

Organophosphate (OP) pesticides are among the most toxic and widely used pesticides in the world (Mearns, Dunn, & Lees-Haley, 1994; Michalek & Pintor, 1990; Overstreet & Schiller, 1992). In 1983, OPs poisoned 2 million people worldwide and resulted in 40 thousand deaths (Mearns et al., 1994). The current widespread use of OP in agriculture provides for direct exposure of production workers, applicators, and field workers. A much wider population is at risk for low level exposure through food and drinking water that contain OP residues (Mearns et al., 1994; Michalek & Pintor, 1990; Overstreet & Schiller, 1992).

An extensive literature exists describing the effects of acute OP poisoning in adult organisms. Organophosphates alter neurotransmission in cholinergic systems and affect both behavioral and immune system responses (Mearns et al., 1994). Considerably less investigation has focused on the developmental effects of low level OP exposure. In particular, little is known of possible perinatal effects of prenatal OP exposure. Maternal exposure to various environmental pollutants during embryogenesis can adversely affect perinatal behavior of offspring (Michalek & Pintor, 1990). Behavioral changes occur in offspring at doses that do not produce overt maternal behavioral or physiological indications (Hughes, Belser, & Brett, 1975).
Characterization of perinatal and postnatal consequences of low level OP exposure is an important consideration in the development of strategies designed to prevent and mitigate adverse effects of OP neurotoxicity.

Organophosphates can enter the body via inhalation, ingestion, or transdermal absorption (Mearns et al., 1994, Michalek & Pintor, 1990). Organophosphates increase concentrations of the neurotransmitter acetylcholine (ACh) in the synaptic cleft by inhibiting acetylcholinesterase (AChE). Acetylcholinesterase degrades ACh released at synapses, terminating its activity (Feldman, Meyer, & Quenzer, 1997; Rang, Dale, Ritter, & Gardner, 1995). A number of OP compounds (e.g., diisopropyl fluorophosphate, parathion, soman) irreversibly inhibit the activity of AChE by phosphorylating the enzyme (Feldman et al. 1997; Overstreet & Schiller, 1992). Although OPs slowly dissociate from AChE, recovery of enzyme function depends on new synthesis, a process that can take weeks (Feldman et al., 1997; Rang et al., 1995). Inhibition of the enzyme results in excessive amounts of ACh and prevents repolarization of the membrane of the postsynaptic cell (Michalek & Pintor, 1990). The apparent irreversibility of AChE inhibition by OPs (Overstreet & Schiller, 1992) presents a potential for cumulative developmental effects of low level pre- and perinatal OP exposure.
The distribution of cholinergic neurons and receptors throughout the nervous and immune systems suggests a potential for a wide array of OP poisoning effects. Cholinergic synapses are found peripherally at neuromuscular junctions and in the autonomic nervous system, and centrally in the basal forebrain, reticular formation, striatum, and hippocampus (Fibiger & Vincent, 1987). Additionally, cholinergic receptors and/or AChE have been found in abundance in the spleen, thymus, lymph nodes, and other lymphoid accumulations (Felten & Felten, 1991).

Onset of symptoms associated with acute OP poisoning occurs within 4 to 8 hours and can endure for several weeks. Physical effects of mild OP intoxication include nausea, vomiting, abdominal pain, numbness in the extremities, fatigue, headaches, excessive salivation, and diarrhea. Victims of moderate intoxication experience more intense manifestation of the above symptoms accompanied by generalized weakness, constriction of pupils, and muscle spasms. Severe intoxication may result in blurred vision, unconsciousness, paralysis, respiratory depression, and death. Psychological effects associated with OP poisoning include cognitive impairment (e.g., memory, concentration, and visuospatial information processing deficits), anxiety, depression, irritability, and restlessness (Mearns et al., 1994).
Cognitive impairments have been demonstrated not only in individuals suffering acute OP poisoning, but also in otherwise symptom-free individuals. Farmers and pesticide applicators routinely exposed to OPs scored significantly higher on the Taylor Manifest Anxiety scale than matched controls (Mearns et al., 1994, Levin et al., 1976). In a 1992 study, 21 migrant farm workers that were twice poisoned, 1 and 5 years prior to the study, scored lower on motor speed and visuospatial memory and higher on anxiety than did controls (Mearns et al., 1994, Reidy et al., 1992). Individuals poisoned an average of 9 years prior to testing exhibited a higher incidence of depression and poorer performance on the three-pairs-of-items memory test. Subjects also scored higher on the Minnesota Multiphasic Personality Inventory (MMPI) validity scales for paranoia and introversion. The results of studies reporting human cognitive impairments suffer from confounding factors such as involvement in litigation (a possible source of anxiety), determination of exposure doses, and absence of blind evaluation procedures. Controlled laboratory studies using animal models found cognitive impairments similar to those reported in humans. Rats that received daily exposure to OPs for 14 days exhibited spatial memory impairment and downregulation of central muscarinic ACh receptors. Male rats showed impairment of behaviors correlated with

Organophosphate exposure also alters immune system responses in adults. Interleukin 2 (IL2) signaling is disrupted by OP pesticide exposure. Organophosphates inhibit IL2-dependent proliferation of human natural killer (NK) cells, mouse CTLL2 cells, and enhancement of target cell killing by human NK cells in vitro (Casale, Vennerstrom, Bavari, & Wang, 1993). Also, human bone marrow cells exposed to active OP metabolites in vitro showed dose-dependent suppression of colony formation by erythrocytes and granulocyte-macrophage progenitors (Gallicchio, Casale, & Watts, 1987). Depression of hematopoiesis of lymphoid and myeloid cells would be expected to compromise immunoreactivity in response to an immunogenic challenge. This evidence suggests that OP-induced alteration of immunoreactivity could result in a greater susceptibility to infectious diseases and cancer. A literature search yielded no information regarding prenatal OP effects on perinatal immune system function.

There is an extensive literature describing adverse embryo morphological and physiological effects of OP exposure. The first report of human malformation associated
with prenatal OP exposure involved a pregnant (4 weeks) farm worker who was among 35 workers poisoned by OPs (Romero, Barnett, & Midtling, 1989). At birth the female infant exhibited brain atrophy, multiple cardiac defects, facial anomalies, and died after 14 days. There was no family history of birth defects. Human fetal brains (8-10 weeks) exposed to OP in vitro exhibited inhibition of AChE (Banerjee, Ghosh, Mitra, & Bhattacharya, 1991). A study of cultured chick dorsal root ganglia demonstrated that OP significantly altered cell morphology and cell membrane integrity (Tuler & Bowen, 1989). An in vivo study of prenatal OP exposure in chicks demonstrated perinatal ataxia (Farage-Elawar & Francis, 1988). Physical anomalies (tibiotarsal angulations and poor feathering) were observed on day 17 of incubation in chicks exposed in ovo to OP on day 3 of incubation (Uyeki, Doull, Cheng, & Misawa, 1982). In vitro studies have demonstrated increased sensitivity of muscle tissue to AChE inhibitors and downregulation of muscarinic ACh receptors. These more subtle effects have occurred in the absence of overt behavioral indications of OP poisoning (Melchers & Van Helden, 1990). These data suggest that pregnant women exhibiting no overt behavioral effects of OP poisoning might have physiologically significant levels of OP contamination that could adversely affect fetal development.
The present study was designed to examine perinatal consequences of prenatal OP exposure in a precocial avian species. Precocial domestic fowl chicks are particularly well suited for perinatal behavioral evaluation independent of maternal confounds (Rogers, 1995); i.e., the eggs and not the hen are exposed. The embryology of domestic fowl is well defined due to its widespread use in developmental biology. The extensive embryological data-base makes the chick embryo an excellent model for toxicity screening. Chemicals have been injected into embryos as early as day 0 and as late as day 15 of incubation. Additionally, the chicken model is preferred over rodent models regarding the clinical signs of OP poisoning. The chicken model better reflects the diverse central and peripheral effects of OPs induced in humans than do rodent models (Farage-Elawar & Francis, 1988). The current study was designed to investigate perinatal effects of prenatal exposure to an OP, diisopropyl fluorophosphate (DFP), using the chick model. Our laboratory has previously studied behavioral effects of prenatal methyl mercury (Hughes, Belser, & Brett, 1975), and more recently effects of prenatal cocaine on perinatal behavior and immune system function in domestic fowl (Cunnick et al., 1994; Hughes et al., 1994a; Hughes et al., 1994b). Cocaine injected into the airspace of fertile eggs at the midpoint of embryogenesis altered perinatal social
(activity and isolation-induced vocalizations) and defensive behaviors (tonic immobility), as well as immunoreactivity to an immunogenic challenge (Hughes et al., 1994a; Hughes et al., 1994b).

Administration of DFP during embryogenesis might also reveal alterations in these responses. Exposure to OPs alter cholinergic systems (Feldman et al., 1997; Rang et al., 1995). In the present study, prenatal DFP effects on perinatal activity and distress vocalizations (DVs) were evaluated during a 5-min isolation period on posthatch day (PHD) 1 and on a tonic immobility (TI) test on PHD 14.

Acute OP poisoning produces anxiety in humans that is evident up to 5 years after exposure (Mearns et al., 1994; Levin et al., 1976). The activity and distress vocalizations (DVs) measures were chosen because social stress alters these responses in chicks. Chicks imprint on cohorts shortly after hatching. Social isolation is stressful for chicks and DVs are an index of stress (Fullerton, Berryman, & Slutkin, 1959; Panksepp, Herman, Conner, Bishop, & Scott, 1978; Sufka & Hughes, 1991). In addition to the stress-induced effects, activity in chicks is also sensitive to cholinergic manipulation. Cholinergic agonists decrease and antagonists increase activity during open field behavior (Genty, Faure, & Mills, 1985; Ksir, 1978; Sanberg, 1983).
The tonic immobility (TI) response is a state of somatic immobility induced by brief physical restraint (Pestrude, 1977; Gallup, 1977). This TI response is sensitive to cholinergic manipulation (Gallup et al., 1983; Hatton, Tinkle, Lanthorn, & Meyer, 1978; Hicks, 1976; Hughes, 1982; Meyer, 1975; Thompson, Piroch, Fallen, Hatton, 1974; Woodruff, 1976) and has been observed in a wide variety of invertebrate (Ratner, 1977) and vertebrate (Pestrude, 1977) species. When an animal is physically restrained it will struggle briefly and then become immobile. The immobile posture persists after restraint is terminated for a period of minutes to hours (Gallup, 1977). Tonic immobility is thought to be an adaptive response by prey during a predatory encounter. Struggling and vocalizing by prey are important stimuli for maintenance of a predatory attack. Attack behaviors of predators are inversely related to the duration of immobility of prey (Thompson, Foltin, Boylan, Sweet, Graves, & Lowitz, 1981; Gallup, Boren, Suarez, Wallnau, 1983). Gallup (1977) characterizes tonic immobility as a fear response. Humans exhibit similar behavior associated with rape-induced paralysis (Suarez & Gallup, 1979) and catatonic schizophrenia (Maser & Gallup, 1974).

Behavioral manipulations that induce fear increase immobility duration and those that attenuate fear reduce TI
duration. Immobility duration is enhanced by predatory encounters (including exposure to humans as potential predators), simulated predatory encounters (e.g., a stuffed hawk, artificial eyes), loud noises, electric shock, suspension over a visual cliff, and conditioned aversive stimuli (Gallup, 1977). Habituation to induction, handling, conditioned safety cues, and tranquilizer administration decrease immobility duration (Crawford, 1977; Gallup, 1977; Gallup et al., 1983; Thompson, 1977).

Tonic immobility is sensitive to manipulation of neurotransmitter systems other than the cholinergic system. Drugs that alter monoaminergic (Gallup et al., 1983; Maser, Gallup, & Hicks, 1975; Hughes, 1985; Thompson & Joseph, 1978) and opioid (Wallnau & Gallup, 1979; Peters & Hughes, 1978) activity also alter immobility duration to varying degrees. The strongest evidence for monoaminergic involvement implicates serotonergic mechanisms of tonic immobility (Gallup et al., 1983). Centrally administered serotonin (5-HT) agonists increase and antagonists decrease immobility duration in chickens (Boren, Gallup, Suarez, Wallnau, & Gagliardi, 1979; Gallup et al., 1983; Gallup & Wallnau, 1977; Hatton, Tinkle, Lanthorn, & Meyer, 1978) and have opposite effects in rabbits (Hatton, Woodruff, & Meyer, 1975).
Cholinergic manipulations exhibit the same avian-mammalian reversal of effects. Physostigmine (Gallup et al., 1983; Thompson et al., 1974) and pilocarpine (Sanberg, 1983), cholinergic agonists, potentiate TI in chickens and ducks and attenuate TI in rabbits and guinea pigs (Hatton et al., 1975; Woodruff, Hatton, Frankl, & Meyer, 1976). Cholinergic antagonists (e.g., scopolamine, atropine) decrease immobility duration in these avian species (Hicks, 1976; Hennig, McIntyre, Moriarity, & Picerno, 1988; Hughes, 1982; Thompson et al., 1974) and enhance TI in mammals (Hatton et al., 1975; Woodruff et al., 1976). Further, cholinergic manipulation of TI is mediated by central muscarinic mechanisms (Thompson et al., 1974). Scopolamine and atropine, both muscarinic receptor antagonists, attenuate TI in chickens, but their methyl analogs, which act only peripherally, do not (Hughes, 1982; Thompson et al., 1974). Cholinergic antagonists have similar effects on locomotor behavior. Scopolamine decreased locomotor behavior in chickens (Ksir, 1978; Sanberg, 1983) and rats (Sanberg, Pisa, & Fibiger, 1981). Atropine decreased open field behavior of chicks (Genty et al., 1985).

Because there has been little research on postnatal effects of prenatal DFP exposure, it is difficult to predict the direction of behavioral effects in this model. Drugs often have opposite effects depending on variables such as
dose and time course, as well as age and physiological state of the organism. However, given the known effects of cholinergic activity on tonic immobility in chicks and the potentiation of acetylcholine by DFP, some tentative predictions can be made. Diisopropyl fluorophosphate increases cholinergic concentrations by irreversibly inhibiting AChE. Cholinergic neurons are present in chicken embryos by embryological day (E) 7 (Rogers, 1995). Normal nervous system development depends on appropriate levels of embryonic neural activity. Prenatal exposure to DFP would be expected to increase embryonic cholinergic activity and might ultimately alter activity-dependent central nervous system development. If prenatal DFP-induced potentiation of cholinergic activity persists into postnatal life, tonic immobility duration in exposed chicks should be enhanced compared to controls. Similarly, locomotor activity in exposed chicks should be inhibited by prenatal DFP exposure. Because cholinergic mechanisms have not been implicated in control of vocalizations, prediction of prenatal DFP effects would be speculative. However, DVs are negatively correlated with activity during isolation. Therefore, if prenatal DFP decreases activity during isolation, DVs might be expected to increase (Sufka & Hughes, 1991).
EXPERIMENT 1

The purpose of this experiment was to determine DFP dose parameters and possible sex differences in hatch success. Additionally, behavioral and immunological effects of prenatal DFP were evaluated for those treatment groups in which sufficient chicks hatched and survived (longer than 3 days). The responses (DVs, activity changes, TI, and immunoreactivity) evaluated in this study are considered adaptive responses.

Diisopropyl fluorophosphate in sterile saline vehicle (0, 50, 100, 200, 400, 800, 1600, or 3200 mg/kg; n = 30 per cell) was injected into the airspace of fertile eggs on E11. Day 11 is the midpoint of embryogenesis and was chosen because it corresponds to a period of rapid central nervous system (Romanoff, 1960) and immune system (Sharma, 1991) development in this species.

Method

Subjects

Fertile White Leghorn eggs (n=240) were obtained from Hyline Inc., Dallas Center, IA. Upon hatch, chicks (N=80) were housed in 46 x 26 x 31 cm opaque plastic cages (n=6-7 per cage) with floors and lids constructed of 2x2 hardware cloth. The colony room was maintained at a temperature of 32.0 ± 1.0 °C during the first week, and at 29.0 ± 1.0 °C, thereafter. Overhead fluorescent lights provided continuous
illumination. Food (Broiler Ration 3281-18C Medicated, Master Mix Poultry Feed, Fort Wayne, IN) and water were available ad lib.

**Apparatus**

**Incubation.** Incubation of eggs occurred in a dark forced air incubator (model 1202, G.Q.F. Manufacturing, Savannah, GA) equipped with an electronic thermostat-regulated heating element, an electronic thermometer/hygrometer, an automatic egg turner, and a capacity of 270 chicken eggs. The incubator was maintained at 38.0 ± 0.5 °C and 56% relative humidity. Chicks were hatched (last 3 days of incubation) in an incubator identical to the one above but without an egg turner (model 1250, G.Q.F. Manufacturing, Savannah, GA) maintained at 37.0 ± 0.5 °C and 66% relative humidity.

**Isolation.** Two test units (38 x 21 x 16 cm) were constructed of 2 x 2 hardware cloth. Three photocells (6.5 cm apart, 5 cm from the floor) monitored activity in each unit. A microphone suspended from the top-center of each unit monitored vocalizations. Each unit was housed in a fan-ventilated, sound-attenuated chamber (BRS/LVE, model LEC-006) illuminated by a 20 W fluorescent light centered on the ceiling of the BRS/LVE chamber. Computer interface modules and data collection software (MED Associates, East Fairfield, VT) recorded vocalizations and photocell
interruptions. White noise masked extraneous environmental noises in the experimental room.

**Tonic immobility.** Tonic immobility tests occurred in plastic tubs (50 x 38 x 20 cm) lined with newspapers. White noise masked environmental noises.

**Procedure**

The experimental design for weight data obtained on PHD 1 and 14 was a 4 (Dose) x 2 (Sex) x 2 (Age) factorial. The design for behavioral data was a 4 (Dose) x 2 (Sex) factorial. Age was not a factor in the latter design because each behavioral test was conducted at only one age for each chick. The design for the immunological portion of the experiment was a 2 (Dose) x 2 (Sex) factorial, because time constraints allowed assays for only 2 prenatal DFP groups.

**Incubation/Hatch.** A 1-mm diameter hole was drilled adjacent to the airspace of each egg. A 0.5-cm square piece of surgical tape was placed over each access hole. All eggs were then placed into a temperature and humidity controlled incubator. Chick embryos (n=30 per injection group) received DFP (0, 50, 100, 200, 400, 800, 1600, or 3200 μg/kg; 0.9% sterile saline vehicle; volume=1 ml/kg) injected into the airspace of eggs on E 11, the midpoint of incubation. Upon hatching chicks were weighed, banded for
identification, and housed in cages according to drug treatment group.

**Vocalizations and Activity.** Locomotor behavior was evaluated on PHD 1. Individual chicks underwent a 5-min isolation period during which distress vocalizations (DVs) and photocell interruptions (activity) were monitored. Chicks were weighed prior to being returned to their home cages.

**Tonic Immobility.** Defensive behavior was examined on PHD 14 using restraint-induced tonic immobility. This age was chosen because TI in younger chicks is not robust and previous data were available for this age (Gallup et al., 1983; Hughes, 1982). An experimenter who was blind to treatment conditions induced TI. A chick was quickly inverted, placed on its back, and physically restrained for 15 seconds. A timer was started upon the experimenter's release of the chick. The criterion for a successful induction was 10 sec of immobility. If a subject did not meet this criterion, induction was attempted again (3 attempts maximum). Latency to the chick righting itself from this dorsally recumbent position was recorded (300 sec cutoff) by trained observers. Chicks were weighed prior to return to their home cages.
Immune Assays. On PHD 16, 2 days after the TI test, the chicks were sacrificed. Blood, thymus, and spleen were harvested to obtain leukocytes for a mitogenic stimulation assay. Sex was determined by confirmation of presence of the ovary or testes. Prenatal DFP effects on perinatal mitogen-induced lymphocyte proliferation were assayed by exposure of immune tissues to T-cell and B-cell mitogens. The mitogens used for immune assays were the T-cell mitogen concanavalin A (Con A), B-cell mitogen lipopolysaccharide (LPS) from *Salmonella typhimurium* and pokeweed mitogen (PWM) which stimulates B-cells and T-cells. The proliferation of lymphocytes in response to these mitogens was used to assess immunological function in previous studies (Cunnick et al., 1994; Hughes et al., 1994a; Hughes et al., 1994b).

Data were analyzed by Pearson chi-square or ANOVA and t-tests for planned comparisons.

Results

Hatch

Prenatal DFP prevented hatches at doses higher than 200 μg/kg (Figure 1). Pearson Chi Square analysis of hatch data indicated no significant difference in the number of live hatches at 0, 50, 100, and 200 μg/kg DFP (n = 19, 21, 23, 19, respectively) and no significant sex differences (assuming equal initial frequencies per group).
Figure 1. Percent of eggs that hatched and chicks survived longer than 3 days as a function of prenatal DFP dose and sex. Embryos received 0, 50, 100, 200, 400, 800, 1600, or 3200 μg/kg DFP injected into the airspace of the egg on E11, the midpoint of embryogenesis. There were no hatches in the groups that received doses greater than 200 μg/kg DFP.
Weight

Chicks that received prenatal DFP weighed less than controls at hatch (Figure 2). By PHD 14 this trend reversed; DFP groups weighed significantly more than controls. Analysis of the weight data revealed a significant main effect of Sex \( [F(1, 73) = 8.136, p = 0.0057] \) and Age \( [F(1, 73) = 5382.184, p < 0.0001] \), but not Dose. Additionally, there were significant interactions of Dose x Age \( [F(3, 73) = 11.352, p < 0.0001] \), Sex x Age \( [F(1, 73) = 7.817, p = 0.0067] \). At hatch, female controls weighed significantly more than the DFP groups \((0 > 50, 100, 200 \mu g/kg, p = 0.0115; 0.0141; 0.0376, \text{respectively})\) as did males groups \((0 > 50, 100, 200 \mu g/kg, p = 0.0052; 0.0005; 0.0003, \text{respectively})\). By PHD 14, the female \((50, 100, 200 > 0 \mu g/kg, p = 0.0637; 0.0028; 0.0034, \text{respectively})\) and male \((50, 100, 200 > 0 \mu g/kg, p = 0.007; 0.0317; 0.0598, \text{respectively})\) DFP chicks weighed significantly more than controls.

Distress Vocalizations and Activity

Prenatal DFP decreased DVs for females, but did not significantly alter DVs in males during the 5-min isolation period (Figure 3). Due to positively skewed distributions, DV data were log-transformed prior to analysis. Analysis of log-transformed distress vocalization data revealed a significant interaction of Dose and Sex \( [F(3, 73) = 3.076, p \)
Figure 2. Mean body weight (+ and - SEM) as a function of prenatal DFP dose, sex, and age (PHD 1 & 14). Embryos received 0, 50, 100, 200 µg/kg DFP injected into the airspace of the egg on El1, the midpoint of embryogenesis.
Figure 3. Mean vocalizations (+ and - SEM) during a 5-min isolation test on PHD 1 as a function of prenatal DFP dose and sex. Embryos received 0, 50, 100, 200 µg/kg DFP injected into the airspace of the egg on E11, the midpoint of embryogenesis.
Prenatal DFP reduced DVs at 50 µg/kg and increased DVs at 100 & 200 µg/kg DFP, but not significantly. Females that received 100 µg/kg vocalized less than females that received 0, 50, and 200 (p=0.0073; 0.0864; 0.0115, respectively). Males vocalized significantly more than females at 100 µg/kg DFP (p=0.0014). Analysis of activity data (Figure 4) indicated no significant differences for mean photocell interruptions between treatment groups during the 5-min isolation period.

**Tonic Immobility**

Prenatal DFP increased tonic immobility duration compared to control groups (Figure 5). Immobility data were log-transformed prior to analysis because they were positively skewed. Analysis of tonic immobility duration data showed a significant main effect of Dose [F(3, 73)=3.594, p=0.0115] and interaction of Dose x Sex [F(3, 73)=3.245, p=0.0269]; there was no significant main effect of Sex. There were no significant differences between sexes for mean tonic immobility duration among control groups. Prenatal DFP increased immobility duration in males at all doses compared to controls (50, 100, 200 > 0; p=0.0052; 0.0115; 0.0083, respectively). Only females that received 100 µg/kg showed increased immobility duration compared to controls (100 > 0, p=0.0115). Males exhibited
Figure 4. Mean photocell interruptions (+ and - SEM) during a 5-min isolation test on PHD 1 as a function of prenatal DFP dose and sex. Embryos received 0, 50, 100, 200 μg/kg DFP injected into the airspace of the egg on E11, the midpoint of embryogenesis.
Figure 5. Mean tonic immobility duration (+ and - SEM) induced by brief physical restraint (15 sec) during a 5-min test on PHD 14 as a function of prenatal DFP dose and sex. Embryos received 0, 50, 100, 200 μg/kg DFP injected into the airspace of the egg on E11, the midpoint of embryogenesis.
significantly longer tonic immobility duration than females only at 50 μg/kg DFP (p=0.034).

Immune Assays

Prenatal DFP increased LPS-induced blood lymphocyte proliferation and negated the sex difference exhibited in controls (Figure 6). Analysis of LPS-induced blood lymphocyte proliferation revealed main effects (at p values > 0.05 & < 0.1) of Dose [F(1, 8)=3.592, p=0.0972] and Sex [F(1, 8)=4.413, p=0.0689]. Planned comparisons indicated significantly greater lymphocyte proliferation in males compared to females at 0 μg/kg DFP (p=0.0249). Prenatal DFP produced significantly greater proliferation in females (200 > 0 μg/kg; p=0.0243) which was not significantly different than that of males at 200 μg/kg DFP. There were no significant differences in PWM- or Con A-induced blood lymphocyte proliferation. Additionally, there were no significant differences between groups for mitogen-induced lymphocyte proliferation in spleen or thymus.

Discussion

Experiment 1 was designed to determine single exposure dose parameters for prenatal DFP exposure and to evaluate possible effects on perinatal responses. Doses that did not prevent hatches or produce morphological abnormalities would be used in subsequent experiments to assess more subtle
Figure 6. Mean blood stimulation index (+ and - SEM) as a function of prenatal DFP dose and sex. The B-cell mitogen LPS induced lymphocyte proliferation in blood harvested on PHD 16. The blood stimulation index represents the ratio of lymphocytes in blood samples exposed to LPS and control samples. Embryos received 0, 50, 100, 200 μg/kg DFP injected into the airspace of the egg on E11, the midpoint of embryogenesis.
behavioral effects of prenatal DFP exposure. Since sufficient numbers of chicks hatched at 200 µg/kg DFP and less, behavioral and immunological effects of DFP exposure were examined.

Hatch data indicated that the lethal dose for a single prenatal DFP exposure at the midpoint of embryogenesis in domestic fowl was between 200 and 400 µg/kg. Further research is required to more accurately determine the lethal dose and the LD_{50}. However, the aim of this experiment was to determine a range of sub-lethal doses at which more subtle behavioral and immunological effects might occur.

Prenatal DFP produced lower hatch weights at all doses compared to control animals. This relationship was reversed by PHD 14 when all DFP groups weighed significantly more than controls. The low hatch weight is consistent with the effects of many teratogens. Prenatal exposure to lead, cadmium, alcohol, nicotine, opiates, and cocaine reduces birth weight (Ali, Murthy, & Chandra, 1986; Middaugh, Lawrence, Randall, Favara, 1988). Weight gains associated with neurotoxin exposure have also been observed in humans. Children who received chronic lead exposure were more likely to be obese as young adults (Kim, Hu, Rotnitzky, Bellinger, & Needleman, 1995).

A single prenatal DFP exposure differentially affected DVs in females and males. These data indicated that
prenatal DFP decreased DVs in females and increased DVs in males. Distress vocalizations vary as a function of stress or fear. Chicks (both isolated and with cohorts) placed into a novel environment similar to their own home cage emit more DVs than chicks that remain in their home cage and DVs continue to increase as environmental dissimilarity increases (Fullerton et al., 1959). Chicks that received handling before isolation emitted more DVs than chicks that were not handled (Sufka & Hughes, 1991). These data suggest that DVs vary in an inverted U-shaped function as fear increases (Sufka & Hughes, 1991). The biphasic pattern of fear-induced DVs makes it difficult to interpret these vocalization data. However, these data suggest that DVs may be sensitive to prenatal DFP exposure.

The existing literature suggests that increased central cholinergic activity should produce decreased activity compared to controls. Although prenatal DFP tended to decrease activity for females there were no significant differences among treatment groups for either sex.

Tonic immobility duration data were consistent with enhancement of TI by cholinergic agonists described in the existing literature (Gallup et al., 1983; Hennig et al., 1988; Sanberg, 1983; Thompson et al., 1974; Woodruff et al., 1976). Prenatal DFP significantly increased tonic immobility duration in all 3 treatment groups in males but,
significantly increased immobility duration in females only at 100 μg/kg DFP. Although there was no significant main effect for sex, these data suggest that females might be less sensitive to a single prenatal DFP exposure compared to males. Prenatal exposure to 50 μg/kg DFP did not increase immobility duration of females but significantly increased duration for males. Prenatal DFP significantly increased immobility duration of both sexes at 100 μg/kg DFP.

Immune data indicated sex differences in LPS-induced blood lymphocyte proliferation. Prenatal DFP (200 μg/kg) increased B-cell proliferation in females so that they were not significantly different from males. These data suggest a differential risk of immunological disregulation for females compared to males that may index greater susceptibility to autoimmune diseases. Experiment 1 demonstrated significant behavioral and immunological consequences of a single prenatal DFP exposure and these effects are relatively durable. Prenatal DFP altered TI and immunological responses on PHD 14 and 16, respectively. The prenatal DFP effects are evident 26 days after the single exposure on E 11. The persistence of DFP effects on TI and immunological responses suggests that DFP may alter the development of regulatory processes in embryos and that the effects are relatively durable.
EXPERIMENT 2

Because AChE deactivation by DFP is long-lasting, a potential exists for cumulative effects of multiple DFP exposures. Therefore, several low-level exposures to DFP might have more profound developmental effects than a single exposure. Experiment 2 was designed to determine cumulative effects of repeated prenatal DFP exposures on perinatal behavioral and immune responses in chicks. Embryos received a single daily exposure to the nonfatal DFP doses in Experiment 1 on E 10-12, a period of rapid brain development.

Method

Subjects and Apparatus

All subject (N=74) and apparatus characteristics were identical to Experiment 1.

Procedure

The experimental design for weight data obtained on PHD 1 and 14 was a 4 (Dose) x 2 (Sex) x 2 (Age) factorial. The design for behavioral data was a 4 (Dose) x 2 (Sex) factorial. Age was not a factor because each behavioral test was conducted only once for each chick. A 2 (Dose) x 2 (Sex) design was used for immunological data, because time constraints allowed assays for only 2 prenatal DFP groups. Diisopropyl fluorophosphate (0, 50, 100, or 200 µg/kg) was injected into the airspace of fertile eggs on 3 successive
days (E 10-12) at midpoint of incubation. Treatment groups were females that received 0 (n=13), 50 (n=13), 100 (n=6), or 200 (n=5) µg/kg DFP and males that received 0 (n=12), 50 (n=7), 100 (n=12), or 200 (n=7) µg/kg DFP. All other procedures were identical to those described in Experiment 1. Hatch data were evaluated by Pearson Chi-square analysis. All other data were analyzed by ANOVA and t-tests for planned comparisons.

Results

Hatch

Repeated prenatal DFP exposure on E 10-12 reduced hatch success and survival, but did not disproportionately affect females or males (Figure 7). Analysis of hatch data indicated a dose-dependent decrease in live hatches as a function of Dose ($X^2 = 9.286, p = 0.024$). Although male hatches tended to be lower than females at 50 µg/kg and higher at 100 µg/kg, there were no significant sex differences.

Weight

Prenatal DFP produced decreased weight at hatch and increased weight on PHD 14 that depended on dose and sex (Figure 8). Analysis of variance of weight data revealed a main effect of Dose [$F(3,67)=6.189, p = 0.0009$], Age [$F(1, 67)=4420.5, p < 0.0001$], and interaction of Dose and Age [$F(3, 67)=10.165, p < 0.0001$]. Prenatal DFP decreased hatch
Figure 7. Percent of eggs that hatched and chicks survived longer than 3 days as a function of repeated (3 exposures) prenatal DFP dose and sex. Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12.
Figure 8. Mean body weight (+ and - SEM) as a function of repeated (3 exposures) prenatal DFP dose, sex, and age (PHD 1 & 14). Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12.
weights of males, 0 > 100 and 200 µg/kg (p=0.0629; 0.0281, respectively), but not females. On PHD 14, weight of chicks that received 200 µg/kg DFP was significantly greater than that of controls for males, (200 > 0 µg/kg (p=0.0013) and females (200 > 0 µg/kg; p=0.0042).

**Distress Vocalizations and Activity**

Repeated prenatal DFP exposure did not differentially affect DVs (Figure 9). Analysis of vocalization data revealed no significant effects of prenatal DFP. Although DFP increased DVs (at p values > 0.05 & < 0.1) in females at 50 µg/kg (50 > 0, p=0.06), ANOVA F test values revealed no significant main effects or interactions for these data.

Prenatal DFP increased activity during isolation on PHD 1 depending on dose and sex (Figure 10). Analysis of activity data indicated a main effect of Dose [F(3, 67)=3.95, p = 0.0118] and interaction of Dose and Sex [F(3, 67)=4.972, p=0.0036]. There was no significant main effect of Sex. Females that received 100 µg/kg exhibited significantly more photocell interruptions than controls (100 > 0 µg/kg, p=0.0011) and the other DFP groups (100 > 50 and 200 µg/kg, p=0.0009; 0.0071, respectively). There were no significant differences in activity of females between the 0, 50, and 200 µg/kg groups. Males that received 50 µg/kg were significantly more active than controls (50 > 0
Figure 9. Mean vocalizations (+ and - SEM) during a 5-min isolation test on PHD 1 as a function of prenatal DFP dose and sex. Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12.
Figure 10. Mean photocell interruptions (+ and - SEM) during a 5-min isolation test on PHD 1 as a function of prenatal DFP dose and sex. Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12.
μg/kg, p=0.012) and the other DFP groups (50 > 100 and 200 μg/kg, p=0.0533; 0.0317, respectively). There were no significant differences in activity of males between the 0, 100, and 200 μg/kg DFP groups.

**Tonic Immobility**

Repeated prenatal DFP exposure increased tonic immobility duration of females but not males (Figure 11). Analysis of tonic immobility data indicated a main effect of Sex [F(1, 66)=4.479, p=0.0382], but no significant main effect of Dose or interaction of Sex and Dose. Females that received 100 μg/kg DFP remained immobile significantly longer than controls (100 > 0 μg/kg, p=0.0398). Immobility duration of females that received 50 and 200 μg/kg DFP was intermediate to that of controls and the 100 μg/kg group. Females that received 50 or 200 μg/kg DFP were not significantly different from either the 0 or 100 μg/kg DFP groups. There were no significant differences in immobility duration for males.

**Immune Assays**

Prenatal DFP decreased PWM-induced blood lymphocyte proliferation (Figure 12). Analysis of blood lymphocyte proliferation data indicated a main effect (at p values > 0.05 & < 0.1) of Dose [F(1, 8)=4.492, p=0.067], but no significant main effect of Sex or interaction of Dose and Sex.
Figure 11. Mean tonic immobility duration (+ and - SEM) induced by brief physical restraint (15 sec) during a 5-min test on PHD 14 as a function of prenatal DFP dose and sex. Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12.
Figure 12. Mean blood stimulation index (+ and - SEM) as a function of prenatal DFP dose and sex. The mitogen PWM induced lymphocyte proliferation in blood harvested on PHD 16. The blood stimulation index represents the ratio of lymphocytes in blood samples exposed to LPS and control samples. Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12.
Prenatal DFP increased LPS-induced lymphocyte proliferation in the thymus (Figure 13). Analysis of variance of lymphocyte proliferation revealed a main effect (at p values > 0.05 & < 0.1) of Dose \[F(1, 8)=4.024, p=0.0798\]. There were no significant main effect of Sex or interaction of Dose and Sex.

Discussion

Hatch data from Experiment 2 demonstrated cumulative effects of DFP exposure on E 10-12. Repeated prenatal DFP exposure reduced hatch success and survival. This outcome is in contrast to effects of a single exposure in Experiment 1, where there were no significant differences between groups that received 200 µg/kg DFP or less. As in Experiment 1, prenatal DFP did not disproportionately affect mortality of males or females.

Prenatal DFP produced effects on weight similar to those observed in Experiment 1. Hatch weights were reduced for males and unaffected for females. However, on PHD 14 males and females that received 200 µg/kg DFP weighed significantly more than controls. These data are consistent with those obtained in Experiment 1 where prenatal DFP reduced hatch weights of all chicks compared to controls and this relationship was also reversed by PHD 14.
Figure 13. Mean thymus lymphocyte proliferation (+ and - SEM) as a function of prenatal DFP dose and sex. The B-cell mitogen LPS induced lymphocyte proliferation in thymus harvested on PHD 16. Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12.
Activity data revealed a substantial dose-dependent increase in activity for each of the sexes. These data are in contrast with the results of Experiment 1 where a single prenatal DFP exposure did not significantly affect activity. Nor was it consistent with the literature on cholinergic agonists that describes decremental effects on locomotor behavior (Genty et al., 1985; Ksir, 1978; Sanberg, 1983).

In Experiment 2, repeated prenatal DFP exposure produced significant increased activity in males only at 50 µg/kg and in females only at 100 µg/kg DFP. The small number of males that received 50 µg/kg (n=7) and females that received 100 µg/kg DFP (n=6) might cause the means for these groups to be more susceptible to the influences of extreme activity counts of some individuals. The extensive variability in male 50 µg/kg and female 100 µg/kg DFP groups suggests an extreme influence of outliers on the means for these small groups. Other than the 2 groups discussed above, prenatal DFP administered on E 10-12 did not significantly affect activity in the second experiment. Emergence of DFP effects on activity (c.f., Experiments 1 & 2) may require substantially greater exposure.

Repeated prenatal DFP exposure differentially affected tonic immobility duration of males and females. The pattern of DFP effects was different than that of Experiment 1 in which repeated prenatal DFP increased TI at all doses for
males and at 100 μg/kg for females. There was a dose-dependent increase in immobility duration for females, but not for males. Prenatal DFP decreased immobility duration of males, although not significantly. The DFP-induced increase in TI for females is consistent with the literature in which cholinergic agonists enhances TI. These data are also compatible with the proposition that females may be hyposensitive to DFP effects on TI compared to males. Repeated exposure to DFP could produce increased immobility duration in less sensitive females, while decreasing duration in males. Repeated prenatal DFP exposure might produce excessive cholinergic activity in males and thereby attenuate TI. Excessive cholinergic activity at neuromuscular synapses can desensitize postsynaptic receptors, functionally blocking neurotransmission (Feldman, et al., 1997; Taylor, 1980). Serotonergic agonists have shown similar effects on TI as those produced by DFP in Experiment 2. Boren et al. (1979) demonstrated that pargyline, a monoamine oxidase inhibitor (MAOI), and tryptophan, a serotonin precursor, administered separately increased TI, but when given in combination attenuated TI.
EXPERIMENT 3

Experiments 1 and 2 demonstrate that prenatal DFP exposure alters tonic immobility duration. Tonic immobility duration has been shown to be mediated by central cholinergic mechanisms (Hughes, 1982; Thompson et al., 1974) and DFP deactivates AchE, thereby increasing cholinergic activity (Feldman et al., 1997; Rang et al., 1995). If increases in TI duration are mediated by prenatal DFP-induced potentiation of cholinergic activity (Gallup et al., 1983; Sanberg, 1983; Thompson et al., 1974), then a cholinergic antagonist should reverse or attenuate the effect. Scopolamine, a cholinergic antagonist that primarily blocks muscarinic receptors reduced TI (Hughes, 1982; Thompson et al., 1974). Cholinergic agonists increase DVs (Sufka & Hughes, 1991) and TI (Gallup et al., 1983; Sanberg, 1983; Thompson et al., 1974), and decrease activity (Genty et al., 1985; Ksir, 1978; Sanberg, 1983). Therefore, scopolamine administered to chicks, 30 min prior behavioral assays should attenuate or reverse effects of prenatal DFP. Experiment 3 was designed to test this hypothesis, as well as evaluate DFP effects on hatch success and weight.

Method

Subjects and Apparatus

All subject (N=117) and apparatus characteristics were identical to Experiments 1 and 2.
Procedure

The design of this experiment was a 3 (prenatal DFP dose) x 3 (postnatal scopolamine dose [SCO]) factorial design for the behavioral tests. A 3 (prenatal DFP dose [0, n=45; 100, n=51; 200, n=22]) x 2 (Age, PHD 1 & 14) factorial design was used for weight data. Sex data were not available for analysis due to a technical error.

Diisopropyl fluorophosphate (0, 100, or 200 µg/kg; volume=1 ml/kg; ip) was injected into the airspace of fertile eggs on 3 successive days (E 10-12) at midpoint of incubation. Thirty minutes prior to isolation and tonic immobility tests chicks received scopolamine hydrobromide (0.0, 0.5, & 1.5 mg/kg). These scopolamine doses altered tonic immobility duration in a previous study (Hughes, 1982). Immune responses were not evaluated. Treatment groups and the number of subjects per cell are represented in Table 1.

Table 1. Treatment groups for Experiment 3.

<table>
<thead>
<tr>
<th>DFP Dose [µg/kg]</th>
<th>Scopolamine Dose [mg/kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>0</td>
<td>n=15</td>
</tr>
<tr>
<td>100</td>
<td>n=14</td>
</tr>
<tr>
<td>200</td>
<td>n=6</td>
</tr>
</tbody>
</table>
Other procedures were identical to those described in Experiment 1. Hatch data were evaluated by Pearson Chi-square analysis. All other data were analyzed by ANOVA and t-tests for planned comparisons.

Results

Hatch

Repeated prenatal DFP (200 µg/kg) reduced the number of chicks that hatched and survived longer than 3 days (Figure 14). Chi square analysis indicated a significant effect of DFP on hatch success ($X^2 = 35.429, p < 0.0001$).

Weight

There were no significant effects of repeated DFP on hatch weights or weights on PHD 14 (Figure 15).

Distress Vocalizations and Activity

Repeated prenatal DFP reduced DVs and scopolamine reversed this effect on a dose-dependent basis (Figure 16). Analysis of vocalization data indicated a significant main effect of DFP [$F(2, 117) = 4.269, p=0.0162$]. There was no significant main effect of SCO or interaction of DFP and SCO. Prenatal DFP significantly reduced DVs compared to controls ($100 < 0 \mu g/kg$ DFP, $p=0.0076$). Scopolamine produced a dose-dependent reversal of this effect. The DFP-induced decrease in vocalizations was reversed by $1.5 \text{ mg/kg}$ scopolamine ($1.5 > 0.0 \text{ mg/kg}$, $p=0.0098$) but not by $0.5 \text{ mg/kg}$
Figure 14. Percent of eggs that hatched and chicks survived longer than 3 days as a function of repeated (3 exposures) prenatal DFP dose. Sex data was not obtained. Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12.
Figure 15. Mean body weight (+ and - SEM) as a function of repeated (3 exposures) prenatal DFP dose and age (PHD 1 & 14). Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12.
Figure 16. Mean vocalizations (+ and - SEM) during a 5-min isolation test on PHD 1 as a function of prenatal DFP dose and postnatal scopolamine dose. Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12. Chicks received 0.0, 0.5, or 1.5 mg/kg scopolamine (volume=1 ml/kg; ip) 30 minutes before the behavioral test.
There were no significant effects of DFP or SCO on mean photocell interruptions (Figure 17).

**Tonic Immobility**

Postnatal scopolamine decreased tonic immobility duration as a function of DFP dose (Figure 18). Analysis of tonic immobility duration revealed a main effect (p values > 0.05 & < 0.1) of SCO \(F(2, 108)=2.747, p=0.0686\), but no main effect of DFP. The main effect of SCO was qualified by the interaction (p values > 0.05 & < 0.1) of DFP and SCO \(F(4, 108)=2.177, p=0.0763\). Scopolamine decreased tonic immobility duration of control chicks and those that received 100 \(\mu g/kg\) DFP (0.5 < 0.0 mg/kg scopolamine, p=0.0468; 0.0362, respectively), but did not reduce immobility duration in chicks that received 200 \(\mu g/kg\) prenatal DFP.

**Discussion**

Hatch data from Experiment 3 were consistent with data obtained in Experiment 2, in which 200 \(\mu g/kg\) DFP administered on E 10-12 significantly reduced hatch success. Repeated prenatal DFP exposure significantly decreased hatch success and survivability of chicks at 200 \(\mu g/kg\) DFP but did not at 100 \(\mu g/kg\).

Weight data did not indicate any differences due to prenatal DFP exposure. These data were not consistent with Experiment 1 and 2, in which prenatal DFP chicks weighed...
Figure 17. Mean photocell interruptions (+ and - SEM) during a 5-min isolation test on PHD 1 as a function of prenatal DFP dose and postnatal scopolamine dose. Embryos received 0, 100, or 200 µg/kg DFP injected into the airspace of the egg on E10-12. Chicks received 0.0, 0.5, or 1.5 mg/kg scopolamine (volume=1 ml/kg; ip) 30 minutes before the behavioral test.
Figure 18. Mean tonic immobility duration (+ and - SEM) induced by brief physical restraint (15 sec) during a 5-min test on PHD 14 as a function of prenatal DFP dose and postnatal scopolamine dose. Embryos received 0, 100, or 200 μg/kg DFP injected into the airspace of the egg on E10-12. Chicks received 0.0, 0.5, or 1.5 mg/kg scopolamine (volume=1 ml/kg; ip) 30 minutes before the behavioral test.
significantly more than controls on PHD 14. The lack of sex data may have masked potential DFP effects on weight in this experiment.

Vocalization and activity data during the isolation test continued to vary across experiments. Prenatal DFP produced similar effects on DVs and activity in Experiments 1 (single exposure) and 3 (3 exposures), but were in contrast to effects on these measures in Experiment 2 (3 exposures). A single prenatal DFP exposure in Experiment 1 significantly decreased DVs, but did not affect activity and in Experiment 2, 3 DFP exposures significantly increase activity, but had no effect on DVs.

In Experiment 3 prenatal DFP on E 10-12 decreased DVs at the 100 μg/kg dose, but not at 200 μg/kg. Scopolamine (1.5 mg/kg) reversed this effect. The DFP effects on DVs were similar to DFP effects in Experiment 1, where a single DFP exposure reduced DVs in females 50 and 100 μg/kg, and in males only at 50 μg/kg. Prenatal DFP on E 10-12 did not significantly alter DVs in Experiment 2. Scopolamine, a cholinergic antagonist, reversed the DFP-induced reduction of DVs in Experiment 3. The attenuation of DFP effects on DVs by scopolamine suggests that the DFP-induced dose-dependent reduction in DVs was due to increased cholinergic activity.
Effects of prenatal DFP on activity also varied across Experiments 1-3. A single DFP exposure in Experiment 1 and repeated DFP exposure on E 10-12 in Experiment 3 did not affect activity. Additionally, in Experiment 3, scopolamine administered 30 min before isolation did not affect activity. In Experiment 2, repeated DFP exposure (E 10-12) produced unexpected increases in activity. Activity, as measured herein, is evidently not very sensitive to 1 or 3 exposures to prenatal DFP.

Prenatal DFP significantly increased tonic immobility duration in Experiments 1 and 2. If the increased immobility duration was mediated by DFP-induced increases in cholinergic activity, the cholinergic antagonist scopolamine should reverse the effect. In Experiment 3, scopolamine reduced immobility duration of controls and chicks that received 100 μg/kg, but did not decrease immobility duration in chicks that received 200 μg/kg DFP. These data demonstrate the involvement of cholinergic mechanisms for DFP-induced effects on tonic immobility duration. Further, scopolamine’s failure to decrease immobility duration in chicks that received 200 μg/kg DFP, is consistent with increased cholinergic activity due to prenatal DFP exposure. The lack of significant DFP effects might be due to a ceiling effect. Many test animals reached the 300 sec
criterion during the TI test. A number of animals reached criterion in Experiments 1 and 2, also.
EXPERIMENT 4

Experiment 4 was designed to further characterize the nature of prenatal DFP-induced behavioral effects using a cholinergic agonist, physostigmine. Administration of physostigmine 30 min before behavioral tests was expected to increase cholinergic activity. Physostigmine, like DFP, increases cholinergic activity by inhibiting AChE, but is not as long-acting as DFP (Quenzer et al., 1997; Rang et al., 1995). According to the existing literature, physostigmine-induced increases in cholinergic activity should decrease activity (Genty, Faure, & Mills, 1985, Ksir, 1978; Sanberg, 1983), and increase DVs and TI duration (Gallup et al., 1983; Thompson et al., 1974; Sanberg, 1983). Administration of physostigmine in Experiment 4 should have the same effects, unless cholinergic activity is excessive. If physostigmine administration produces excessive cholinergic activity opposite effects are possible (Carleton, 1983; Taylor, 1980). Prenatal DFP effects on hatch success and weight were also evaluated in Experiment 4.

Method

Subjects and Apparatus

All subject (N=92) and apparatus characteristics were identical to Experiments 1-3.
Procedure

The experimental design for weight data was a 3 (prenatal DFP dose) x 2 (sex) factorial. For the behavioral tests the experimental design was a 3 (prenatal DFP dose) x 3 (postnatal physostigmine [PHY] dose) x 2 (Sex) factorial. Diisopropyl fluorophosphate (0, 150, or 200 µg/kg) was injected into the airspace of fertile eggs on 3 successive days (E 10-12) at midpoint of incubation. The 150 µg/kg DFP dose was chosen to maximize potential DFP effects and minimize hatch failure. Thirty minutes before the isolation and tonic immobility tests chicks received physostigmine hemisulfate (0.0, 0.25, & 0.5 mg/kg; volume=1 ml/kg; ip). These physostigmine doses were chosen because they were comparable to doses used in the Thompson et al. (1974) study. As in Experiment 3, no immunological assays were conducted. Treatment groups and number of animals per cell are represented in Table 2.

Chicks received handling and habituation to experimenters on PHD 14-17. During handling and habituation, a pair of chicks was removed from the home cage and transported to the weighing area in an opaque plastic container. Each chick was individually picked up, handled briefly, weighed, and returned to the plastic container. After both chicks had been handled and weighed, they were returned to the home cage. Handling and habituation to
Table 2. Treatment groups for Experiment 4.

<table>
<thead>
<tr>
<th>DFP Dose [µg/kg]</th>
<th>Physostigmine Dose [mg/kg]</th>
<th>Females</th>
<th>Males</th>
</tr>
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<td>n=4</td>
<td>n=7</td>
</tr>
<tr>
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</tr>
<tr>
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<td>n=3</td>
<td>n=3</td>
</tr>
<tr>
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<td></td>
<td>n=4</td>
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<td>n=8</td>
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Experimenters decreases TI duration (Gallup et al., 1983). Due to schedule constraints TI evaluation occurred on PHD 18. In addition, the cutoff criterion for TI duration was increased to 600 sec (10 min). Chicks were handled and the cutoff for TI was changed to 600 sec to avoid possible ceiling effects. All other procedures were identical to those described in Experiment 1. Hatch data were evaluated by Pearson Chi-square analysis. All other data were analyzed by ANOVA and t-tests for planned comparisons.

Results

Hatch

Repeated prenatal DFP exposure tended to reduce hatch and survival of chicks at 150 µg/kg, but this effect was not
significant (Figure 19). Analysis of hatch data indicated no significant differences in hatch success across groups as a function of DFP ($X^2 = 1.294, p = 0.5237$).

**Weight**

Females and males that received prenatal DFP weighed significantly more than controls by PHD 14 (Figure 20). Analysis of weight data revealed a significant main effect of Sex [$F(1, 87) = 6.241, p = 0.0145$], DFP [$F(2, 87) = 13.226, p < 0.0001$], Age [$F(1, 87) = 5692.1, p < 0.0001$], but no significant main effect of DFP. The main effects were qualified by a significant interaction of DFP and Age [$F(2, 87) = 19.278, p < 0.0001$]. On PHD 14, females (150 & 200 > 0 µg/kg DFP, $p = 0.0008; 0.0008$, respectively) and males (150 & 200 > 0 µg/kg DFP, $p = 0.0002; 0.0001$, respectively) that received prenatal DFP weighed significantly more than controls. Planned comparisons revealed significant sex differences for hatch weight only at 200 µg/kg DFP (males > females, $p = 0.0303$). On PHD 14, males weighed more than females at all DFP doses, but approached significance only at 200 µg/kg DFP ($p = 0.0628$).

**Distress Vocalizations and Activity**

Repeated prenatal DFP produced an increase in DVs that was dependent on postnatal PHY dose (Figure 21). There were no significant effects of sex, therefore data were analyzed as a 3 (prenatal DFP) x 3 (postnatal PHY) factorial.
Figure 19. Percent of eggs that hatched and chicks survived longer than 3 days as a function of repeated (3 exposures) prenatal DFP dose and sex. Embryos received 0, 150, or 200 µg/kg DFP injected into the airspace of the egg on E10-12.
Figure 20. Mean body weight (+ and - SEM) as a function of repeated (3 exposures) prenatal DFP dose and age (PHD 1 & 14). Embryos received 0, 150, or 200 μg/kg DFP injected into the airspace of the egg on E10-12.
Figure 21. Mean vocalizations (+ and − SEM) during a 5-min isolation test on PHD 1 as a function of prenatal DFP dose and postnatal physostigmine dose. Embryos received 0, 150, or 200 μg/kg DFP injected into the airspace of the egg on E10-12. Chicks received 0.0, 0.25, or 0.5 mg/kg physostigmine (volume=1 ml/kg; ip) 30 minutes before the behavioral test.
Analysis of vocalization data indicated a significant interaction of DFP and PHY \([F(4, 83)=2.938, p=0.0252]\). Physostigmine tended to decrease DVs at 0 and 200 \(\mu g/kg\) DFP, but not significantly. At 150 \(\mu g/kg\) DFP, 0.25 mg/kg PHY significantly increased DVs compared to chicks that received 0.0 or 0.5 mg/kg PHY (0.25 > 0.0 and 0.5, \(p=0.0222; 0.0006, \) respectively). There were no significant effects of DFP or PHY on photocell interruptions (Figure 22) during the 5-min isolation period.

**Tonic Immobility**

Postnatal physostigmine increased tonic immobility duration depending on sex and prenatal DFP dose (Figure 23). Analysis of tonic immobility duration revealed a significant main effect of DFP \([F(2, 73)=3.977, p=0.0229]\), interaction (at \(p\) value > 0.5 & < 0.1) of DFP and PHY \([F(4, 73)=2.341, p=0.0629]\), and interaction of DFP, PHY, and Sex \([F(4, 73)=3.007, p=0.0236]\). Prenatal DFP significantly increased immobility duration in females (150 & 200 > 0 \(\mu g/kg\) DFP, \(p=0.0095; 0.0574, \) respectively). Physostigmine administered 30 min before the tonic immobility test significantly increased immobility duration in chicks that received 0 \(\mu g/kg\) DFP (0.5 > 0.0 mg/kg physostigmine, \(p=0.0169\)). There were no significant differences in TI duration between male treatment groups.
Figure 22. Mean photocell interruptions (+ and - SEM) during a 5-min isolation test on PHD 1 as a function of prenatal DFP dose and postnatal physostigmine dose. Embryos received 0, 150, or 200 μg/kg DFP injected into the airspace of the egg on E10-12. Chicks received 0.0, 0.25, or 0.5 mg/kg physostigmine (volume=1 ml/kg; ip) 30 minutes before the behavioral test.
Figure 23. Mean tonic immobility duration (+ and − SEM) induced by brief physical restraint (15 sec) during a 10-min test on PHD 18 as a function of prenatal DFP dose, postnatal physostigmine dose, and sex. Embryos received 0, 150, or 200 μg/kg DFP injected into the airspace of the egg on E10–12. Chicks received 0.0, 0.25, or 0.5 mg/kg physostigmine (volume=1 ml/kg; ip) 30 minutes before the behavioral test.
Discussion

Prenatal DFP did not significantly decrease hatch success and survival in Experiment 4. These data were in contrast with Experiments 2 and 3 in which DFP on E 10-12 adversely affected hatch success. The basis of this discrepancy is unknown.

Prenatal DFP did not affect hatch weight of chicks. However, on PHD 14 chicks that received prenatal DFP weighed significantly more than controls. Prenatal DFP effects on weight at PHD 14 in Experiment 4 are consistent with data from Experiments 1 and 2 in which DFP groups weighed significantly more than controls at that same age.

The TI data support predictions made for prenatal DFP and postnatal phsyostigmine effects in females. Prenatal DFP (3 exposures) significantly increased TI duration for females but not males. These results were similar to Experiment 2, in which 3 prenatal DFP exposures also significantly increased TI in females and did not affect males. The TI effects of 3 DFP exposures in Experiments 2 and 4 are in contrast to effects in Experiment 1 wherein, a single prenatal DFP exposure increased TI in males at all doses and in females at only the intermediate DFP dose. Physostigmine enhanced TI in female chicks that received 0 μg/kg DFP. Physostigmine-induced potentiation of cholinergic activity produced a mean immobility duration
similar to that of chicks that received 150 and 200 µg/kg DFP.

Attempts to avoid ceiling effects via increased handling and 600 sec criterion for TI were not effective. The experimenter that induced TI was not involved in handling of chicks prior to testing. Predatory encounters increase TI duration (Gallup, 1977). Exposure to the novel experimenter as a predator may have produced similar effects. The TI data demonstrate that prenatal DFP enhancement of TI in females endures at least through PHD 18. The persistence of DFP effects suggests that prenatal DFP effects may be developmental rather than transient changes in cholinergic activity.
GENERAL DISCUSSION

Organophosphate compounds, potent AChE inhibitors, are widely used as agricultural pesticides (Mearns, Dunn, & Lees-Haley, 1994; Michalek & Pintor, 1990; Overstreet & Schiller, 1992). Little is known of possible perinatal consequences of prenatal OP exposure. The present series of experiments was designed to evaluate perinatal behavioral and immunological effects of prenatal exposure to DFP, an OP.

Prenatal DFP reduced hatch weight in Experiments 1 and 2, but had no significant effects on hatch weight in Experiments 3 and 4. However, in Experiments 1, 2, and 4, DFP chicks weighed significantly more than controls on PHD 14. These data suggest that prenatal DFP altered either metabolism or feeding behavior in chicks. The DFP-induced low hatch weight in Experiments 1 (single exposure) and 2 (3 exposures) are consistent with the effects of various teratogens. Prenatal DFP did not significantly reduce hatch weights in Experiments 3 and 4. The reason for this discrepancy is unknown. Prenatal exposure to lead, alcohol, nicotine, opiates, and cocaine reduces birth weight (Ali et al., 1986; Middaugh et al., 1988).

Prenatal DFP-induced weight gains by PHD 14 were observed in Experiments 1, 2, and 4. Similar weight gains associated with neurotoxin exposure have been observed in
humans. Children who received chronic lead exposure were more likely to be obese as young adults (Kim, Hu, Rotnitzky, Bellinger, & Needleman, 1995). It is possible that current weight data reflect DFP-induced, cholinergically mediated effects. Prenatal DFP effects on body weight are also consistent with effects of hyperinsulinemia on body weight. Ventromedial hypothalamic (VMH) lesions cause obesity and hyperphagia in rats (Brobeck, Tepperman, & Long, 1943; Hetherington & Ranson, 1940). Hustvedt & Lovo (1972; cited in Pinel, 1997) demonstrated that VMH lesion-induced obesity and hyperphagia were due to elevated insulin levels. The pancreas secretes insulin in response to ACh released from parasympathetic neurons of the vagus nerve. Sawchenko, Eng, Gold, & Simson (1977; cited in Pinel, 1997) eliminated obesity and hyperphagia in VMH lesioned animals by severing the vagus nerve. Elevated blood insulin concentrations triggers the storage of nutrients as fat in adipose tissue. Inhibition of AChE by DFP increases ACh activity in the parasympathetic nervous system. The increased weight gain by DFP groups through PHD 14 in Experiments 1, 2, and 4, is consistent with the interpretation that prenatal DFP exposure increased cholinergic activity.

The data from the current study suggest that DVs emitted during a 5-minute isolation period on PHD 1 may be sensitive to cholinergic manipulation. A single prenatal
DFP exposure differentially affected DVs of females and males in Experiment 1, but there were no significant sex differences in Experiments 2-4 (3 exposures). Distress vocalizations are an index of fear and vary in an inverted U-shaped fashion as fear increases (Fullerton et al., 1959; Sufka & Hughes, 1991). In Experiments 1 (a single exposure) and 3 (3 exposures), 100 µg/kg DFP reduced DVs. Also, in Experiment 3, 1.5 mg/kg scopolamine administered 30 min before isolation reversed the DFP effect. Prenatal DFP on E 10-12 did not significantly affect DVs in Experiment 2. Although there were no significant effects of DFP on E 10-12 in Experiment 4, physostigmine, a cholinergic agonist, produced biphasic effects on DVs at 150 µg/kg DFP. Females that received 0.25 mg/kg emitted significantly more DVs than controls and 0.5 physostigmine tended to decrease DVs compared to controls, but not significantly. These data tend to support the proposition that DVs are sensitive to cholinergic manipulation in young domestic fowl. The nature of stress effects (Fullerton et al., 1959; Sufka & Hughes, 1991) and drug dose (Carlton, 1983) on DVs, both vary in an inverted U-shaped fashion, may be factors contributing to the variability of vocalization data in this study. More consistent prenatal DFP effects on DVs may require different exposure parameters such as more than 3 exposures at
different doses, embryonic ages, and/or an evaluation session longer than 5 min.

The existing literature regarding cholinergic effects on activity suggested that cholinergic agonists would decrease and antagonists increase activity in chicks. The data collected (concurrently with DVs) in this study during the 5-min isolation period on PHD 1 did not reveal consistent DFP effects on the activity measure. In Experiment 1, a single DFP exposure at the midpoint of embryogenesis tended to decrease activity in females and increase activity in males, but neither significantly. In Experiments 3 and 4, repeated DFP on E 10-12 did not affect activity as did postnatal scopolamine and physostigmine, respectively. In Experiment 2, prenatal DFP on E 10-12 significantly increased activity in males at 50 μg/kg and females at 100 μg/kg. However, the substantial variability in males at 50 μg/kg and females at 100 μg/kg DFP suggests the influence of outliers. The relatively small number of subjects in each group (n=7 and 6, respectively) might render the means for groups more susceptible to extreme activity counts. As with DVs more consistent prenatal DFP effects on activity may require different exposure and measurement parameters. Additionally, isolation-induced stress decreases activity. Activity levels may have been reduced by isolation stress and the low baseline activity
levels may have prevented emergence of prenatal DFP-induced effects.

The current study demonstrated prenatal DFP effects on tonic immobility at PHD 14 and 18 in chicks and indicate that these effects are mediated by increased cholinergic activity. Further, DFP effects on TI differentially affected females and males. The TI response is considered an adaptive anti-predatory response (Ratner, 1977) and deviations from baseline responses might put the animal at greater risk. The TI data were collected on PHD 14 in Experiments 1-3 and in Experiment 4 on PHD 18. In Experiment 1, a single prenatal DFP exposure at midpoint of embryogenesis significantly increased TI duration in males at 50, 100, and 200 μg/kg doses and females at 100 μg/kg DFP. In Experiment 2, DFP exposure on E 10-12 significantly enhanced TI at 100 μg/kg in females and tended to decrease TI in males, but not significantly. Prenatal DFP on E 10-12 did not significantly increase TI duration in Experiment 3. However, the interaction of postnatal scopolamine, a cholinergic antagonist, with prenatal DFP suggests that DFP altered cholinergic activity at 200 μg/kg DFP. Scopolamine (0.5 mg/kg) significantly reduced TI in controls and the 100 μg/kg DFP group, but did not attenuate TI at 200 μg/kg. These data suggest that DFP effects on TI were due to increased cholinergic activity. In Experiment 4, DFP on E
10-12 significantly increased TI in females at 150 and 200 μg/kg, but did not significantly affect males. Physostigmine (0.5 mg/kg), a cholinergic agonist, significantly increased TI in females that received 0 μg/kg DFP, producing TI duration similar to that of females at 150 and 200 μg/kg DFP.

In Experiment 1, a single prenatal DFP exposure produced a robust TI increase in males at 50 μg/kg, but females required 100 μg/kg to significantly enhance TI. These data suggest that females may be less sensitive to DFP-induced TI effects. Results from Experiment 2, in which embryos received 3 DFP exposures, appear to contradict a female hyposensitivity hypothesis. However, prenatal DFP produced dose-dependent TI increases at 50 and 100 μg/kg in females, suggesting a more robust effect at 50 μg/kg. Similarly in Experiment 4, DFP on E 10-12 produce robust TI increases in females, but had no significant effect on males. The absence of significant TI increases in males could be consistent with greater sensitivity to the cholinergic effects of DFP compared to females. Excessive ACh at neuromuscular junctions can prevent repolarization of the postsynaptic membrane and functionally block neurotransmission (Taylor, 1980). Increased cholinergic activity increases TI, but if excessive ACh blocked neurotransmission it might attenuate TI duration. The Boren
et al. study (1979) demonstrated that modest increases in 5-HT can increase and excessive 5-HT can decrease TI. When administered individually, pargyline, a MAOI, and tryptophan, a serotonin precursor, increase 5-HT and enhance TI. However, when given in combination these serotonergic agonists decreased immobility duration and pretreatment with p-chlorophenylalanine, a 5-HT antagonist prevented the pargyline + tryptophan attenuation of TI. A similar phenomenon in the cholinergic system might explain the robust TI effect in males due to a single exposure in Experiment 1 and the lack of TI in males after 3 prenatal DFP exposures in Experiments 2 and 4. A single DFP exposure (Experiment 1) may moderately increase cholinergic activity and enhance TI, while 3 exposures (Experiments 2-4) might produce excessive cholinergic increases, which may attenuate TI.

Prenatal DFP differentially altered mitogen-induced lymphocyte proliferation in the current study. In Experiment 1, a single prenatal DFP exposure at midpoint of embryogenesis, produced sex- and dose-dependent increases in LPS-induced blood lymphocyte proliferation. At 0 μg/kg DFP, lymphocyte proliferation in females was significantly less than males. Prenatal DFP, 200 μg/kg, increased lymphocyte proliferation in females, but not males, and abolished the sex difference observed at 0 μg/kg. The mitogen LPS,
stimulates proliferation of B-cells. In Experiment 2, prenatal DFP on E 10-12 altered lymphocyte proliferation in blood and the thymus. Prenatal DFP decreased PWM-induced blood lymphocyte proliferation in females and males. Pokeweed mitogen stimulates both B- and T-cells. Although DFP alterations in blood lymphocyte proliferation were induced by different mitogens in Experiments 1 (LPS) and 2 (PWM), both stimulate B-cells. However, PWM also stimulates T-cells. Because PWM affects both B- and T-cells, additional assays are required to determine whether the prenatal DFP alteration of PWM-induced lymphocyte proliferation reflects changes in B-cell populations, T-cell populations, or both.

The LPS-induced B-cell proliferation in the thymus was increased by prenatal DFP on E 10-12. Normally, not many B-cells are present in the thymus. These data might indicate increased B-cell function or inappropriate homing of B-cells to the thymus. Flow cytometry assays could determine whether thymus B-cell populations are actually enhanced or whether the response reflects enhanced functioning of B-cells.

Immune data from the current study suggest that B-cell proliferation in blood was increased by a single DFP exposure and B- and/or T-cell proliferation is decreased by 3 exposures at the midpoint of embryogenesis. Disturbances
in baseline immune responses may be maladaptive. Deficient immune responses might put an animal at greater risk of infectious disease and excessive responses might increase risk of autoimmune diseases.

The results of the current study demonstrated that prenatal DFP exposure altered weight gain, hatch success, perinatal behavior, and immunoreactivity in young domestic fowl. Further, the data suggest that prenatal DFP-induced TI effects were a result of increased cholinergic activity. Prenatal DFP effects may be due to irreversible deactivation of embryonic AChE which produced a relatively permanent (up to 27 days) increase in ACh activity. It is also possible that more than 4 weeks are required for AChE synthesis to restore AChE concentrations to normal levels. Chicks tested later than PHD 18 might exhibit normal responses for measures evaluated in this study. However, prenatal DFP might induce permanent changes in the development of the regulatory mechanisms for these responses. Prenatal DFP might permanently reduce AChE concentrations, which would increase synaptic ACh concentrations. The TI data are consistent with the literature describing effects of cholinergic agonists and antagonists on duration of the response (Hicks, 1976; Hennig et al., 1988 Hughes, 1982; Sanberg, 1983; Sanberg et al., 1981; Thompson et al., 1974). Vocalization and activity data did not reveal clear-cut
effects of prenatal DFP. More consistent effects on DVs and activity might require more than 3 exposures or alteration of other exposure parameters.

The current study demonstrated that DFP at midpoint of embryogenesis significantly altered hatch success, weight gain, DVs, activity, tonic immobility, and immune responses. The baseline responses for these measures, by definition, are adaptive. Therefore, deviations from the baseline are abnormal, and might compromise survival. Further, consequences of prenatal DFP exposure persist into the third week of postnatal life in chickens. Prenatal DFP altered weight gain at PHD 14, TI at PHD 14 and 18, and immune responses on PHD 16, up to 27 days after exposure. Weight gain, TI, and immune responses were the most reliable correlates of prenatal DFP. The TI data suggest that prenatal DFP, a potent AChE inhibitor, produced increased cholinergic activity that persists at least through PHD 18. Under the conditions of this study, DVs and activity were not reliable indices of prenatal DFP exposure. Different doses, critical periods, and patterns of exposure might reveal greater effects on DVs and activity. These factors and other processes sensitive to cholinergic manipulation (e.g., learning and memory) should be investigated in future research.
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